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THE ANTITUBERCULAR PROPERTIES OF A SERIES OF THIOLS AND SULPHIDES

BY

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The antitubercular activity of a series of thiols, dithiolans, thiol esters, dimercaptopropyl esters, and episulphides has been examined *in vitro* and *in vivo* in mice infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. Most of the thiol compounds were inactive, although dimercaprol (2,3-dimercaptopropanol; B.A.L.) and a few closely related compounds showed slight activity *in vivo*, the only exception being 2,3-dimercaptopropyl chloride which was very active. The dithiolans were inactive, but some of the thiol esters were moderately active, in particular 2,3-di(acetylthio)propyl acetate and 1,2,3-tri(acetylthio)propane. The majority of the dimercaptopropyl esters had significant activity, the most active compounds being 2, 3 - dimercaptopropyl benzoate, 1, 3 - dimercapto - 2 - propyl benzoate, 2, 3 - dimercaptopropyl *o*-chlorobenzoate, and 2,3-dimercaptopropyl *p*-chlorobenzoate. All the *S*-acyl derivatives of 3-mercaptopropylene sulphide had good antitubercular activity, some being more active than streptomycin. The most active compound of the series was 3-(2-furoylthio)propylene sulphide. The activity of the compounds is believed to be due to their conversion *in vivo* to 3-mercaptopropylene sulphide and not due to the formation of ethanethiol. Slight deviation from the basic structure abolishes antitubercular activity.

Anderson and Chin (1947) first drew attention to the role of thiol compounds in experimental tuberculosis. These workers found that 2,3-dimercaptopropanol (dimercaprol; B.A.L.) 100 μg./ml. inhibited the growth of *Mycobacterium tuberculosis* grown in Dubos medium. Del Pianto (1950) reported the activity of 2-mercaptobenzothiazole in combination with salts of the *S*-esters of thiosulphonic acid. He suggested that the antitubercular activity of sodium ethyl thiosulphate was due to the formation of ethylmercaptan (ethanethiol). Solotorovsky and his colleagues [Brown, Matzuk, Becker, Conbere, Constatin, Solotorovsky, Winsten, Ironson and Quastel (1954); Solotorovsky, Winsten, Ironson, Brown and Becker (1954); and Solotorovsky, Winsten, Ironson and Brown (1956)] examined a series of ethylthio compounds, in particular *S*-ethyl-L-cysteine, which was reported to be more active than *p*-aminosalicylic acid when administered in the diet. Other workers were unable to confirm these findings and further investigation revealed that the activity of *S*-ethyl-L-cysteine and other members of the series depended upon the formation of the volatile compound ethanethiol [Solotorovsky (1955) (1956); Solotorovsky, Ironson, Winsten (1956); Oginsky, Solotorovsky and Brown (1955) (1956)]. Kushner, Dalalian, Bach, Centola, Sanjurjo and Williams (1955) and Davies, Driver, Hoggarth, Martin, Paige, Rose and Wilson (1956) have investigated more recently the antitubercular activity of series of compounds related to ethanethiol. The latter authors considered that only compounds which were converted to ethanethiol were active.

We have investigated the antitubercular activity of dimercaprol and a number of related compounds and have found some active derivatives. The activity of the compounds does not appear to depend upon the formation of ethanethiol.

METHODS

The *organism* employed was the human strain of *Mycobacterium tuberculosis*, $H_{37}Rv$, reference National Collection of Type Cultures 7416.

Compounds to be tested for *in vitro* activity were treated according to their solubility. Aqueous solutions were used when possible, but compounds which were only sparingly soluble in water were dissolved in 1 ml. ethoxyethanol and diluted 1:20 with distilled water. This ether possesses some growth-inhibitory activity against *Mycobacterium tuberculosis* at a concentration of 1:20, but the amount of ethoxyethanol in the final test concentration was never allowed to exceed 1:200.

Serial concentrations of the dissolved compounds were made in Dubos medium. The tubes were inoculated with two drops of 1:10 concentration of a

suspension, standardized to Wellcome opacity tube 2, of a 10-day-old culture of *Mycobacterium tuberculosis* grown in Dubos, and incubated at 37°. Readings of growth were made at 7 days.

The activity is recorded as the lowest concentration of the compound (μg./ml.) inhibiting growth of the organism, that is, the minimal inhibitory concentration (M.I.C.).

In vivo *Tests.*—Male albino mice weighing 18 to 22 g. were infected by injecting intravenously 0.2 ml./20 g. of a suspension in saline of a 15-day-old culture of $H_{37}Rv$ strain of *Mycobacterium tuberculosis* grown in Proskauer and Beck medium. The suspension opacity was adjusted to a density equivalent to 4 mg. wet weight of organism/ml.

Compounds were administered daily in arachis oil (unless otherwise stated—see Tables) at doses of 1/2, 1/5, 1/10, and 1/20th of their approximate subcutaneous LD50 values to groups of mice, 10 to a group. The arachis oil solution acted as a depot from which the compounds were slowly absorbed. In each experiment one group received no treatment and acted

TABLE I
INACTIVE THIOLS

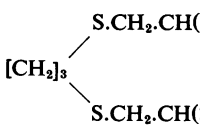
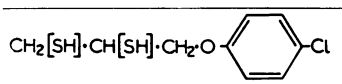
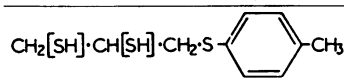
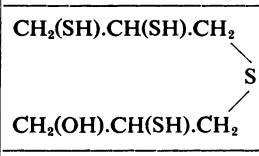
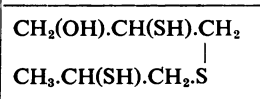
B.R.L. No.	Formula	B.R.L. No.	Formula
215	$CH_2(SH).CH_2.SH$	554	
217	$CH_2(SH).CH_2.CH_2.SH$		
218	$CH_2(SH).CH_2.CH_2.CH_2.SH$	559	$CH_3.CH(SH).CH_2.S.[CH_2]_3.SH$
231	$CH_2(SH).CH(OH).CH_2.SH$	560	$CH_2(OH).CH_2.CH_2.S.CH_2.CH(SH).CH_3$
243	$CH_2(OH).CH_2.SH$	570	$CH_2(SH).CH_2(OH).CH_2.S.CH_2.CH(SH).CH_3$
244	$CH_2(SH).CH(SH).CH_3$	573	$CH_2(SH).CH_2.S.CH_2.CH_2SH$
248	$CH_2(SH).CH(SH).CH_2.SH$	576	$CH_2(SH).CH(OH).CH_2.CH_2SH$
259	$CH_2(OH).CH_2.CH_2.SH$	581	$CH_3.CH(SH).CH(SH).CH_2OH$
264	$CH_2(SH).CH(OH).CH_3$	584	$CH_2(SH).CH(SH).CH_2.S.CH_2.CH(SH).CH_3$
271	$CH_2(OH).CH(OH).CH_2.SH$	614	$CH_2(SH).CH(O.CO.CH_3).CH_2SH$
449	$CH_2(SH).CH(SH).CH_2.OC_2H_5$	622	$CH_2(SH).CH_2.CH(SH).CH_2O.CO.CH_3$
458	$CH_2(SH).CH(SH).CO.OCH_3$	633	$C(CH_2.SH)_2(OH)(CO_2H)$
476	$CH_2(SH).CH(SH).CH(OCH_3).CH_2.OCH_3$	639	$CH_2(OH).CH(SH).CH(SH).CH_2OH$
484	$CH_2(SH).CH(SH).CO_2H$	713	$CH_2(SH).CH(SH).CH_2.O.C_6H_5$
493	$CH_2(SH).CH(OH).CH(SH).CH_3$	735	
496	$CH_2(SH).C(CH_3)(OH).CH_2.SH$	756	
515	$CH_2(SH).CH_2.CH(SH).CH_2.OH$	765	$CH_2(SH).CH(OH).CH(SH).CH_2SH$
522	$CH_2(SH).CH(SH).CH(OH).CH_3$		
552	$CH_3.CH(SH).CH_2.S.CH_2.CH_2OH$		

TABLE II

ACTIVE THIOLS

All compounds were administered subcutaneously in arachis oil.

B.R.L. No.	Formula	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
—	Dimercaprol $CH_2(SH).CH(SH).CH_2OH$	50·0	100	9·5	7·0	—	—
479	$CH_2(SH).CH(SH).CH_2Cl$	6·25	100	—	24·0	16·0	9·0
489		25–50	>500	—	14·0	8·0	3·5
550	$CH_3.CH(SH).CH_2.S.CH_2.CH_2SH$	>100·0	200	10·0	7·0	2·5	—
555		—	250	7·5	4·0	2·0	—
567	$CH_2(SH).CH_2(SH).CH(Cl).CH_3$	50·0	500	11·0	7·0	3·5	—
578	$CH_2(SH).C(CH_3)(Cl).CH_2SH$	>50·0	400	—	8·0	6·0	—
609	$CH_3.CH(SH).CH(SH).CH_2Cl$	>50·0	300	—	4·0	6·0	—
784	$CH_2(SH).[CH_2]_8.CH(SH).CH_2OH$	>50·0	500	—	4·0	0·5	—

as an infected control. Some compounds were tested for oral activity, either by administration by stomach tube or in the diet. The doses administered were again 1/2, 1/5, 1/10, and 1/20th of their approximate acute oral LD50. The water-soluble compounds were given in aqueous solution in a volume of 0.2 ml./20 g. and the insoluble solids as acacia suspension, 0.2 ml./20 g. When the compounds were given in the diet the solution or suspension was added to 250 g., so that the appropriate dose was contained in 5 g., that is, the average daily intake for one mouse. Daily dosing commenced in all cases 24 hr. after infection and continued until the twentieth day of the experiment. The TD50 values (median survival time) were determined by plotting the accumulative percentage mortality against time in days on log probit paper, and in cases where there was an increase in TD50 value over that of the control the results were analysed by the method of Litchfield (1949).

Toxicities.—Only approximate estimates of the acute LD50 values were obtained. The compounds were prepared as oil and water emulsions using acacia as the dispersing agent, and injected subcutaneously into groups of 5 mice per dose level.

RESULTS

Thiols

The inactive thiols are shown in Table I and the active thiols in Table II. The parent compound of the series, dimercaprol (2,3-dimercaptopropanol), and all the active thiols, apart from 2,3-dimercaptopropyl chloride (B.R.L. 479), were only slightly active *in vivo*. *In vitro* their activity was negligible. The compounds which were active possessed free thiol groups and a hydroxyl group, but not necessarily all compounds of this type were active. Acylation of the hydroxyl group or replacement of the hydroxyl group with a thiol, carboxyl or substituted carboxyl group abolished activity. Compounds having only one thiol group were inactive. If the hydroxyl group in the compounds having two vicinal thiol groups was replaced by a chlorine atom, activity was retained and might in fact be enhanced, for example, 2,3-dimercaptopropyl chloride (B.R.L. 479) was appreciably active *in vivo*, giving a considerable extension of the survival time. *In vitro* it had an

activity of 6.25 μg./ml., being the only member of this group which had a significant *in vitro* activity.

The chloro compounds B.R.L. 479, B.R.L. 567 and B.R.L. 609, which were prepared from the hydroxyl analogues and hydrochloric acid, may be contaminated with isomeric structures (Doyle, Holland, Mansford, Nayler, and Queen, 1960).

Dithiolans

Ring closure to form dithiolans resulted in complete loss of activity—Table III.

Thiol Esters

The inactive thiol esters are listed in Table IV and the active thiol esters in Table V. Some of the active compounds showed considerable *in vitro* activity. 2,3-Di(acetylthio)propyl acetate (B.R.L. 403) and 2,3-di(acetylthio)propanol (B.R.L. 464) are active at a concentration of 0.8 μg./ml. 1,2,3-Tri(acetylthiopropane (B.R.L. 411) was also very active *in vitro*. These three compounds also showed good activity *in vivo*. Increasing the size of the acyl group to a butyryl group, however, led to a considerable decrease both of *in vivo* and *in vitro* activity. The substitution of other radicals also led to a diminished activity.

TABLE III

DITHIOLANS

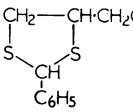
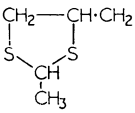
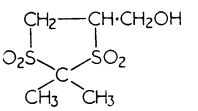
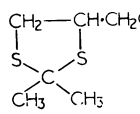
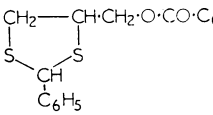
B.R.L. No.	Formula	B.R.L. No.	Formula
401		613	Glucose derivative of dimercaprol
412		647	
414		650	

TABLE IV

INACTIVE THIOL ESTERS

B.R.L. No.	Formula
450	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).CH_2.S.CO.CH_3$
471	$CH_2(O.CH_2COOH)CH(S.CO.CH_3).CH_2S.CO.CH_3$
543	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH_2.CH_2.S.CO.CH_3$
545	$CH_3CH(O.CO.CH_3).CH(S.CO.CH_3).CH_2.S.CO.CH_3$
568	$CH_2(S.CO.CH_3).C(CH_3)(O.CO.CH_3).CH_2.S.CO.CH_3$
583	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).CH_2.CH_2.S.CO.CH_3$
585	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH(S.CO.CH_3).CH_3$
640	$CH_2(O.CO.CH_3).CH(S.CO.CH_3).CH(S.CO.CH_3).CH_2.O.CO.CH_3$
657	$C(CH_2.S.CO.CH_3)_2(O.CO.CH_3)(CO_2H)$
755	$CH_2(S.CO.CH_3).CH(O.CO.CH_3).[CH_2]_4.S.CO.CH_3$
766	$CH_2(S.CO.CH_3).CH(S.CO.CH_3).CH(O.CO.CH_3).CH_2.S.CO.CH_3$
788	$CH_2(S.CO.CH_3).[CH_2]_8.CH(S.CO.CH_3).CH_2O.CO.CH_3$
826	$CH_2(S.CH_3).CH_2.S.CO.CH_3$

TABLE V

ACTIVE THIOL ESTERS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous suspension.

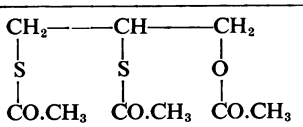
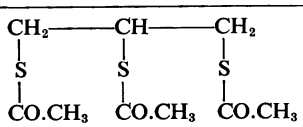
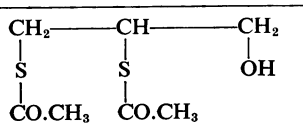
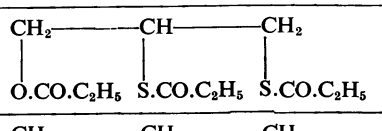
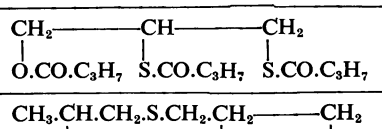
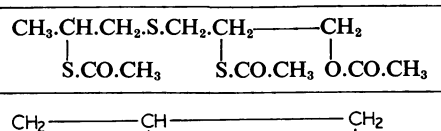
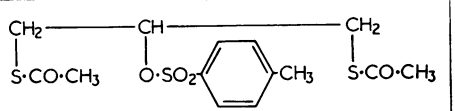
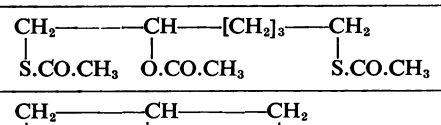
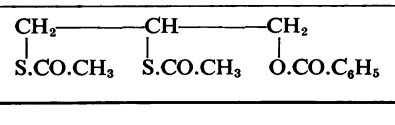
B.R.L. No.	Formula	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50		
				1/2	1/5	1/10
403		0·8	200	15·25	9·0	2·5
411		1·6	99	17·5	9·5	2·5
464		0·8	150	11·5	4·5	2·75
535		—	>500	15·0	2·0	2·0
536		6·25	500	2·0	−0·5	−0·5
562		6·25	500	2·5	−1·0	−1·5
655		6·25–12·5	>500	—	14·0	11·5
			150 (a)	—	2·0	3·0 (a)
758		>50·0	>500	—	4·0	1·5
877		—	>500	—	4·0	2·5

TABLE VI

INACTIVE DIMERCAPTOPROPYL ESTERS

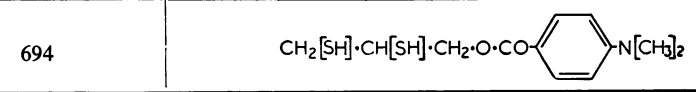
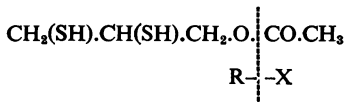
B.R.L. No.	Formula
646	$CH_2(SH).CH(SH).CH_2.O.CO.[CH_2]_{14}.CH_3$
694	

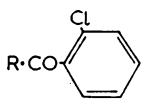
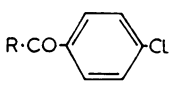
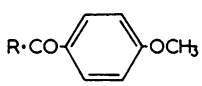
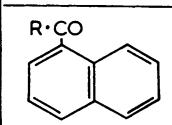
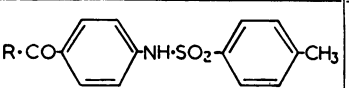
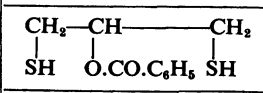
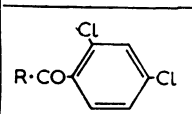
TABLE VII

ACTIVE DIMERCAPTOPROPYL ESTERS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous suspension, (*b*) orally in arachis oil.

General formula:



B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
463	R–CO.CH_3	3·125–6·25	78	15·0	8·0	4·4	—
504	R–CO.C_2H_5	12·5	150	13·0	4·0	—	—
510	R–CO.C_3H_7	25·0	400	13·0	8·5	1·5	—
587	R–CO.C_6H_5	12·5	>500	28·4	18·6	16·4	9·0
			600 (a)	5·2	2·5	1·25 (a)	—
			—	−3·5	11·5	0 (b)	—
636		25·0	>500	20·0	29·0	22·8	10·7
			500 (a)	6·5	2·0	2·5 (a)	—
637		12·5	500	54·0	29·0	16·0	11·25
638	R–CO.CH_2.C_6H_5	25·0	500	—	14·5	8·5	1·0
			>500 (a)	—	3·0	3·5 (a)	—
651		25·0	200	22·5	11·5	5·5	—
668		50·0	>500	—	9·5	2·5	—
670		>50·0	>500	—	8·0	0·5	—
671	R–CO.CH_2.S.C_2H_5	25·0	300		8·0	3·5	—
673	R–CO.CH_2.S.CH_2.C_6H_5	50·0	>500	14·0	10·5	5·0	--
			>500 (a)	3·0	1·0	−0·5 (a)	--
680		1·56	500	34·0	19·0	10·25	3·5
693	R–CO.$[CH_2]_2$.S.CH_3	25·0	500	—	8·5	6·0	—
707		25–50	150	—	3·0	0·75	--

Dimercaptopropyl Esters

The inactive compounds are given in Table VI and the active compounds in Table VII. When the hydroxyl group is esterified there is usually a considerable increase in antitubercular activity, particularly in compounds with vicinal thiol groups, and where the hydroxyl group is adjacent to these. 2,3-Dimercaptopropyl benzoate (B.R.L. 587), 2,3-dimercaptopropyl *o*-chlorobenzoate (B.R.L. 636) and 2,3-dimercaptopropyl *p*-chlorobenzoate (B.R.L. 637) are the most active of this particular type. The precise nature of the ester radical, however, does not seem to be of great importance in determining the degree of activity. All of the *O*-esters of dimercaprol were, however, comparatively inactive *in vitro*.

A notable exception to the general pattern is 2-mercapto-1-mercaptomethylethyl benzoate (B.R.L. 680), which has the two thiol groups separated by the ester group. This compound is active both *in vitro* and *in vivo*. The isomer 2,3-dimercaptopropyl benzoate (B.R.L. 587) is relatively inactive *in vitro*.

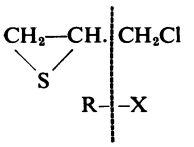
Episulphides

By eliminating the elements of water from thiol compounds containing a vicinal hydroxyl group

TABLE VIII

INACTIVE EPISULPHIDES

General formula:



B.R.L. No.	R.X
509	$R.CH_3$
511	$R.CH_2Cl$
512	$R.CH_2.O.C_2H_5$
513	$R.CH_2.S.C_2H_5$
514	$R.CH_2.N(C_2H_5)_2$
520	$R.CH_2.S.CO.NH.C_6H_5$
723	$R.CH_2.S.CH_3$

TABLE IX

ACTIVE EPISULPHIDES

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (*a*) orally in aqueous solution, (b) orally in arachis oil, (c) in the diet.

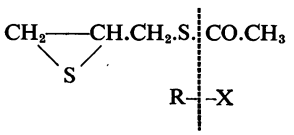
B.R.L. No.	Formula ($R=CH_2—CH—$ with S)	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
482	$R.CH_2SH$	12·5	44 88 (a)	−1·5	−3·0	−3·0 (c)	—
				17·2	17·0	14·5	4·0
				3·0	1·25	1·0 (a)	—
				5·5	−1·0 (b)	—	—
528	$CH_3.CH—CH.CH_2SH$ (S)	100	200	—	2·5	1·5	—
553	$R.CH_2.S.S.CH_2.R$	—	500	3·5	18·0	14·0	—
577	$CH_2—CH.CH_2.CH_2SH$ (S)	50·0	400	—	4·5	6·75	—
599	$R.CH(SH).CH_3$	12·5–25·0	100	2·5	1·5	−1·0	—

TABLE X

EPISULPHIDES WITH *S*-ACYL GROUPS

Compounds were administered subcutaneously in arachis oil except where indicated as follows: (a) orally in aqueous suspension, (b) orally in arachis oil, (c) in the diet, (d) orally in acacia suspension, (e) subcutaneously in acacia suspension.

General formula:



B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
459	$R.CO.CH_3$	0·4–0·8	45	17·25	14·0	12·0	4·5
			110 (a)	9·25	6·25	0·5 (a)	—
				3·5	2·0 (b)	—	—
				−2·5	−1·5	−2·5 (c)	—
540	$R.CO.C_2H_5$	1·56	100	21·0	17·5	11·0	—
546	$CH_2(S)CH.CH_2.CH_2.S.CO.CH_3$	—	300	14·5	5·25	0·5	—
561	$R.CO.C_3H_7$	6·25–12·5	100	14·5	13·5	7·0	—
571	$CH_2(S)C(CH_3).CH_2.S.CO.CH_3$	50·0	300	2·5	2·7	1·5	—
580	$R.CO.CH_2.CH_2CO_2H$	25·0	100	— 7·5	11·5 3·5	5·0 4·0	—
			200 (a)	—	1·5	1·5 (a)	—
590	$R.CO.CH_2Cl$	25·0	60	16·0	17·5	16·3	6·0
			150 (a)	3·5	2·5	2·5 (a)	—
595	$CH_2(S)CH.CH(S.CO.CH_3).CH_3$	25–50	300	5·5	3·5	1·5	—
600	$R.CO.[CH_2]_2.CO.OCH_3$	1·56–3·125	100	17·5	6·5	12·0	—
601	$CH_2(S)CH.[CH_2]_3.S.CO.CH_3$	3·125	>500	17·5	10·0	1·5	—
602	$R.CO.O.C_2H_5$	1·56	150	16·5	18·0	15·0	4·5
605	$R.CO.C_6H_5$	1·56	300	30·5	16·5	20·5	0
			400 (a)	4·0	2·5	2·0 (a)	—
615	$R.CO.CH_2.CO.O.C_2H_5$	3·125	75	22·5	11·0	8·0	—
617	$R.CO.CHCl_2$	25·0	150	—	17·5	10·0	5·0

TABLE X—*continued.*

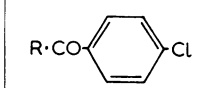
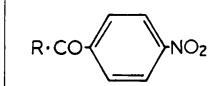
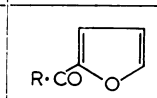
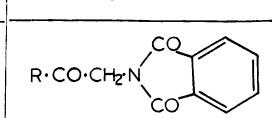
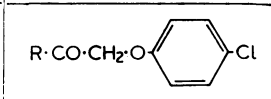
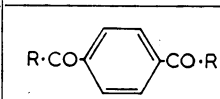
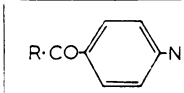
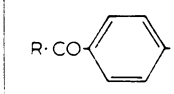
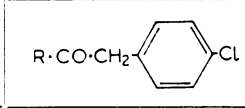
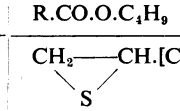
B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg/kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
625	$R.CO.CH_2.CH_2Cl$	6·25	300	—	19·5	17·5	—
629	$R.CO.CH_2.C_6H_5$	12·5	500	22·5	27·5	17·0	11·0
			>500 (a)	13·0	9·0	6·0 (a)	—
			—	—	4·0	0·5 (b)	—
635	$R.CO.CCl_3$	25·0	500	—	—	22·5	21·5
643	$R.CO.CO.O.C_2H_5$	12·5	100	17·5	13·5	10·5	—
649		50·0	200	29·5	12·5	2·5	—
			>1000 (d)	10·0	1·5	0·5 (d)	—
656		6·25	>500	29·5	15·0	11·0	—
			>500 (d)	16·25	5·2	2·75(d)	—
658		0·39	300	—	28·5	26·3	8·5
			500 (a)	—	7·0	0·5 (a)	—
664		>50·0	300	15·0	16·0	15·0	—
			1000 (d)	4·0	2·0	3·0 (d)	—
669		>50·0	200	—	5·5	2·5	—
672	$R.CO.CH(C_6H_5)_2$	>50·0	>500	6·0	1·0	1·0	—
677	$R.CO.CH_2.S.C_2H_5$	6·25	100		18·5	9·0	4·0
681	$R.CO.[CH_2]_{14}.CH_3$	>50·0	>500	4·0	0·5	−1·0	—
690	$R.CO.CH_2.NH.CO.O.CH_2.C_6H_5$	25·0	150	12·5	8·5	1·5	—
710	$R.CO.CH.[CH_2]_4.CH_2$	6·25–12·5	>500	—	34·5	23·5	1·5
711	$R.CO.[CH_2]_2.CO.CH_3$	25·0	100	—	23·5	—	—
			200 (a)	—	7·0	2·5 (a)	—
			—	—	2·5	2·0 (b)	—
721		3·125	>500		25·0	17·0	17·0 (e)

TABLE X—*continued.*

B.R.L. No.	R.X	*In vitro* Activity μg./ml.	LD50 mg./kg.	Extension in TD50 over Control. Dose Fraction of LD50			
				1/2	1/5	1/10	1/20
727	$R.CO.CH_2.NH.CO.C_6H_5$	>50·0	400		17·0	16·0	1·5 (e)
728		3·125	>500	—	8·0	11·0	2·0 (e)
733		3·125–6·25	>500	—	21·0	13·0	5·5 (e)
746	$R.CO.O.CH_3$	6·25–12·5	50	—	8·5	4·5	—
747		25·0		—	12·5	9·0 (e)	—
748	$R.CO.CH(Cl).C_6H_5$	25·0	>500	—	2·5	0·5 (e)	—
753	$R.CO.O.C_4H_9$	3·125	>500	—	22·0	8·0	—
785		—	300	—	2·0	2·0	—

to form episulphides the compounds produced have enhanced antitubercular activity. The inactive compounds are shown in Table VIII and the active compounds in Tables IX and X. Since the synthetic method (Doyle, Holland, Mansford, Nayler, and Queen, *J. chem. Soc.*, in the press) was not structurally definite either or both of the preparations, B.R.L. 528 and B.R.L. 599, may be mixtures of the two isomeric structures. Of the compounds in Table IX only 3-mercaptopropylene sulphide (B.R.L. 482) and di(2,3-epithiopropyl) disulphide (B.R.L. 553) show good activity. On the other hand, the majority of episulphides with *S*-acyl groups (Table X) were highly effective chemotherapeutic agents. The compounds with an episulphide ring must, however, be comparatively simple in order to be active ; compounds with high activity being confined to *S*-acyl derivatives of 3-mercaptopropylene sulphide (B.R.L. 482). Substitution of methyl groups on any of the carbon atoms almost abolishes activity. The addition of groups other than simple acyl radicals also abolishes activity. The nature of the acyl radical, on the condition that it is a simple group, would not appear to be the main determining factor for antitubercular activity, but the acetyl (B.R.L. 459) and furoyl (B.R.L. 658) derivatives both have high *in vivo* and *in vitro* activities.

DISCUSSION

A large number of thiol compounds and related derivatives have been investigated for antitubercular activity, and many have proved to be active *in vivo*. There are, however, a number of discrepancies between the *in vitro* activity and the *in vivo* activity, and an explanation for this must be sought.

Since the majority of the episulphides are very active *in vivo*, it would appear that the episulphide structure is the necessary moiety for antitubercular activity. This view is supported by the investigations of Miles and Owen (1952) and Harding and Owen (1954), who have shown that acetylated thiols with a vicinal hydroxyl group in the presence of mild alkali are deacetylated with accompanying ring closure to form episulphides. The activity of the thiols and thiol *O*-esters can therefore be accounted for by such a mechanism. Further confirmation that an active derivative is formed has been obtained by the findings of Mansford and

Langley (to be published), who have shown that the addition of 3-acetylthiopropylene sulphide (B.R.L. 459) to serum leads to the formation of 3-mercaptopropylene sulphide (B.R.L. 482). If B.R.L. 482 is the active derivative, the absence of activity *in vitro* may be explained by the fact that it is an unstable compound and under the mild alkaline conditions of the *in vitro* test it forms inactive polymers.

The activity of the compounds tested is not due to their conversion to ethanethiol which has been shown to be the active agent produced by other thiol antitubercular agents (Oginsky, Solotorovsky, and Brown, 1955, 1956). We have been unable to detect ethanethiol in the expired air of animals injected with any of the compounds tested, nor has it been possible by using B.R.L. 587 containing radioactive ^{35}S to detect ^{35}S in the breath. Over 80% of the injected ^{35}S was recovered in the urine of rats in 24 hr. as sulphate.

The activity of the compounds which are active *in vitro* can possibly be explained by the assumption that the compound is absorbed on the surface of the organisms and the active metabolite (i.e., B.R.L. 482) is formed in close proximity to it and is therefore able to exert an antibacterial action before it is destroyed chemically. While there is little evidence for this theory, it does provide a likely explanation.

This explanation could also account for the apparent discrepancy between activities of 2,3-dimercaptopropyl benzoate (B.R.L. 587) and 1,3-dimercapto-2-propyl benzoate (B.R.L. 680) *in vitro* and *in vivo*. It is possible that B.R.L. 680 can be readily converted *in vitro* to the parent episulphide whereas B.R.L. 587 cannot. *In vivo*, however, the reaction may readily take place, with the result that both compounds are equally active. It is unlikely that the compounds are simply dealkylated to the hydroxyl compound without ring closure taking place, since the parent hydroxyl compound (B.R.L. 231) of B.R.L. 680 is highly toxic and completely inactive *in vivo*.

We wish to thank Mr. F. P. Doyle and his colleagues for preparing the compounds; Dr. L. N. Owen for the supply of 3,4-dimethoxybutane-1:2-dithiol (B.R.L. 476), 1,4-dihydroxybutane-2:3-dithiol (B.R.L. 639) and 1,4-diacetoxy-2,3-(diacetylthio)butane (B.R.L. 640); Dr. J. H. C. Nayler for guidance on chemical nomenclature; and Mr. D. Wright for technical assistance.

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THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIOPROPYLENE SULPHIDE AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE

BY

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(RECEIVED FEBRUARY 3, 1960)

Two derivatives of 3-mercaptopropylene sulphide (B.R.L. 482), 3-acetylthiopropylene sulphide (B.R.L. 459) and 3-(2-furoylthio)propylene sulphide (B.R.L. 658), have been tested in mice for antitubercular activity. The compounds were administered daily in arachis oil by the subcutaneous route. When tested by a prolongation in survival time B.R.L. 459 was less active than streptomycin, while B.R.L. 658 was of the same order of activity. When assessed by the lesions present in the lungs after 14 days' treatment B.R.L. 658 was as active as isoniazid and B.R.L. 459 was of the same order of activity as streptomycin. Neither compound, however, cured an established infection and resistance developed rapidly to both compounds, both *in vitro* and *in vivo*. It is concluded that, in view of their adverse physical and pharmacological properties, the compounds are unsuitable for clinical use.

Acred and Brown (1960) have tested a series of new antitubercular compounds related to dimercaprol (2,3-dimercaptopropanol) and have produced evidence that the activity of the compounds depends on the formation of 3-mercaptopropylene sulphide. *S*-acyl derivatives of this compound possess considerable antitubercular activity. Two of the most active compounds, 3-acetylthiopropylene sulphide (B.R.L. 459) and 3-(2-furoylthio)propylene sulphide (B.R.L. 658), have been examined in greater detail.

METHODS

The methods and techniques for assessing antitubercular activity *in vitro* and *in vivo* were the same as those used by Acred and Brown in the previous paper. The organism employed in all tests was the human strain ($H_{37}Rv$) of *Mycobacterium tuberculosis*. In all tests the propylene sulphides were administered in arachis oil solution; streptomycin and isoniazid were given in aqueous solution.

Determination of the Extension of the Median Survival Time (TD50).—The antibacterial activity of the compounds was tested in mice (18 to 22 g.) infected with *Mycobacterium tuberculosis* and compared with that of streptomycin and isoniazid. Doses of one-fifth, one-tenth, one-twentieth and one-fortieth of the acute subcutaneous LD50 values were administered daily by the subcutaneous route for 20 days following the infection; the activity being expressed as the extension in days of the median survival time, over that of the infected control.

Lung Lesion Test.—As a further check on activity the procedure described by Solotorovsky, Winsten, Ironson, Brown, and Becker (1954) was employed. Ten mice (18 to 22 g.) were allocated to each group. The mice were infected intravenously with 0.8 mg. wet weight of *Mycobacterium tuberculosis*. The compounds were administered at one-fifth, one-tenth, one-twentieth and one-fortieth of the acute subcutaneous LD50 values. Treatment was stopped when 50% of the infected control animals died. On that day all surviving animals were killed and the lungs removed and kept in 10% formol saline for 48 hr. The degree of lung tuberculous involvement was scored in percentage for each group, the final result being expressed as the mean percentage tuberculous involvement.

Activity Against an Established Infection.—Groups of 10 mice were infected intravenously with 0.8 mg. wet weight *Mycobacterium tuberculosis*. Treatment was commenced after seven days, and continued until the twentieth day. Doses of the propylene sulphides equal to one-fifth and of streptomycin equal to one-tenth the acute subcutaneous LD50 were given by the subcutaneous route. The TD50 values were estimated and compared with an infected control group.

In vivo *Resistance.*—The propylene sulphides and streptomycin were administered daily by the subcutaneous route at one-twentieth of their acute subcutaneous LD50 values. Groups of 10 mice (18 to 22 g.) infected intravenously with 0.8 mg. wet weight *Mycobacterium tuberculosis* were used. When two or three mice remained in each group they were killed, and the lungs and spleens removed and homogenized. The tubercle bacilli were cultured from the

homogenates on Löwenstein-Jensen slopes and in Proskauer and Beck medium. For the next passage a further group of mice were infected with an inoculum prepared from the isolated organism grown on Proskauer and Beck medium. The *in vitro* sensitivity and virulence of the bacilli isolated at the first, third, and fifth passages were determined and compared with the results obtained for bacilli isolated at each passage taken from an infected, untreated control group of mice. The *in vitro* sensitivity was determined in Dubos medium. The virulence to mice was checked by infecting intravenously a group of 10 mice with 0.8 mg. wet weight of the organism isolated, and determining the TD50 in the absence of drug treatment.

Development of Resistance in vitro.—A suspension of a 10-day culture of *Mycobacterium tuberculosis*, standardized to Wellcome opacity tube 2, was diluted 1 : 10 with Dubos medium. Two drops of this suspension were added to tubes, each containing 2.5 ml. of serial dilutions of B.R.L. 459 and B.R.L. 658, streptomycin, and isoniazid in Dubos medium. Incubation was carried out at 37° for 7 days. The concentration of drug in μg./ml. which just inhibited growth (minimal inhibitory concentration) was noted. The inoculum for the subsequent passage was taken after a further 7 days' incubation from the tube containing the highest concentration of drug in which growth occurred. Passaging was continued until a high degree of resistance to the compounds had developed.

Results

Extension of Median Survival Time.—The results obtained are given in Table I. B.R.L. 459 is less active than streptomycin at all dose levels. B.R.L. 658 also appeared to be less active, but in 1 experiment with streptomycin a rather unusually long extension of the TD50 was recorded. If this test is not taken into account the activity of B.R.L. 658 and streptomycin would be of the same order. Isoniazid is considerably more active than both the compounds under test.

Lung Lesion Test.—The results obtained are given in Table II. When tested in this way B.R.L. 459 was of the same order of activity as streptomycin. At the dose levels employed all the lungs showed advanced tuberculous involvement. B.R.L. 658, on the other hand, was more active than streptomycin. At one-tenth of the subcutaneous LD50 dose the tuberculous involvement was slightly greater than minimal, while at one-twentieth of the LD50 there was only an average of 25% involvement. However, at one-fortieth of

TABLE I

THE ANTITUBERCULAR ACTIVITY OF 3-ACETYLTHIOPROPYLENE SULPHIDE (B.R.L. 459) AND 3-(2-FUROYLTHIO)PROPYLENE SULPHIDE (B.R.L. 658), STREPTOMYCIN AND ISONIAZID IN MICE

The extension of the TD50 value is the increase in survival time of the treated mice over the untreated mice, measured from the regression of percentage mortality against time on log-probit paper at the 50% mortality level. The *in vitro* activity was determined in Dubos medium seven days after inoculation.

Compound	Subcutaneous LD50 mg./kg.	*In vitro* Activity (M.I.C.) μg./ml.	Expt. No.	Extension in TD50 over Control—Days. Dose Fraction of Subcutaneous LD50			
				1/5	1/10	1/20	1/40
B.R.L. 459 ..	45·0	0·4–0·8	1		13·0	1·0	1·0
			2		7·0	2·5	2·5
			3		2·5	2·5	0
			4	14·0	12·0	4·5	0
B.R.L. 658 ..	300·0	0·8	1		17·0	9·0	3·0
			2		7·5	5·5	5·5
			3		4·5	0	1·0
			4	28·5	26·5	8·5	—
Streptomycin ..	520·0	0·4	1	—	16·5	9·0	4·0
			2	—	16·0	7·0	7·0
			3	59·0	38·0	15·0	6·5
			4	12·5	10·0	9·0	5·5
Isoniazid ..	182·0	0·05	1	—	89·0	44·0	38·0
			2			70·0	75·0

TABLE II

LUNG LESION TEST

The mice were killed and the lungs examined on the day 50% of the control mice died.

Compound	Expt. No.	% Lung Tuberculous Involvement. Dose Fraction of Subcutaneous LD50		
		1/10	1/20	1/40
B.R.L. 459	1	57	79	71
	2	100	100	100
	3	72	82	100
	Mean	76·3	87·0	90·3
B.R.L. 658	1	12	37	92
	2	8	27	50
	3	16	12	83
	Mean	12	25	75
Streptomycin	1		100	
	2		86	
	3		100	
	4		100	
	5	66	90	
	6	57	90	
	Mean	61·5	94·3	
Isoniazid	1	0		
	2	5	50	

the LD50 there was advanced involvement, but this was not a great deal worse than that seen in mice treated with one-tenth the subcutaneous LD50 of streptomycin. From the limited data with isoniazid, B.R.L. 658 appeared to have the same order of activity.

TABLE III

DELAYED THERAPY

Compound	TD50—Days. Dose Fraction of Subcutaneous LD50	
	1/5	1/10
B.R.L. 459	21·0	
B.R.L. 658	20·5	
Streptomycin ..		100% survival after 30 days
Infected control (no treatment)	20·0	

Action Against Established Infection.—Neither B.R.L. 658 nor B.R.L. 459 gave any protection to mice which had been infected 7 days previously. On the other hand, streptomycin had a pronounced antitubercular action ; all the mice still survived 30 days after the infection (see Table III).

In vivo *Resistance.*—The *in vitro* sensitivities of the bacilli isolated from mice at the end of the first, third and fifth passages are given in Table IV.

TABLE IV

VIRULENCE AND *IN VITRO* SENSITIVITIES OF *MYCOBACTERIUM TUBERCULOSIS* PASSAGED IN MICE

Mice infected with *Mycobacterium tuberculosis* were treated daily with one-twentieth the subcutaneous LD50 of B.R.L. 459, B.R.L. 658, and streptomycin for twenty days. At the end of each passage the organism was isolated on Löwenstein and Jensen slopes from the mice and the *in vitro* sensitivity (minimum inhibitory concentration—μg./ml.) determined in Dubos medium. The virulence was determined by infecting groups of ten mice and estimating the TD50 in the usual manner.

Passage	1		3		5	
Compound	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days	*In vitro* Activity M.I.C. μg./ml.	Virulence TD50 Days
B.R.L. 459	0·78	19·0	1·56–3·13	18·0	12·5	12·0
Control ..	1·56	18·0	—	13·0	0·78	15·0
B.R.L. 658	0·78	22·0	6·25	20·0	3·13	14·0
Control ..	0·39	18·0	—	13·0	0·39	15·0
Streptomycin	0·78	—	0·39	14·0	0·2	19·0
Control ..	0·78	18·0	—	13·0	0·2	15·0

Resistance of the bacilli to B.R.L. 459 and B.R.L. 658 had developed markedly by the end of the fifth passage, while no change in the sensitivity to streptomycin was detectable. In every instance the virulence of all the isolated bacilli was fully maintained.

In vitro *Resistance.*—Resistance induced by *in vitro* transfer to B.R.L. 658 and B.R.L. 459 developed with exceptional rapidity (see Fig. 1). The minimal inhibitory concentration at the commencement of the test for B.R.L. 658 and B.R.L. 459 was 0.39 μg./ml., and within five passages this

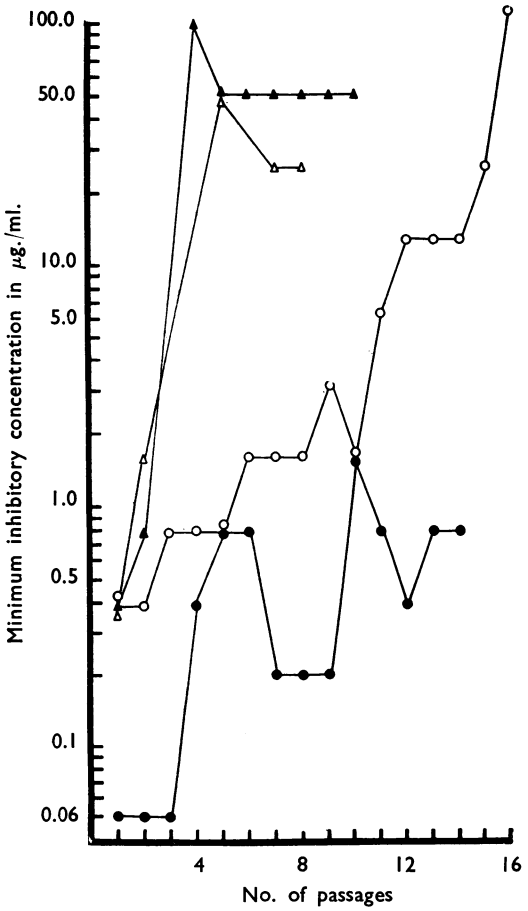

FIG. 1.—Development of resistance of *Mycobacterium tuberculosis* ($H_{37}Rv$) in Dubos medium to B.R.L. 459, B.R.L. 658, streptomycin, and isoniazid. ▲ B.R.L. 459. △ B.R.L. 658. O Streptomycin. ● Isoniazid.

rose to 50 to 100 μg./ml. for B.R.L. 459, and 25 to 50 μg./ml. for B.R.L. 658. In the control series resistance to streptomycin began to develop within twelve passages, the minimal inhibitory concentration rising from 0.39 to 6.25 μg./ml. With isoniazid resistance began to develop at the fourth passage, but did not increase to the degree shown by the propylene sulphides.

DISCUSSION

B.R.L. 459 and B.R.L. 658 are highly active antitubercular compounds when tested in mice infected with the $H_{37}Rv$ strain of *Mycobacterium tuberculosis*. B.R.L. 658 is the more active of the two compounds, comparing favourably with streptomycin by the TD50 test, and is of the same order of activity as isoniazid by the lung lesion test. B.R.L. 459 shows a corresponding difference in relative activity to streptomycin and isoniazid by the two methods. The difference in relative activity observed using the two methods is probably due to the compounds having a bacteriostatic and not bactericidal action *in vivo*. In the TD50 test administration of the compounds is stopped after 20 days when a generalized lethal infection develops. On the other hand, in the lung lesion test the mice are still under treatment when they are killed. The growth of the organism is well suppressed at this stage and the lungs are practically devoid of lesions. The lung lesion test therefore gives a more favourable impression of relative activity.

Resistance to the compounds develops exceptionally rapidly *in vitro* and also *in vivo*. They are also incapable of curing an established infection and are ineffective orally. When administered daily over a period of six months to guinea-pigs infected with *Mycobacterium tuberculosis* they give rise to ulcers of such severity that they render an assessment of antitubercular activity in guinea-pigs impracticable. In addition, the compounds are odorous, oily, water-insoluble liquids which are unstable in the presence of water and in mild alkaline conditions. In spite, therefore, of their initial highly promising activity in mice, their adverse physical and pharmacological properties would seem to rule out their possible clinical use.

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INTERACTION BETWEEN HISTAMINE AND DICHLOROISOPROTERENOL, HEXAMETHONIUM, PEMPIDINE, AND DIPHENHYDRAMINE, IN NORMAL AND RESERPINE-TREATED HEART PREPARATIONS

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Histamine stimulated the isolated auricles and heart of the guinea-pig. The effect was best seen in auricles which had been previously depressed by treatment with reserpine. Ganglionic blocking drugs (hexamethonium and pempidine), applied to auricles which had been previously treated with reserpine, abolished the diphasic effect of nicotine, but did not alter the response to histamine. Dichloroisoproterenol did not modify the stimulant action of histamine in isolated auricles, either before or after treatment with reserpine; nor did it alter the response of the isolated heart. Diphenhydramine reduced or blocked the stimulant action of histamine in auricles which had been previously treated with reserpine. The results support the hypothesis that histamine stimulates the myocardium by a direct action on specific receptors.

Histamine is known to stimulate the isolated heart of the rabbit and cat (Dale and Laidlaw, 1910; Gunn, 1926; Staub and Grossmann, 1930), of the guinea-pig (Went and Lissak, 1935; Penna, Illanes, Ubilla, and Mujca, 1959), and of the rat and frog (Went, Varga, Szucs, and Feher, 1952). It also stimulates isolated auricles of the rabbit (Dews and Graham, 1946) and initiates contractions in isolated strips of ox ventricle (Iwao, 1938).

The stimulant action of histamine on cardiac tissue may be direct on myocardial receptors or indirect through the local release of catecholamines (Truex, 1950; Kottegoda, 1953). Evidence suggesting a direct action was recently obtained by Giotti and Jngianna (1959) and Pepeu, Mannaioni, and Giotti (1959) in experiments with isolated auricles which had been previously stopped by treatment with reserpine. Histamine, adrenaline, and noradrenaline restored the activity of the auricles but nicotine and ephedrine did not, presumably because the auricles had been partially depleted of catecholamines (Giotti, 1954; Pepeu, Masi, and Giotti, 1959).

The introduction of 1-(3,4 dichlorophenyl)-2 isopropyl aminoethanol hydrochloride (dichloroisoproterenol) (Powell and Slater, 1958), a specific antagonist of the catecholamines on the heart (Moran and Perkins, 1958), provided an opportunity to re-investigate the problem of the site of action of histamine on the heart.

METHODS

Isolated guinea-pig auricles were suspended in 50 ml. oxygenated Tyrode solution at 29°, according to the method of Giotti (1954). The auricles were connected to an isotonic lever with magnification of 5; the load on the auricles was 1.5 g. Contractions were recorded on a smoked drum.

The isolated guinea-pig heart was prepared by the method of Langendorff (1895) and Porter (1898) and was suspended in a modified Langendorff apparatus. The perfusion pressure was kept constant at 40 cm. water. The *p*H of the Ringer-Locke solution was 8.2 and of the coronary effluent 7.5. The temperature was adjusted to 38°. The apex of the ventricles was connected to an isotonic lever, which wrote on a smoked drum. The heart rate was taken from a dipolar surface electrogram. The coronary outflow was followed with the recording system of Giotti and Beani (1957).

The drugs used were: (−)-adrenaline bitartrate and (−)-noradrenaline bitartrate (Recordati); nicotine as supplied by the Italian State Monopoly of Tobacco, and crystallized by us as the bitartrate;

TABLE I

EFFECT OF DICHLOROISOPROTERENOL ON THE RESPONSE OF ISOLATED GUINEA-PIG AURICLES TO STIMULANT DRUGS

The experiments were carried out on the same preparations. Dichloroisoproterenol (10 μg./ml.) was allowed to act for 30 min. Each value is the mean±S.D. of 6 experiments.

Treatment of Auricles	Drug	Dose μg./ml.	Contraction Maximum Height in mm.		Rate per min.	
			Before Dose	After Dose	Before Dose	After Dose
Nil	Adrenaline	1	37·3±6	49·6±11	87±5	98±5
Dichloroisoproterenol ..	,,	1	14·5±6	17·6±2	83±5	83±5
Nil	Noradrenaline	1	37·5±6	49·8±11	86±4	96±2
Dichloroisoproterenol ..	,,	1	15·1±5	16·1±4	83±4	83±4
Nil	Histamine	2	37·7±6	47·2±8	89±4	95±3
Dichloroisoproterenol ..	,,	2	13·5±7	30·6±7	82±4	88±5

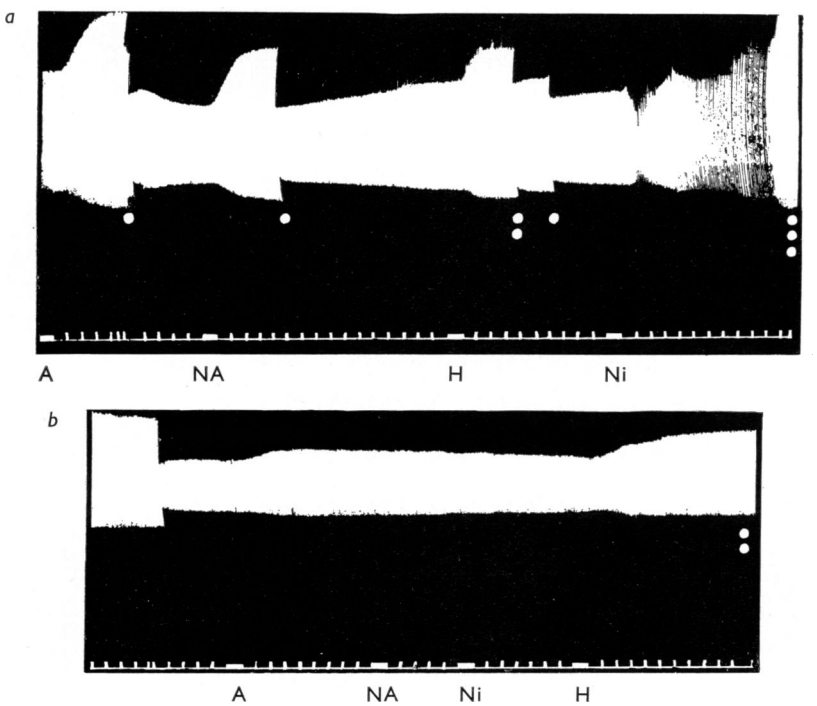

FIG. 1.—Dichloroisoproterenol 1×10^{-4} prevents the effects of the catecholamines and nicotine in guinea-pig isolated auricles; the effect of histamine is still present. (*a*) The effect of adrenaline 1×10^{-7} (*A*), noradrenaline 1×10^{-7} (*NA*), nicotine 5×10^{-5} (*Ni*), and histamine 1×10^{-6} (*H*). (*b*) The effect of the same substances 60 min. after treatment with dichloroisoproterenol. Upper tracing: height of contraction. Lower tracing: time, 10 sec. The washings are indicated by the white dots.

TABLE II

EFFECT OF RESERPINE AND DICHLOROISOPROTERENOL ON THE RESPONSE OF ISOLATED GUINEA-PIG AURICLES TO STIMULANT DRUGS

Reserpine (5 μg./ml.) was allowed to act for 5 hr.; dichloroisoproterenol (1 μg./ml.) for 10 min. before applying the stimulant drug. Experiments were carried out on the same preparations. Each value is the mean±S.D. of 6 experiments.

Treatment of Auricles	Drug	Dose μg./ml.	Contraction Maximum Height in mm.		Rate per min.	
			Before Dose	After Dose	Before Dose	After Dose
Reserpine	Adrenaline	1	2·4±2	39·8±6	19·2±19	75·8±16
,, +dichloroisoproterenol	,,	1	0·8±1	5±5	6·6±8	11·4±13
Reserpine	Histamine	2	3·2±4	22·4±9	14·4±15	70·2±11
,, +dichloroisoproterenol	,,	2	0·6±1	20·4±9	4·8±7	50·2±3

histamine dihydrochloride (Roche); reserpine was kindly supplied by Ciba Ltd., and used as the phosphate; pempidine (1,2,2,6,6,-pentamethylpiperidine) gratefully acknowedged as a gift from Dr. A. Banchetti, Istituto Gentili, Pisa; Benadryl (diphenhydramine) Parke Davis; dichloroisoproterenol kindly supplied by Dr. N. J. Giarman and Dr. G. Pepeu, of Yale University.

The drugs were dissolved in the appropriate physiological solution and concentrations (w/v) are expressed as the salt. Dose is quoted as the concentration of drug in the bath.

The stimulant drug was applied to the auricles in successive doses. When the response was maximal, the bath fluid was changed three times. The antagonist was then allowed to act on the auricles for a period of 30 to 60 min. before retesting the effect of the stimulant drugs. Reserpine, however, was left in contact with the auricles for 4 to 6 hr.

RESULTS

Effect of Histamine, Adrenaline, Noradrenaline, and Nicotine on Isolated Guinea-pig Auricles Before and After Treatment with Dichloroiso-

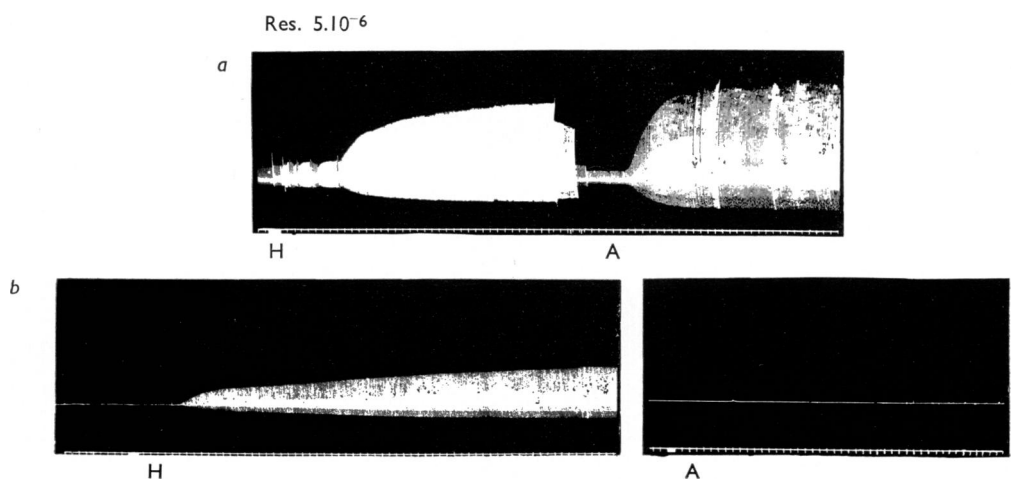

FIG. 2.—(*a*) Effects of histamine 1×10^{-6} and adrenaline 1×10^{-7} on guinea-pig auricles which had been previously treated with reserpine. (*b*) Effects of the same substances after treatment with dichloroisoproterenol 10^{-6} in addition to the previous treatment with reserpine. Upper tracing: height of contraction. Lower tracing: time, 10 sec.

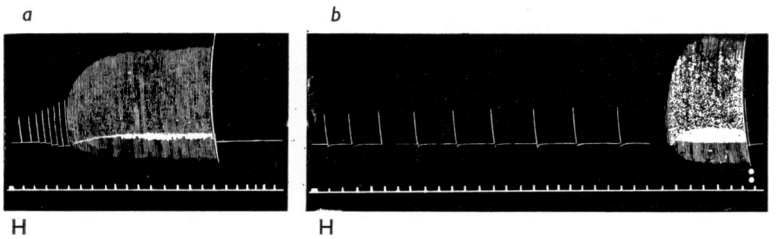

FIG. 3.—(*a*) Response of guinea-pig auricles, arrested by reserpine 5×10^{-6}, to histamine 1×10^{-6} (*H*). (*b*) Treatment with ganglionic-blocking agents (hexamethonium 1×10^{-4}) for 30 min. does not affect the histamine stimulation. Upper tracing: height of contraction. Lower tracing: time, 20 sec. The washings are indicated by the white dots.

proterenol (Table I, Fig. 1). — Dichloroisoproterenol (10 μg./ml.) abolished the stimulant effect of adrenaline (1 μg./ml.) and of noradrenaline (1 μg./ml.) on the rate, and greatly reduced the effect on the contraction, of the auricles. It also abolished the diphasic action (inhibition followed by stimulation) of nicotine (50 μg./ml.). Dichloroisoproterenol, however, did not abolish the response to histamine: the difference (17 mm.) between the mean for the contraction before the dose and the mean after the dose was significant ($P<0.05$).

Effect of Histamine and Adrenaline Before and After Dichloroisoproterenol on Isolated Guinea-pig Auricles Previously Treated with Reserpine (Table II, Fig. 2).—When dichloroisoproterenol (1 μg./ml.) was applied to auricles which had been previously treated with reserpine, the rate slowed and the contraction became smaller; in some experiments the auricles stopped. Under these conditions, the auricles

TABLE III

EFFECT OF ADRENALINE, NORADRENALINE, AND HISTAMINE ON THE ISOLATED HEART OF THE GUINEA-PIG, BEFORE AND AFTER TREATMENT WITH DICHLOROISOPROTERENOL

Experiments were carried out on the same preparations; dichloroisoproterenol was added to the perfusion fluid 30 min. before testing adrenaline, noradrenaline, and histamine. The values for the contraction are expressed as percentage increase of the height of the kymographic record. Each value is the mean±S.D. of 6 experiments.

Stimulant Drug	Dose μg.	Percentage Increase in Contraction			
		Dichloroisoproterenol			
		Dose (μg./ml.): 0	1	5	10
Adrenaline	0·1	135±30	0		
		115±27		0	
		103±20			0
Noradrenaline ..	0·1	129±6	0		
		94±35		0	
		108±16			0
Histamine	0·1	25±13	20±15		
		18±7		26±23	
		35±4			6±11
	0·5	46±14	57±16		
		41±14		67±22	
		77±40			71±42
	1·0	145±51	91±25		
		103±36		121±23	
		127±31			137±79

responded only slightly, or not at all, to adrenaline, but still retained their sensitivity to histamine. Thus, the mean values for the effect of adrenaline on the contraction (5.0 ± 5 mm.) and on the rate (11.4 ± 12 per min.), after the combined treatment, differed significantly ($P<0.05$) from the corresponding mean values (39.8 ± 6 mm. and 75.8 ± 16 per min.) obtained after treatment with reserpine alone. Dichloroisoproterenol (1 μg./ml.) did not reduce the effect of histamine on the contraction in the same auricles, but appeared slightly to depress its stimulant effect on the rate.

Effects of Histamine Before and After Diphenhydramine and Pempidine on Isolated Guinea-pig Auricles Previously Treated with Reserpine (Figs. 3 and 4).—When ganglionic-blocking agents (hexamethonium 100 μg./ml.; pempidine 0.1 μg./ml.) were allowed to act for 30 min. on auricles which had been previously treated with reserpine, the response to histamine was not affected. When they were allowed to act for 90 min., the stimulant action of histamine on the auricles was no longer obtained. Diphenhydramine (5 μg./ml.), applied to auricles which had been previously treated with reserpine (5 μg./ml. for 5 hr.), reduced or abolished the stimulant effect of histamine on the rate and force of contraction, but did not affect the response of the auricles to adrenaline.

Effects of Histamine, Adrenaline, Noradrenaline, and Nicotine on the Isolated Heart of the Guinea-pig, Before and After Treatment with Dichloroisoproterenol (Table III, Fig. 5).—Dichloroisoproterenol acting in a concentration of 1 μg./ml. was without effect on the heart. Concentrations of 5 to 10 μg./ml. reduced the contraction to $59.8\% \pm 11$, and the rate to $95.3\% \pm 4$, of the original values.

The stimulant effects of adrenaline and noradrenaline and the diphasic effect of nicotine all disappeared in hearts treated with dichloroisoproterenol in concentrations of 1 to 10 μg./ml. Within this range of concentrations, dichloroisoproterenol did not depress the response to small doses of histamine.

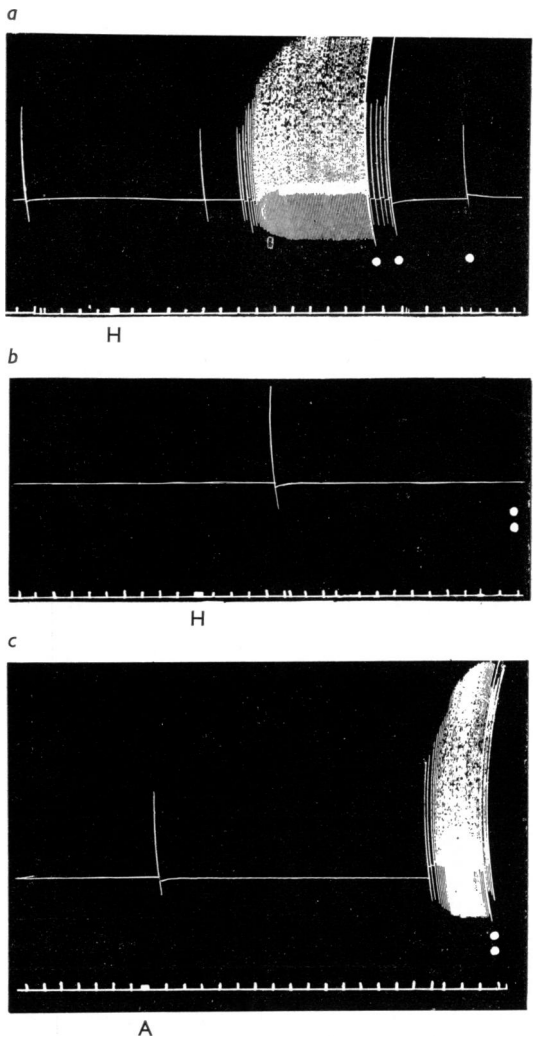

FIG. 4.—Diphenhydramine 5×10^{-6} prevents the response to histamine of guinea-pig auricles previously treated with reserpine. (*a*) Response to histamine 1×10^{-6} (*H*) on guinea-pig auricles previously treated with reserpine 5×10^{-6}. (*b*) Histamine 1×10^{-6} (*H*) 1 hr. after treatment with diphenhydramine 5×10^{-6}. (*c*) Adrenaline 2×10^{-7} (*A*) 90 min. after the treatment with diphenhydramine 5×10^{-6}. Upper tracing: height of contraction. Lower tracing: time, 20 sec. The washings are indicated by the white dots.

DISCUSSION

The results obtained in the present experiments suggest that histamine stimulates isolated cardiac tissue directly, and not through the release of catecholamines. They show that the stimulant action of histamine persisted in auricles which were first treated with reserpine, and then rendered insensitive to adrenaline and noradrenaline by suitable doses of dichloroisoproterenol. Went *et al.* (1952) reported that ergotamine abolished the stimulant effect of histamine on the isolated mammalian heart, and

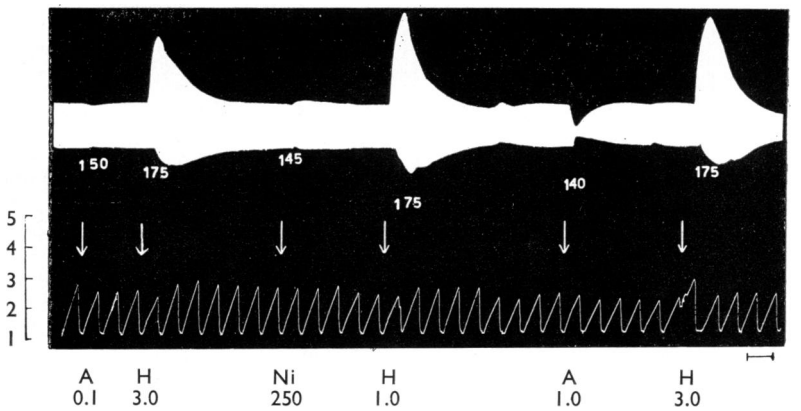

FIG. 5.—Dichloroisoproterenol 1×10^{-6} inhibits the effect of the adrenaline (*A*) and nicotine (*Ni*) in isolated guinea-pig heart, while histamine (*H*) stimulation is still present. Upper tracing: height of contraction. Lower tracing: flow of the perfusion fluid. The numbers indicate heart rate per min. Time, 2 min.

took this as indirect evidence for the release of adrenaline. However, not only is the anti-adrenaline action of ergotamine and related substances on the isolated mammalian heart open to some doubt (Nickerson, 1949), but the results of the present work fail to support the conclusion that histamine acts through the release of adrenaline.

It is unlikely that histamine exerted an effect on ganglia since its action was unaffected by ganglionic blocking agents which were applied in concentrations that abolished the diphasic action of nicotine. The action of ganglionic blocking drugs in abolishing the effect of histamine, after prolonged contact with the preparations, has been discussed elsewhere (Pepeu, Mannaioni, and Giotti, 1958, 1959). The fact that anti-histaminic drugs antagonized the stimulant effect of histamine without modifying the action of adrenaline and noradrenaline may be taken as further evidence for the existence of specific receptors for histamine in cardiac muscle. These findings, however, do not exclude the possibility that histamine acts through the release of stimulating substances which are not antagonized by dichloroisoproterenol. Went *et al.* (1954) have reported that when isolated perfused hearts are stimulated with histamine, substances which inhibit plain muscle appear in the perfusate. These substances have not yet been identified. The appearance of catecholamines in the perfusate during stimulation by histamine would suggest, in the light of the present findings, that their formation or release was not the cause, but the result, of the direct stimulant action of histamine on the heart.

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NEUROMUSCULAR BLOCKADE BY STREPTOMYCIN AND DIHYDROSTREPTOMYCIN

BY

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Both streptomycin and dihydrostreptomycin in large doses produced neuromuscular blockade in different types of skeletal nerve-muscle preparations. Streptomycin had a quicker, more marked and a longer-lasting action than dihydrostreptomycin. Neostigmine reversed a partial block of streptomycin, but a complete neuromuscular block remained unaffected. Calcium ions antagonized the neuromuscular block of streptomycin rapidly but incompletely.

Both streptomycin and dihydrostreptomycin may cause a number of untoward phenomena, particularly injury to the nervous system and hypersensitivity reactions. The main toxic side-effect in the chronic use of streptomycin is damage to the vestibular mechanisms and the eighth cranial nerve, which was first described in man by Hinshaw and Feldman (1945) and in experimental animals by Molitor and his collaborators (1946, 1950). The latter authors also showed that streptomycin in high dosage caused death in experimental animals as a result of respiratory failure. Brazil and Corrado (1957) studied the motor effects of streptomycin in dogs and in pigeons, and they found that streptomycin possesses neuromuscular blocking properties which are similar to those produced by magnesium. Calcium and neostigmine suppressed the motor effects of acute streptomycin intoxication. Brazil and Corrado (1957) employed a very high dose (110 mg./kg.) to demonstrate the neuromuscular blocking effect of streptomycin in dogs, and the effect was not compared with that of dihydrostreptomycin, which is known to differ from streptomycin in toxicity in certain respects. Recently Loder and Walker (1959) have reported 3 cases of muscular paralysis in patients who were kept on streptomycin treatment for a period varying from 3 days to 3 months with usual dosage. It was therefore thought necessary to investigate the pharmacological actions of streptomycin on various types of skeletal muscles and compare its effects with those of dihydrostreptomycin. The results of such a study are reported in this paper.

METHODS

Streptomycin and dihydrostreptomycin were purchased locally of " Hindustan Antibiotics " make; the purity was stated by the manufacturers to conform to the standards prescribed by the *British Pharmacopoeia.*

Rat Phrenic Nerve-Diaphragm Preparation.—This was prepared as described by Bülbring (1946). Ten adult rats were used. The isolated tissues were suspended in a 60 ml. bath, containing Tyrode solution with double the amount of dextrose. Single shocks at rates not exceeding 8/min. were applied to the nerve from a square wave stimulator, at 10 to 12 volts and 0.5 to 1.0 msec. duration. Drugs were added directly to the bath through a very fine polythene tube attached to a hypodermic needle.

Dog Sciatic-Gastrocnemius Preparation.—Experiments were performed in 10 dogs anaesthetized with sodium pentobarbitone (35 mg./kg.) injected intraperitoneally. One leg was immobilized and the sciatic nerve was exposed between the hamstring muscles and sectioned. The peripheral stump of this nerve was placed on a non-polarizing bipolar electrode. The contractions of the gastrocnemius muscle were recorded with an isometric lever. The muscle was stimulated through the sciatic nerve by a square wave stimulator at a rate of 8/min. at 10 to 12 volts and 0.5 to 1.0 msec. duration. Retrograde injections of drugs were made through the cannulated common iliac artery.

Frog Rectus Abdominis Muscle Preparation.—Frogs were pithed and the rectus abdominis muscle was dissected and suspended in a 10 ml. bath containing oxygenated frog-Ringer solution at room temperature. The contractions produced by acetylcholine chloride (0.10 to 1.0 μg./ml.), when added to the bath and left in contact with the tissue for 90 sec., were recorded. Streptomycin and dihydrostreptomycin were added to the bath 30 sec. before a subsequent addition of acetylcholine and the contractions again recorded for 90 sec. The bath was then washed out. Between successive additions of acetylcholine there was at least an interval of 8 min., or until the muscle recovered from the effect of the streptomycins.

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RESULTS

Effect on Rat Diaphragm Preparations. — Streptomycin and dihydrostreptomycin were tested in concentrations ranging from 10 μg. to 1.5 mg./ml. Neither substance affected the contractions in concentrations up to 200 μg./ml. However, at 0.25 mg./ml. streptomycin sulphate produced a mean reduction of 44%, and dihydrostreptomycin at the same dose produced a mean reduction of 19%. Higher concentrations produced proportionately greater effects, a maximum of 94% reduction with streptomycin 1.5 mg./ml., and of 71% with the same dose of dihydrostreptomycin (Table I). It was observed that there was a short latent period in the onset of action with dihydrostreptomycin which was not seen with streptomycin. Recovery of the diaphragmatic contractions was complete after washing out the drug, the recovery time varying according to the dose. Higher concentrations took a longer time for the complete recovery of the muscle. Muscle treated with dihydrostreptomycin took a shorter time for recovery in comparison with streptomycin. The response to direct excitation of the rat diaphragm did not alter in any of the experiments.

TABLE I

THE MEAN REDUCTION IN MUSCULAR CONTRACTIONS PRODUCED BY STREPTOMYCIN AND DIHYDROSTREPTOMYCIN IN THREE DIFFERENT PREPARATIONS

Muscle Preparation	Dose	% Reduction in Contraction	
		Streptomycin	Dihydrostreptomycin
	mg./ml.		
Rat phrenic nerve-diaphragm	0·25	44	19
	0·5	57	52
	1·0	94	71
	mg./kg.		
Dog gastrocnemius-sciatic nerve	10	20	13
	20	83	16
	40	100	24
	mg./ml.		
Frog rectus abdominis	5	37	11
	10	40	27
	20	46	38

Effect on Dog Gastrocnemius Sciatic Preparation. — Streptomycin and dihydrostreptomycin were injected intravenously in doses ranging from 1 mg. to 40 mg./kg. body weight. Doses below 8 mg./kg. did not affect the contractions of the gastrocnemius, whereas higher doses produced graded reductions in the amplitude of the contractions (Table I and Fig. 1*a*). The effect of dihydrostreptomycin was not only slower to develop but the overall depression in muscular contraction was much less (Table I and Fig. 1*b*). As in the rat diaphragm, recovery was quicker with dihydrostreptomycin.

Neostigmine given intravenously in 1.5 mg. doses antagonized the effect of a partial neuromuscular block of streptomycin (Fig. 2*a*), but in the presence of total neuromuscular block neostigmine failed to restore the contractions (Fig. 2*b*). Calcium chloride, 0.5 mg./kg., on the other hand, antagonized the neuromuscular block of streptomycin quickly, but not completely (Fig. 2*b*).

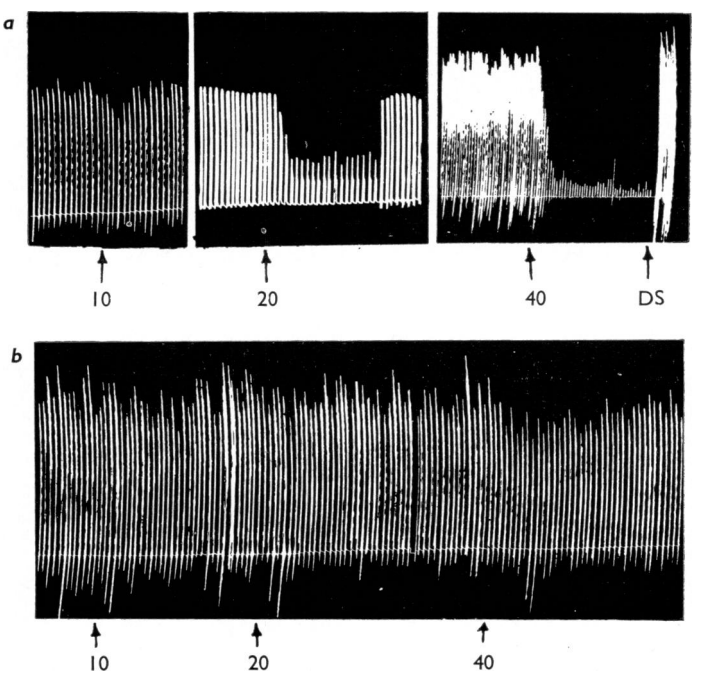

FIG. 1.—Dog gastrocnemius-sciatic nerve preparation. Contractions induced by indirect stimulation of the sciatic nerve or by direct stimulation of the muscle. In (*a*) effect of streptomycin 10, 20, and 40 mg./kg. and of direct stimulation (DS). In (*b*) effect of dihydrostreptomycin 10, 20, and 40 mg./kg.

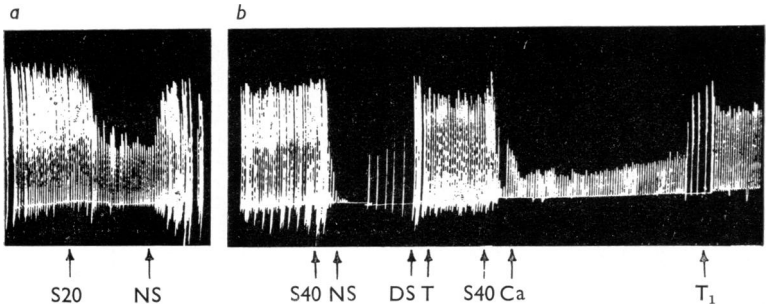

FIG. 2.—Dog gastrocnemius-sciatic nerve preparation. Contractions induced by indirect stimulation of the sciatic nerve or by direct stimulation of the muscle. Effect of streptomycin and antagonists. At S20, 20 mg./kg. streptomycin. NS, neostigmine 0.15 mg./kg. S40, 40 mg./kg. streptomycin. DS, direct stimulation. T, time of recovery—30 min. after first injection of streptomycin. T_1, time of recovery—25 min. after second injection of streptomycin. Ca, calcium chloride 10%—0.5 ml./kg.

Effect on Frog Rectus-Abdominis Muscle Preparation.—The effects of streptomycin and dihydrostreptomycin were tested on the acetylcholine-induced contractions of the rectus abdominis muscle. Graded doses of streptomycin and dihydrostreptomycin were given ranging from 1 mg. to 20 mg./ml. Doses from 5 to 20 mg./ml. inhibited the contractions of the rectus muscle to a varying degree, and the effect was proportional to the concentration of drug in the bath. Streptomycin had a greater effect than dihydrostreptomycin; 20 mg./ml. of streptomycin depressed the muscle so as to prevent its recovery for as long as 2 hr. even after repeated washings of the muscle. In contrast to this, recovery of the muscle was complete in the case of dihydrostreptomycin after a period of 1 hr. (Fig. 3*a* and *b*).

DISCUSSION

It has been clearly demonstrated that streptomycin can cause muscular paralysis in high doses, thus confirming the observations of Brazil and Corrado (1957). The muscular paralysis is much more marked with streptomycin than dihydrostreptomycin when equal doses are used. The onset of action is more rapid with streptomycin and the recovery time of muscle is definitely shorter with dihydrostreptomycin. The muscular paralysis seems to be due to neuromuscular blockade produced by streptomycin, since the direct excitability of the different muscle preparations was never found to be decreased in any of the experiments. Neuromuscular blockade was demonstrated in three different types of skeletal muscle preparations, which included mammalian as well as amphibian skeletal muscles. This lends support to the view that all types of muscles are susceptible to the neuromuscular blocking action of streptomycin in high doses. In contrast to Brazil and Corrado (1957), who studied only one type of skeletal muscle preparation (the sciatic-tibialis anticus of the dog) and used a high dose (110 mg./kg.), in these experiments we found that a dose of 40 mg./kg. produced complete paralysis and smaller doses had proportionately a smaller effect. It is therefore not unlikely that streptomycin in ordinary therapeutic doses may be capable of producing neuromuscular blockade in certain isolated cases, as has been observed by Loder and Walker (1959). The neuromuscular blockade with streptomycin can possibly occur as

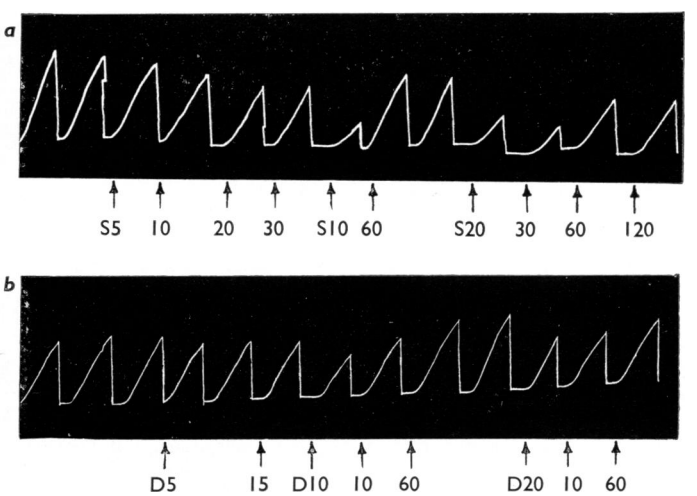

FIG. 3.—Frog rectus abdominis suspended in a 10 ml. bath containing oxygenated frog-Ringer solution at room temperature. Contractions to acetylcholine (1.0 μg./ml.). In (*a*) effects of streptomycin and in (*b*) effects of dihydrostreptomycin. Numbers indicate time intervals in min. Acetylcholine was left in the bath for 90 sec. and then washed out. At S5, 5; S10, 10; and S20, 20 mg./ml. streptomycin. At D5, 5; D10, 10; and D20, 20 mg./ml. dihydrostreptomycin.

a result of cumulative action of the drug which is usually administered over a prolonged period.

Neostigmine antagonizes the effect of streptomycin only when there is a partial neuromuscular block. In the presence of complete neuromuscular block the effect of neostigmine is negligible. Calcium ions can antagonize quickly but less completely the effect of streptomycin on skeletal muscles. Similar results have been reported by Corrado, Ramos, and De Escobar (1959) for the neuromuscular blocking action of neomycin. It is thus likely that the neuromuscular blocking actions of neomycin and streptomycin involve a common mechanism of action.

Another interesting feature of the present investigation is the difference in action of streptomycin and dihydrostreptomycin. Clinically streptomycin differs from dihydrostreptomycin regarding the injury it is likely to cause to the nervous system in general and damage to the eighth cranial nerve in particular. We have found that neuromuscular blockade produced by dihydrostreptomycin is not only slow to develop, but the depression of muscular contraction is less and the time for recovery of the muscles is quicker, as compared with streptomycin.

The present experiments have thus not only confirmed the clinical case reports of Loder and Walker (1959), but also suggest that the danger of neuromuscular blocking action of streptomycin would be greatly diminished by using dihydrostreptomycin. On the other hand, dihydrostreptomycin may cause a greater incidence of auditory damage than streptomycin (Crofton, 1960). The antagonists to the toxicity on skeletal muscles resulting from the use of streptomycin, as suggested from these observations, should be both neostigmine and calcium. Simultaneous administration of calcium throughout streptomycin therapy is suggested as a prophylactic measure against the toxicity of streptomycin on skeletal muscles.

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THE EFFECT OF LEVALLORPHAN TARTRATE AND OF ADIPHENINE HYDROCHLORIDE ON THE ANTIDIURETIC ACTION OF MORPHINE AND NICOTINE

BY

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Levallorphan antagonizes the antidiuretic effect of morphine. It is at least 25 times as potent as nalorphine in this respect. However, neither levallorphan nor adiphenine antagonized the antidiuretic action of nicotine.

Morphine and nicotine are both known to inhibit the diuretic response to water, an action mediated through the hypothalamic neurohypophysial system (De Bodo, 1944; Burn, Truelove, and Burn, 1945; Bisset and Walker, 1957). Nalorphine has been shown to antagonize the antidiuretic effect of one of these compounds—namely, morphine (Winter, Gaffney, and Flataker, 1954; Schnieden and Blackmore, 1955) and levallorphan is known to be a very potent antagonist of certain of the actions of morphine (Fromherz and Pellmont, 1952). It therefore seemed of interest to see if this latter compound antagonized the antidiuretic effects of both morphine and nicotine. In addition, adiphenine hydrochloride ("Trasentin") has also been reported to have certain antinicotinic actions (Tripod, 1949), and this compound was therefore also tested to see if it would antagonize the antidiuretic effects of nicotine.

METHODS

Adult albino rats weighing approximately 250 g. were used. The effect of drugs on the diuretic response to water was tested by the method of Schnieden and Blackmore (1955) on groups of 16 animals. Initially the animals in the group were randomly allocated to one of the drug treatments, and cross-over experiments were then carried out at weekly intervals until all 16 animals in the group had received all treatments. All drugs except adiphenine in a dose of 31 mg./kg. (Table II) were given subcutaneously 45 min. after the second dose of water was administered. The water load remaining at this time was considered the initial water load. For the next 90 min. urine volumes were noted at 30 min. intervals. The following drugs were injected: nicotine hydrogen tartrate 7.5 mg./kg., morphine sulphate 10 mg./kg., nalorphine hydrobromide 25 mg./kg., levallorphan tartrate 0.1 or 1 mg./kg., adiphenine hydrochloride 21 or 31 mg./kg. Control groups received corresponding injections of 0.9% sodium chloride solution. In all instances except one (Table II) the antidiuretic drug and its possible antagonist were injected simultaneously into different subcutaneous sites. Analgesic effect in rats was estimated by the time required from exposure to a standard thermal stimulus until the rat moved its tail (Davies, Raventós, and Walpole, 1946). To avoid injury to the animal's tail exposure was limited to 15 sec.; failure of the animals to react in this time was considered to indicate "complete analgesia."

RESULTS

Levallorphan and nalorphine are capable of antagonizing the inhibition of the renal response to a water load produced by morphine as illustrated in Table I. This Table also shows that 1 mg./kg. levallorphan produces approximately the same degree of antagonism as 25 mg./kg. nalorphine. Neither levallorphan nor nalorphine had an antidiuretic effect in rats in the doses used; when comparing the effect of 0.9% sodium chloride solution with nalorphine, $t = 1.7$ $P<0.1>0.05$.

Neither adiphenine nor levallorphan antagonized the antidiuretic effect of nicotine, and adiphenine had no significant effect on the renal response to a water load (Table II); when comparing the effect of 0.9% sodium chloride solution with adiphenine, $t = 1.9$ $P<0.1>0.05$. However, adiphenine in a dose of 21 mg./kg. enhanced the antidiuretic effect of nicotine; when comparing nicotine with nicotine plus adiphenine, $t = 2.4$ $P<0.05>0.01$.

TABLE I

THE EFFECT OF NALORPHINE AND LEVALLORPHAN ON THE ANTIDIURETIC ACTION OF MORPHINE

Figures represent percentage of initial water load excreted during the 90 min. period after injection of the drugs (mean±S.E. for 16 animals)

Saline	Nalorphine	Levallorphan	Morphine	Morphine/ Nalorphine	Morphine/ Levallorphan	Dose (per kg. Body Wt.) Levallorphan
91·7± 9·3	73·3±5·4	—	5·3±2·1	69·7±6·2	—	—
123·7±17·6	—	109·5±13·2	18·0±2·9	—	48·6±17·6	0·1 mg.
109·2±11·2	—	107·6±19·2	4·8±1·2	—	65·0±10·6	1·0 ,,

TABLE II

THE EFFECT OF LEVALLORPHAN AND ADIPHENINE ON THE ANTIDIURETIC ACTION OF NICOTINE

Figures represent percentage of initial water load excreted during the 90 min. period after injection of the drugs (mean±S.E. for 16 animals). Adiphenine 31 mg. was given 45 min. before nicotine.

Saline	Nicotine	Nicotine/ Adiphenine	Nicotine/ Levallorphan	Adiphenine	Dose (per kg. Body Wt.)
88·6±8·6	34·7±6·4	—	41·9±7·5	—	Levallorphan 1 mg.
67·4±4·9	19·3±3·4	8·7±2·4	—	56·2±3·2	Adiphenine 21 ,,
70·8±8·6	24·3±9·8	24·4±8·7	—	57·4±9·2	,, 31 ,,

TABLE III

EFFECT OF LEVALLORPHAN ON MORPHINE ANALGESIA

The number of animals which showed "complete analgesia" (>15 sec.) to a standard thermal stimulus.

Drug	No. of Animals Tested	Time after Injection	
		40 min.	70 min.
Morphine 10 mg./kg.	16	13	12
Morphine 10 mg./kg./ levallorphan 0·1 mg./kg.	16	10	11
Morphine 10 mg./kg.	8	8	8
Morphine 10 mg./kg./ levallorphan 1 mg./ kg.	8	0	0

Increasing the dose of levallorphan not only antagonized the antidiuresis of the morphine-treated animals but also decreased the analgesic effect of morphine. Levallorphan 0.1 mg./kg. had no appreciable effect in antagonizing the analgesic action of morphine; in doses of 1 mg./kg., however, its effect was more pronounced (Table III).

DISCUSSION

Morphine and nicotine both stimulate neurohypophysial secretion (De Bodo, 1944; Giarman, Mattie, and Stephenson, 1953; Duke, Pickford, and Watt, 1951; Bisset and Walker, 1957) presumably by hypothalamic stimulation. It appears likely from the work of Pickford (1939, 1947, 1953) that the pathway for the antidiuretic response involves a synapse at the supraoptic nucleus. Bisset and Walker (1957) have suggested that such synapses differ from synapses at autonomic ganglia. They noted that hexamethonium in doses sufficient to block the pressor and convulsant actions of nicotine had no effect on its antidiuretic effect. Adiphenine has been shown by Tripod (1949) to have marked antinicotinic and anti-acetylcholine effects. Like hexamethonium, it can also inhibit the convulsant actions of nicotine (Tripod, 1949) but not its antidiuretic effects.

Pickford (1939) noted that acetylcholine injected intravenously inhibited a water diuresis but that

pretreatment with atropine did not prevent this inhibition. More recently Supek and Eisen (1953) have shown that the antidiuretic effect of nicotine also is not blocked by atropine. Since adiphenine has an atropine-like action the present findings are consistent with those of Supek and Eisen (1953). These workers also noted that other atropine-like compounds and ganglionic blocking compounds such as pentamethonium, parpanit, and diparcol were also without effect.

An injection of morphine into the supraoptic nucleus has been shown to inhibit water diuresis. Since levallorphan is a competitive antagonist of morphine it presumably acts by competing with it for tissue receptors. Beckett, Casy, Harper, and Phillips (1956) are of the opinion that the stereochemical structure of these antagonists are important in determining the goodness of fit of such compounds for the analgesic receptor site. It is likely that nicotine acts in a different way from morphine, since Pickford (1953) showed that the injection of dyflos into the supraoptic nucleus abolished the antidiuretic action of acetylcholine (which presumably has an action similar to nicotine) but not that of morphine—hence the inability of levallorphan to antagonize nicotine might be expected.

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THE EFFECT OF DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE SMALL INTESTINE AND OTHER ORGANS IN RATS AND MICE

BY

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(RECEIVED MAY 31, 1960)

In rats, a diet of meat or liver caused a rise in the amount of 5-hydroxytryptamine in the small intestine, an increase in the weight of the intestine and a 10-fold increase in anaerobic bacteria in the rectum. A diet containing added tryptophan did not affect tissue levels of 5-hydroxytryptamine in rats, but in mice there was a rise of 5-hydroxytryptamine in various tissues. The significance of these findings is discussed together with the possible role of intestinal bacteria in mediating the effects caused by a meat diet. No change was observed in the 5-hydroxytryptamine content of brain or in the distribution of argentaffin cells in the jejunum as a result of these diets.

Cordier (1926) reported that the argentaffin cells in the intestine of mice were increased in number 5 to 10 times and showed increased granularity when the animals received a diet exclusively of raw meat for 7 to 10 weeks. Since argentaffin cells are believed to secrete 5-hydroxytryptamine (Erspamer, 1954 ; Benditt and Wong, 1957), Stacey and Sullivan (1957) studied the effect of a diet of raw meat on the 5-hydroxytryptamine content of the small intestine of rats, and reported that it was raised. They also found that the 5-hydroxytryptamine content of the small intestine in mice was raised when tryptophan was added to the diet, and concluded that the high tryptophan intake in an exclusively meat diet might account for the rise of intestinal 5-hydroxytryptamine. The present paper reports further studies on the effect of protein feeding on 5-hydroxytryptamine metabolism in rats and mice.

METHODS

Groups of adult male rats weighing 120 to 200 g. or mice weighing 20 to 25 g. were matched according to age and weight. Controls received a mixed balanced diet supplied by J. Murray and Sons, London, S.E.8, in cube or powder form, and treated groups received the experimental diet. Both groups were given unrestricted water and a vitamin supplement (Abidec drops ; Parke, Davis and Co.) in their drinking water twice weekly. Both groups remained healthy, although differences in weight between control and treated groups sometimes developed during treatment.

Aliquots of a homogenate of the whole small intestine from the pyloric sphincter to the ileo-caecal junction of mice were assayed. Since the whole rat small intestine is bulky, tough and difficult to homogenize completely, the mucous membrane, which contains all the 5-hydroxytryptamine (Feldberg and Toh, 1953), was scraped off with a scalpel and homogenized ; aliquots were then assayed.

The 5-hydroxytryptamine content of the small intestine and spleen was usually assayed fluorimetrically by the methods of Udenfriend, Weissbach and Clark (1955) or Weissbach, Waalkes, and Udenfriend (1958). Sometimes, as indicated in the text, 5-hydroxytryptamine was assayed biologically. Fluorescence was measure in 3N hydrochloric acid, in which tryptamine and tryptophan do not fluoresce (Udenfriend, Bogdanski, and Weissbach, 1955). For bioassay, 5-hydroxytryptamine was extracted with 95% acetone and assayed on the atropinized oestrous rat uterus by the superfusion technique (Gaddum, 1953). Brain 5-hydroxytryptamine was assayed by the method of Bogdanski, Pletscher, Brodie, and Udenfriend (1956).

Since the treated animals lost weight during the experiment, results expressed on the basis of tissue weight or final body weight are fallacious. However, as body weights at the beginning of the experiment were similar for the two groups, the concentrations of 5-hydroxytryptamine are expressed in μg./kg. initial body weight except where stated otherwise.

Some variation will be seen between the results obtained for control groups because of differences between them in the initial body weight. There was closer agreement when the results were expressed as μg./g. tissue.

For estimations of the 5-hydroxytryptamine content of platelets, rats were lightly anaesthetized with ether, and bled from the carotid artery either through a piece of polythene tubing not more than 2.5 cm. long or a siliconed glass cannula. Mice were decapitated after light ether anaesthesia and the blood collected via a siliconed glass funnel into a siliconed glass tube containing 1 ml. of disodium edetate solution (1.0 g./100 ml. 0.7% sodium chloride). The volume of blood obtained was about 1 ml. from mice and 6 ml. from rats. Platelet-rich plasma was prepared by the method of Hardisty and Stacey (1955), except that the blood was centrifuged for 10 min. at 160 *g*. The relatively high proportion of sodium edetate present did not interfere with the fluorimetric estimation since it neither fluoresced nor caused quenching of the fluorescence due to 5-hydroxytryptamine.

Urinary excretion of 5-hydroxyindoleacetic acid was estimated on the pooled urine of groups of rats by the method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus, and Smith (1956) after collecting urine from the rats in metabolism cages for 16 to 18 hr. overnight. For estimation of the percentage recovery of injected 5-hydroxytryptamine as 5-hydroxyindoleacetic acid, rats were injected subcutaneously with 6 mg./kg. 5-hydroxytryptamine as the creatinine sulphate after which urine was collected for 16 to 18 hr. overnight. Controls received 0.5 ml. saline subcutaneously. Urinary creatinine was estimated by the microchemical method of Folin (1914).

For observation of the rate at which 5-hydroxytryptamine was lost from the small intestine, consecutive pieces of jejunum starting from the duodenal junction, each 1.8 to 2.5 cm. long, were incubated for 0.5, 1.0, 1.5, and 2.0 hr. in Krebs Ringer phosphate solution at 37° with gentle shaking. The pieces were then washed in fresh, ice-cold Krebs solution and assayed for 5-hydroxytryptamine.

In some experiments rectal swabs were taken from animals on the day they were killed. They were plated on blood agar and MacConkey's media. The former plates were incubated aerobically and the MacConkey's plates anaerobically in a McIntosh and Fildes' jar. After 24 hr. the colonies were counted.

Sections of small intestine taken for histology of the argentaffin cells were fixed in Bouin's fluid, or in 10% formol, and embedded in paraffin. The sections were stained with Masson's silver stain or by the activated Protargol method (Schofield, 1951).

Results

Experiments with Meat Diet

Rats.—Rats were fed on a diet of raw lean beef for either 10 to 23 days or 60 to 67 days before 5-hydroxytryptamine was assayed fluorimetrically. Table I shows the results of these experiments. There was a significant rise of intestinal 5-hydroxytryptamine in the meat-fed rats after both periods of treatment. The 5-hydroxytryptamine content of spleen, platelets and brain was not affected after 10 to 23 days of treatment, however.

These results were confirmed when the intestinal 5-hydroxytryptamine content of groups of rats was assayed biologically after 20, 43 and 76 days on a meat diet. At the end of each of these periods of treatment the 5-hydroxytryptamine level was higher in treated than in control rats. The overall mean rise in 7 treated rats was 102%, but since the numbers of animals in the 3 experiments were not strictly comparable, the overall results have not been statistically analysed.

Urinary excretion of 5-hydroxyindoleacetic acid was estimated at various times during these

TABLE I

THE EFFECT OF A MEAT DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OF RATS IN 2 EXPERIMENTS OF DIFFERENT DURATION

Mean results are expressed as μg. 5-hydroxytryptamine/kg. body weight ± standard error, except for platelets, where results are expressed as μg. 5-hydroxytryptamine/ml. blood. n.s.=difference not significant ($P>0.05$).

Length of Treatment (days)	No. of Rats		5-Hydroxytryptamine Content			Tryptophan Intake mg./kg./day	
10–23	Controls	Treated	Controls	Treated	Significance	Controls	Treated
Small intestine	16	16	67·0 ±3·65	89·0 ±3·78	$P<0.001$		
Brain	10	10	6·0 ±0·55	5·1 ±0·37	n.s.		
Spleen	16	15	26·3 ±3·26	27·4 ±5·3	n.s.	116	808
Platelets	4	5	1·42±0·09	1·67±0·14	n.s.		
60–67							
Small intestine	5	5	106·0 ±8·68	157·0 ±14·9	$P<0.02$		

experiments. Four estimations were carried out during the first 10 days of treatment and showed no difference between the groups; the mean excretion for 6 control rats was 234 ± 30 μg./kg./24 hr., and for 6 meat-fed rats 239 ± 26 μg./kg./24 hr. Since it was possible that there might be a time lag before changes in 5-hydroxyindoleacetic acid excretion occurred, estimations were carried out after further periods of meat feeding. The results are shown in Table II. Although the excretion of 5-hydroxyindoleacetic acid by meat-fed rats was lower throughout, excretion by both groups varied considerably. Excretion of creatinine, though lower in meat-fed rats, was less variable.

TABLE II

URINARY EXCRETION OF 5-HYDROXY-INDOLEACETIC ACID AND CREATININE BY 6 CONTROL AND 6 MEAT-FED RATS AT VARIOUS TIMES DURING TREATMENT

Duration of Treatment (days)	Group	5-Hydroxy-indole-acetic Acid μg./kg./24 hr.	Creatinine mg./kg./24 hr.	5-Hydroxy-indole-acetic Acid/ Creatinine Ratio
11	Control	334	34	10·0
	Meat-fed	283	28	10·1
17	Control	367	29	12·9
	Meat-fed	247	21	12·1
51	Control	214	29	7·5
	Meat-fed	166	26	6·5
80	Control	187	30	6·2
	Meat-fed	134	26	5·3
169	Control	166	32	5·2
	Meat-fed	122	29	4·2

To investigate whether control and meat-fed rats metabolized injected 5-hydroxytryptamine differently, the percentage recovery of injected 5-hydroxytryptamine as urinary 5-hydroxyindoleacetic acid was studied. From 9 control rats the recovery was 28.5%, and from 9 rats treated for 20 days 30.5%. Erspamer (1955) gave similar figures for the recovery of injected 5-hydroxytryptamine.

In all these experiments it was found that the intestinal mucous membrane of rats which were meat-fed weighed more than in controls. The figures are shown in Table III. In the first experiment, lasting 10 to 23 days, the difference was significant. When the mucosal and muscle layers of the intestine were weighed separately it was found that in both control and treated groups the mucosal layer formed approximately the same proportion of the total weight of the intestine, namely 25 to 30%, indicating that the weight increase involved both mucosal and muscle layers.

This phenomenon was not a reflection of differences in body weight between control and treated groups. As shown in Table III, the mean body weights of the groups were very close. In the second experiment, lasting 60 to 67 days, the final body weight of the meat-fed rats was a little lower than that of controls, although their intestinal mucosa was heavier.

The weight increase of the intestine did not account for the rise in 5-hydroxytryptamine content, because the mucosa of meat-fed rats

TABLE III

THE EFFECT OF A MEAT DIET ON THE WEIGHT AND 5-HYDROXYTRYPTAMINE CONTENT OF THE SMALL INTESTINAL MUCOSA AND THE BODY WEIGHT OF RATS

All results given as means $\pm$ standard error. n.s.=difference not significant ($P>0.05$).

Duration of Treatment (days)	Group	No. of Rats	Wt. of Intestinal Mucosa (g.)	5-Hydroxytryptamine μg./g. Mucosa	Body Weight (g.)	
					Initial	Final
10 to 23	Control	16	$3{\cdot}84 \pm 0{\cdot}16$	$3{\cdot}30 \pm 0{\cdot}14$	$188 \pm 5{\cdot}8$	$210 \pm 7{\cdot}6$
	Meat-fed	15	$4{\cdot}52 \pm 0{\cdot}13$	$3{\cdot}81 \pm 0{\cdot}16$	$194 \pm 6{\cdot}9$	$215 \pm 6{\cdot}2$
			$P<0{\cdot}01$	$P<0{\cdot}05$		
60 to 67	Control	5	$3{\cdot}53 \pm 0{\cdot}21$	$3{\cdot}57 \pm 0{\cdot}19$	$120 \pm 2{\cdot}3$	$241 \pm 0{\cdot}6$
	Meat-fed	5	$4{\cdot}14 \pm 0{\cdot}30$	$4{\cdot}93 \pm 0{\cdot}18$	$131 \pm 3{\cdot}7$	$231 \pm 3{\cdot}5$
			n.s.	$P<0{\cdot}001$		

contained more 5-hydroxytryptamine per g. of tissue than did control mucosa (Table III).

Since it was thought possible that alterations of intestinal bacterial flora due to meat-feeding might contribute towards the rise in weight of the intestine or in its 5-hydroxytryptamine content, rectal swabs were taken from 9 control and 9 meat-fed rats. The growth of aerobes was qualitatively and quantitatively similar in treated and control groups, but the anaerobic growth was much more profuse in meat-fed rats. The ratio of the number of anaerobic colonies after 24 hr. incubation between meat-fed and control rats was of the order of 10:1. Qualitative differences were not investigated.

In view of the histological findings in meat-fed mice reported by Cordier (1926), sections of jejunum from control and treated rats were stained for argentaffin cells. In control specimens under the oil immersion (magnification ×1,000) 425 argentaffin cells were counted in 400 fields, or 1.06 cells/field. In the intestine of meat-fed rats there were 392 argentaffin cells in 420 fields, or 0.94 cells/field. No difference in the number of granules in the argentaffin cells of the two groups was observed. An attempt was made to count the number of granules per cell, but in many cases the intensity of granulation made it impossible to distinguish individual granules. No other histological differences between the intestines of meat-fed and control animals were seen.

Mice.—An attempt was made to repeat the experiment of Cordier on the effect of meat-feeding for 7 to 10 weeks on the argentaffin cells of mice, and to estimate intestinal 5-hydroxytryptamine in addition. By the end of 9 weeks, however, only 2 of the meat-fed mice remained alive. Very few argentaffin cells were seen in stained sections of jejunum. Under the oil immersion (magnification ×1,000) 8 cells were seen in 50 fields in the controls, and 2 in 50 fields in meat-fed mice. The number of surviving mice was too small for any comparison of intestinal 5-hydroxytryptamine content to be made.

Experiments with Tryptophan Feeding

Rats.—By measuring the food intake and using the analytical data of Block and Weiss (1956) it was calculated that the mean dietary tryptophan intake of meat-fed rats was considerably higher than that of the controls (Table I).

If the effect of raising the intestinal 5-hydroxytryptamine of rats as a result of meat-feeding was due to the increased dietary tryptophan intake, it should be possible to reproduce the effect by adding tryptophan to the ordinary diet. Treated rats therefore received the normal diet of rat cubes crushed into powder form to which 1% DL-tryptophan had been added, controls receiving crushed cubes alone.

Three experiments of varying duration were carried out. No change in intestinal 5-hydroxytryptamine was found. Results are shown in Table IV in which rats treated for 22, 28, 40, 60, and 61 days are grouped together for convenience. It will be seen that the tryptophan intake of treated rats was many times greater than that of controls. The 5-hydroxytryptamine content of spleen and brain was also assayed in these animals but no differences were found between treated and controls. No difference in the weight of the intestine was found between the two groups.

Recovery of injected 5-hydroxytryptamine and serial 5-hydroxyindoleacetic acid estimations did not reveal any differences between controls and tryptophan-fed rats.

TABLE IV

THE EFFECT OF 1% TRYPTOPHAN ADDED TO THE CONTROL DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE SMALL INTESTINE OF RATS IN 3 EXPERIMENTS OF DIFFERENT DURATION

Results are expressed as means ± standard error.

Duration of Treatment (days)	No. of Rats		5-Hydroxytryptamine Content μg./kg. Body Weight		Tryptophan Intake mg./kg./day	
	Controls	Treated	Controls	Treated	Controls	Treated
10 to 15	6	5	61·0±6·79	59·0±2·49	116	1,246
22 ,, 61	10	10	64·0±3·43	67·5±5·47	112	832
144 ,, 161	5	6	73·5±0·63	78·0±7·02	109	760

Bacterial growth from rectal swabs was studied. No qualitative or quantitative difference was found between the two groups in aerobic growth. Anaerobic growth from swabs of tryptophan-fed rats was 2.5 times that of the controls.

Mice.—Treated mice received 1% DL-tryptophan in their diet in one experiment for 10 to 12 days and in another for 61 to 63 days. Table V shows the results obtained. In both experiments the 5-hydroxytryptamine content of the small intestine and spleen was significantly raised in the treated mice. Brain 5-hydroxytryptamine was unaffected. Platelet 5-hydroxytryptamine was assayed in the shorter experiment and also found to be raised. The mean weight of the intestine was the same in the 2 groups.

Calculation of the dietary tryptophan intake showed that treated mice had a very much higher intake than controls.

TABLE V

THE EFFECT OF 1% TRYPTOPHAN ADDED TO THE CONTROL DIET ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OF MICE IN 2 EXPERIMENTS OF DIFFERENT DURATION

Mean results are expressed as μg. 5-hydroxytryptamine/kg. body weight ± standard error, except for platelets, where results are expressed as μg. 5-hydroxytryptamine/ml. blood. n.s.=difference not significant ($P>0.05$).

Length of Treatment (days)	No. of Mice		5-Hydroxytryptamine Content			Tryptophan Intake mg./kg./day	
10–12	Controls	Treated	Controls	Treated	Significance	Controls	Treated
Small intestine ..	19	19	172 ±14·1	221 ±14·0	$P<0.05$		
Brain	10	10	17·3 ± 0·69	17·3 ± 1·36	n.s.	230	1,132
Spleen	11	11	59 ± 9·42	108 ±10·6	$P<0.01$		
Platelets	10	10	3·25± 0·28	4·78± 0·39	$P<0.01$		
61–63							
Small intestine ..	8	7	206 ±18·8	315 ±35·0	$P<0.01$		
Brain	8	6	16·3 ± 1·42	16·5 ± 2·23	n.s.	248	1,194
Spleen	8	7	100 ±16·1	168 ±19·3	$P<0.02$		

TABLE VI

THE EFFECT OF OTHER HIGH-PROTEIN DIETS ON THE 5-HYDROXYTRYPTAMINE CONTENT OF THE TISSUES OF RATS

Mean results are expressed as μg. 5-hydroxytryptamine/kg. body weight ± the standard error. n.s.=difference not significant ($P>0.05$).

Diets of Groups	No. of Rats	Small Intestine	Spleen	Brain	Tryptophan Intake μg./kg./day
Control	8	100 ± 3·64	15·6±1·36	5·7±0·48	126
Liver	8	151 ±13·5	12·4±1·13	5·0±0·25	324
		$P<0.01$	n.s.	n.s.	
Control	5	91·0± 5·05	16·6±2·24	—	112
Soya Bean	5	91·5±12·4	15·9±1·27	—	265
		n.s.	n.s.		
Control	6	61·0± 6·81	41·5±2·22	—	116
Calcium caseinate ..	5	59·0± 6·65	51·5±9·54	—	481
		n.s.	n.s.		

Other Protein-feeding Experiments

Although meat-feeding of rats produced a significant rise of intestinal 5-hydroxytryptamine, this did not occur in tryptophan-fed rats. The reason for this discrepancy was investigated by feeding high-protein diets in various forms to rats.

Diets composed of ox liver, soya bean flour, and calcium caseinate were fed to groups of rats, matched with controls, for 10 to 20 days, and the results of 5-hydroxytryptamine assays are shown in Table VI.

Only the liver diet produced a rise of the 5-hydroxytryptamine content of the small intestine. As with meat-fed rats, the weight of the intestinal mucosa of liver-fed rats was greater than in controls, the mean weights being 4.52 ± 0.09 g. and 4.07 ± 0.19 g. respectively ($P<0.1$).

Calcium caseinate proved to be a very inadequate diet, since the treated rats lost 15.5% of their body weight while controls gained 9%. This was reflected in the relative weights of the intestinal mucosa, the mean weight for controls being 3.44 ± 0.28 g. and of treated rats 2.55 ± 0.17 g. As shown in Table VI, the intestinal 5-hydroxytryptamine content of rats fed with calcium caseinate expressed as μg./kg. body weight differed little from that of controls. The diminution of intestinal size and weight due to inanition did not affect the total 5-hydroxytryptamine content, but there was a higher concentration per g. of mucosa. The mean 5-hydroxytryptamine content of treated rats was 4.11 ± 0.10 μg./g. and of controls 3.06 ± 0.18 μg./g. of mucosa ($P<0.05$).

No differences between control and treated rats were found in the 5-hydroxytryptamine content of spleen or brain. On all these diets, the tryptophan intake of the treated rats was appreciably higher than that of their controls.

Gut Incubation Experiments

To investigate whether there was any difference between treated and control animals in the rate at which the small intestine gave up 5-hydroxytryptamine into the surrounding medium, pieces of jejunum were incubated as described in "Methods." Experiments were performed on intestine from 3 meat-fed rats, 2 tryptophan-fed rats and 2 tryptophan-fed mice, all of which were matched with controls. In all experiments the rate of loss was about the same for treated and control animals.

Discussion

The results show that a meat diet caused a rise of intestinal 5-hydroxytryptamine in rats and that a liver diet had a similar effect. Since meat feeding in rats produced no effect on the 5-hydroxytryptamine content of the spleen or platelets or on the urinary excretion of 5-hydroxyindoleacetic acid, it seems likely that the effect of this diet was a local one, increasing the storage of 5-hydroxytryptamine in the intestine. The addition of tryptophan to the diet of rats, however, did not affect the 5-hydroxytryptamine content in any of the tissues examined, nor was it affected by feeding the rats on a diet of soya bean flour or calcium caseinate, both of which provided diets rich in tryptophan. The failure of these tryptophan-rich diets to raise intestinal 5-hydroxytryptamine makes it unlikely that the high tryptophan content of the meat diet was wholly responsible for this change. However, the fact that tryptophan supplied in the form of meat may have been better absorbed or better utilized than when added to the ordinary diet cannot be excluded. In this connexion it may be significant that ox muscle contains 77 μg. pyridoxine/100 g. and ox liver 170 μg./100 g., whereas the diet received by control rats was largely composed of cereals and unlikely to contain more than 40 μg. pyridoxine/100 g. (data from Bicknell and Prescott, 1953).

A large increase in the anaerobic bacterial flora was found in the intestine of meat-fed rats. Mitoma, Weissbach, and Udenfriend (1956) have shown that hydroxylation to 5-hydroxytryptophan is one of the major metabolic pathways for tryptophan in *Chromobacterium violaceum*. Many organisms, including the common intestinal organism *E. coli* (Stephenson, 1949), are capable of metabolizing tryptophan, although the pathways involved have so far been incompletely defined, while conversely certain bacteria have been shown to be capable of synthesizing tryptophan (Clifton, 1957). Possibly, therefore, in the altered state of the intestinal bacterial flora in meat-fed rats, bacterial activity might result in both increased formation and hydroxylation of tryptophan.

Another possible mechanism by which bacteria might have affected intestinal 5-hydroxytryptamine is by causing an inflammatory reaction. Zbinden and Pletscher (1958) reported that chronic irritation of the stomach and small intestine of rabbits by continuous administration of ethyl alcohol often led to an increase in the 5-hydroxytryptamine content.

It was also observed that a meat diet caused an increase in the weight of the intestine of rats. It has been reported that the small intestine in germ-free chicks weighs much less than in controls, and also that the intestinal weight could be significantly reduced by antibiotic feeding, while the body

weight of the chicks was increased (Stokstad, 1954). It was suggested that the normal animal has a chronic inflammation of the intestine. In meat-fed rats, the great increase of the anaerobic flora might, conversely, have exacerbated such chronic intestinal inflammation as might exist in the normal rat. In tryptophan-fed rats there was no increase in intestinal weight and a much smaller increase in anaerobic bacteria compared with meat-fed rats.

Eber and Lembeck (1958) reported a significant difference in the intestinal 5-hydroxytryptamine content of groups of rats fed on tryptophan-rich and tryptophan-poor diets. In the experiments described here, the addition of tryptophan to the diet had no effect on the 5-hydroxytryptamine content of rat tissues, but the controls did not receive a tryptophan-poor diet, which perhaps accounts for the lack of any difference between control and treated rats. A possible reason for the poor response of tissue 5-hydroxytryptamine levels to diets rich in tryptophan is that hydroxylation proceeds at a maximal rate even on a normal tryptophan intake (Udenfriend, Titus, Weissbach, and Peterson, 1956). Tryptophan-deficient diets are reported to cause significant reduction in intestinal 5-hydroxytryptamine in a number of species (Eber and Lembeck, 1958; Zbinden, Pletscher, and Studer, 1958) although these authors stress that the decrease in the 5-hydroxytryptamine content develops slowly.

A marked species difference was found in the relative effects in rats and mice of a tryptophan-rich diet. While no effect was produced in rats, there was a widespread rise of the 5-hydroxytryptamine content in mice, affecting the small intestine, spleen and blood platelets. Such a generalized effect on the 5-hydroxytryptamine content of mice suggests that the diet caused an increase in 5-hydroxytryptamine synthesis in these animals.

Tryptophan feeding did not affect the brain concentration of 5-hydroxytryptamine, whereas Zbinden *et al.* (1958) found that brain 5-hydroxytryptamine was reduced in rabbits, guinea-pigs, rats and mice which received a tryptophan-deficient diet.

The observation of Cordier (1926) that the number of argentaffin cells in mice was increased by meat-feeding was not confirmed in these experiments, nor was any increase found in argentaffin cells of rats as a result of meat-feeding. Zbinden and Pletscher (1958) found that in rabbits the increase in 5-hydroxytryptamine caused by ethyl alcohol was only in some instances accompanied by a moderate rise in the number of argentaffin cells. Zbinden *et al.* (1958) found that only in severe tryptophan deficiency were the histochemical reactions of the argentaffin cells diminished. It seems, therefore, that effects upon intestinal 5-hydroxytryptamine are not commonly associated with changes in the argentaffin cell count. This is confirmed by Benditt and Wong (1957), who found that a relatively high 5-hydroxytryptamine concentration, greater than 1 mg./ml., is required before argentaffin cells can be stained by silver and azo dyes.

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THE DISTRIBUTION OF 5-HYDROXYTRYPTAMINE AND ADENOSINETRIPHOSPHATE IN CYTOPLASMIC PARTICLES OF THE DOG'S SMALL INTESTINE

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The distribution of 5-hydroxytryptamine, adenosinetriphosphate, and succinic dehydrogenase in sucrose homogenates of the dog's small intestine has been studied. The adenosinetriphosphate was present in two different layers which could be separated by density gradient centrifugation. The upper layer contained also much succinic dehydrogenase, but no amine; it is probably composed of mitochondria. The lower layer contained not only adenosinetriphosphate but also the major portion of the particle-held 5-hydroxytryptamine. The mean molar ratio, amine : adenosinetriphosphate, in the lower layer was 2.6. The experiments suggest that adenosinetriphosphate in the intestine is of importance in the storage of 5-hydroxytryptamine, resembling the function of adenosinetriphosphate in the storage of the catechol amines of the adrenal medulla.

Many observations suggest that the pharmacologically active amines are stored in specific cell organelles which can be sedimented by centrifugation of tissue homogenates prepared in isotonic sucrose. Earlier work from this laboratory has shown that some of the 5-hydroxytryptamine present in homogenates of the dog duodenal mucosa can be sedimented by the use of a sucrose density gradient; by this method a considerable separation of mitochondria and 5-hydroxytryptamine-storing elements has been achieved (Baker, 1958, 1959).

In their storage location, amines seem to be associated with acidic compounds. For instance, in the mast cells histamine is found together with heparin, and in the chromaffin granules of the adrenal medulla the catechol amines occur together with adenosinetriphosphate. The work of Born (1956a; 1956b) and of Born and Gillson (1957) suggests that adenosinetriphosphate is also associated with 5-hydroxytryptamine in the platelets.

In most mammalian species the bulk of the 5-hydroxytryptamine is found in the epithelial lining of the gastro-intestinal tract. Information on the intracellular localization of the amine in this tissue is still far from complete. This is because the cells which carry the 5-hydroxytryptamine represent only a small fraction of those present in the mucous membrane. It seems likely, therefore, that the granular material rich in 5-hydroxytryptamine as prepared by Baker (1959) is still heterogeneous, with the elements carrying the amine contaminated by other structures. In the present work an attempt has been made to achieve a further purification and to find out if the tissue contains adenosinetriphosphate, and how it is distributed among the fractions isolated.

METHODS

Isolation and Purification of Cytoplasmic Granular Fractions

The procedure adopted followed that of Baker (1958; 1959), except that pentobarbitone sodium was used instead of ether as anaesthetic. Dogs were bled, the small intestine was taken out and the mucous membrane was removed and homogenized in 0.3 M sucrose. The portion used always included the duodenum and extended in different experiments to a varying degree to the lower parts of the small intestine. Unbroken cells, nuclei and coarse cell debris were removed by low-speed centrifugation at 900 *g* for 20 min. The supernatant from the low-speed centrifugation was spun at high speed, 11,000 *g* for 30 min., in order to sediment the amine-carrying material. This sediment was resuspended in 0.3 M sucrose and layered over a sucrose density gradient prepared the previous day and spun at 100,000 *g* for 1 hr. in the swinging bucket rotor SW 39L of the Spinco

ultracentrifuge. The fractions obtained were separated by the help of the Schuster cutter and those found rich in 5-hydroxytryptamine and adenosinetriphosphate were diluted with 0.3 M sucrose, to obtain a final molarity of about 1.0 M. They were then again layered over a sucrose density gradient. After a second centrifugation at 100,000 *g* for 1 hr. the fractions were separated and analysed for 5-hydroxytryptamine and adenosinetriphosphate and also for succinic dehydrogenase activity.

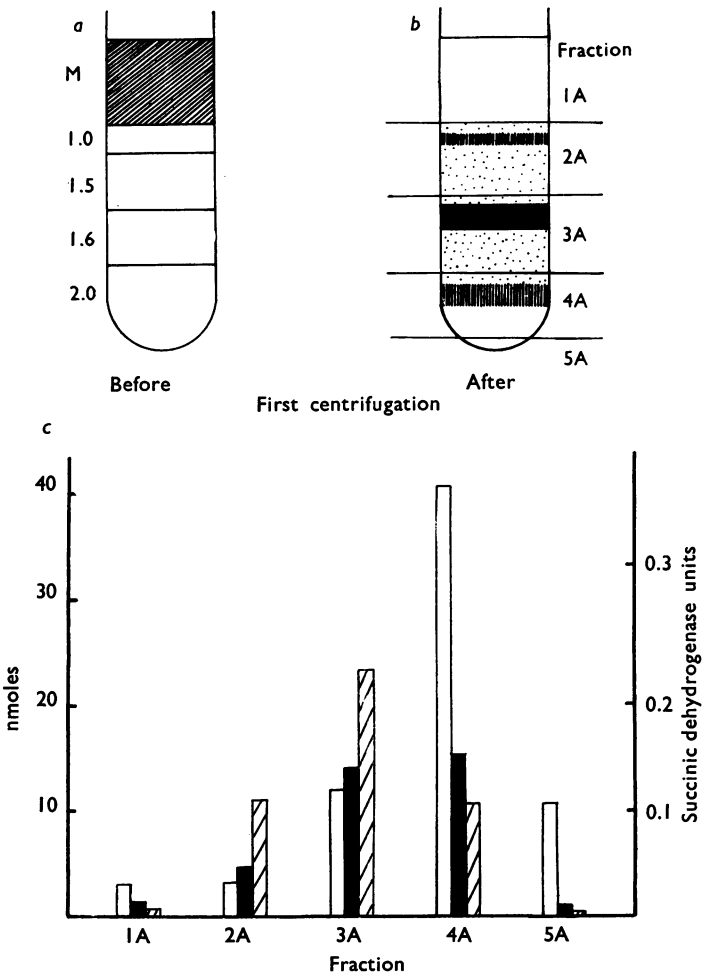

FIG. 1.—First density gradient centrifugation of cytoplasmic particles from the dog's small intestine. (1*a*) Density gradient before centrifugation, with the resuspended large cytoplasmic particles in 0.3 M sucrose at the top of the gradient (shaded area). (1*b*) Diagrammatic representation of the appearance after centrifugation for 60 min. at 100,000 *g*. The numbers at the right refer to the fractions obtained by cutting the tube. (1*c*) Distribution of 5-hydroxytryptamine (white columns), adenosinetriphosphate (black columns), and succinic dehydrogenase (shaded columns) in the particulate fractions.

The unit of succinic dehydrogenase activity is the amount of enzyme that will give an increase in optical density of 1.0 in 10 min.

Methods of Analysis

5-Hydroxytryptamine was extracted with *n*-butanol by the method of Udenfriend, Weissbach, and Clark (1955), as modified by Cargill-Thompson, Hardwick, and Wiseman (1958) and Baker (1959), and assayed on the rat gastric fundus preparation (Vane, 1957). Adenosinetriphosphate was estimated by the firefly luminescence method, as used by Holton (1959). Succinic dehydrogenase activity was determined by measuring the rate of reduction of cytochrome C, as described by Kuff and Schneider (1954).

RESULTS

The separation of the resuspended cytoplasmic particles in a sucrose density gradient is shown in Fig. 1a and 1b. The gradient was adapted from that described by Baker (1959), but the intermediate steps were chosen so as to make the distance between the different particulate fractions convenient for separation. There were three main particulate fractions, one a few mm. below the top of the gradient (2A), a second near the boundary of the 1.5 M and the 1.6 M sucrose (3A), and a third layer below the 1.6 M sucrose (4A).

The distribution of the 5-hydroxytryptamine, adenosinetriphosphate and succinic dehydrogenase in the various fractions is shown in Fig. 1c. The distribution of succinic dehydrogenase and of 5-hydroxytryptamine was essentially as found by Baker (1959); the mitochondrial enzyme had a maximum in fraction 3A, whereas the 5-hydroxytryptamine had a maximum in fraction 4A. The uppermost granular layer (2A) was poor in both enzyme and 5-hydroxytryptamine.

The distribution of adenosinetriphosphate differed from that of the two other constituents. Little was recovered in fraction 2A; the adenosinetriphosphate present was approximately equally divided between the other two particulate

fractions, 3A and 4A. In other experiments the mitochondria (3A) contained up to 3 times the amount of adenosinetriphosphate found in the dense particulate fraction (4A).

In order to determine whether adenosinetriphosphate was associated with the 5-hydroxytryptamine-containing granules, experiments were performed in which the fraction rich in 5-hydroxytryptamine (fraction 4A of Fig. 1b) was diluted with 0.3 M sucrose and again centrifuged in a sucrose density gradient. The gradient used in this centrifugation is shown in Fig. 2a, and the appearance of the gradient tube at the end of the centrifugation in Fig. 2b. The gradient differed from that used in the first gradient centrifugation in that the layer of 1.0 M sucrose at the top of the gradient was omitted. This layer was unnecessary, since the molarity of the resuspended particulate material was adjusted to approximately 1.0 M. Equal volumes of 1.5 M, 1.6 M, and 2.0 M layers of sucrose were used (Fig. 2a).

The distribution of the particulate elements after a centrifugation for 1 hr. at 100,000 *g* is shown in Fig. 2b. There were two layers, of which the larger one, fraction 4B, was present in a level equal in density to that from which the material had been obtained in the first density gradient centrifugation (fraction 4A of Fig. 1b).

Fig. 2c shows the distribution of the components in the different layers. In this figure the % distribution of the material recovered is given. It can be seen that fraction 4B was again rich in both adenosinetriphosphate and 5-hydroxytryptamine, but succinic dehydrogenase activity was still associated with this fraction. Unlike fraction 1A of the first density gradient, the top fraction, 1B, contained about one-quarter of the 5-hydroxytryptamine and also of the succinic dehydrogenase activity; this indicates that some release had accompanied the dilution with 0.3 M sucrose of the particulate material from fraction 4A, which had been recovered in strongly hypertonic sucrose. This is analogous to observations on the chromaffin granules of the adrenal medulla (Hillarp and Nilson, 1954; Eade, 1958).

A second experiment was carried out in which use was made of the experience gained from the results just described. An initial high-speed

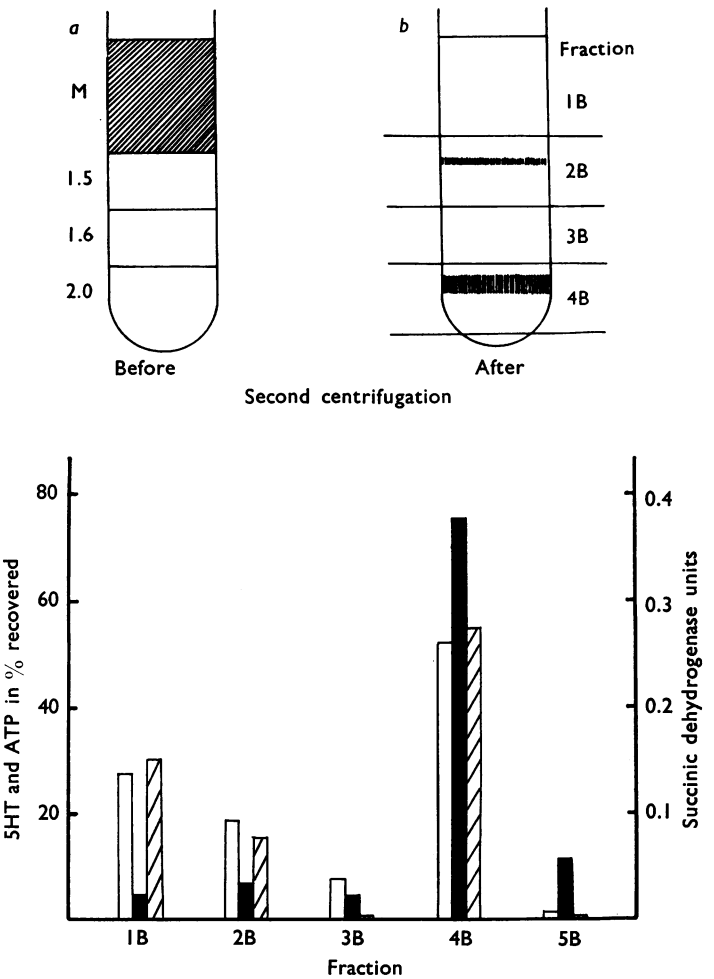

FIG. 2.—Second density gradient centrifugation of the granular fraction (4A) rich in 5-hydroxytryptamine and adenosinetriphosphate obtained in the gradient shown in Fig. 1*b*. (2*a*) Gradient before centrifugation. (2*b*) Appearance of the gradient after centrifugation for 60 min. at 100,000 *g* and position of the fractions collected. (2*c*) Determination of 5-hydroxytryptamine (white columns), adenosinetriphosphate (black columns), and succinic dehydrogenase (hatched columns) in the different fractions. Note that in this diagram 5-hydroxytryptamine and adenosinetriphosphate are expressed in terms of the total material recovered.

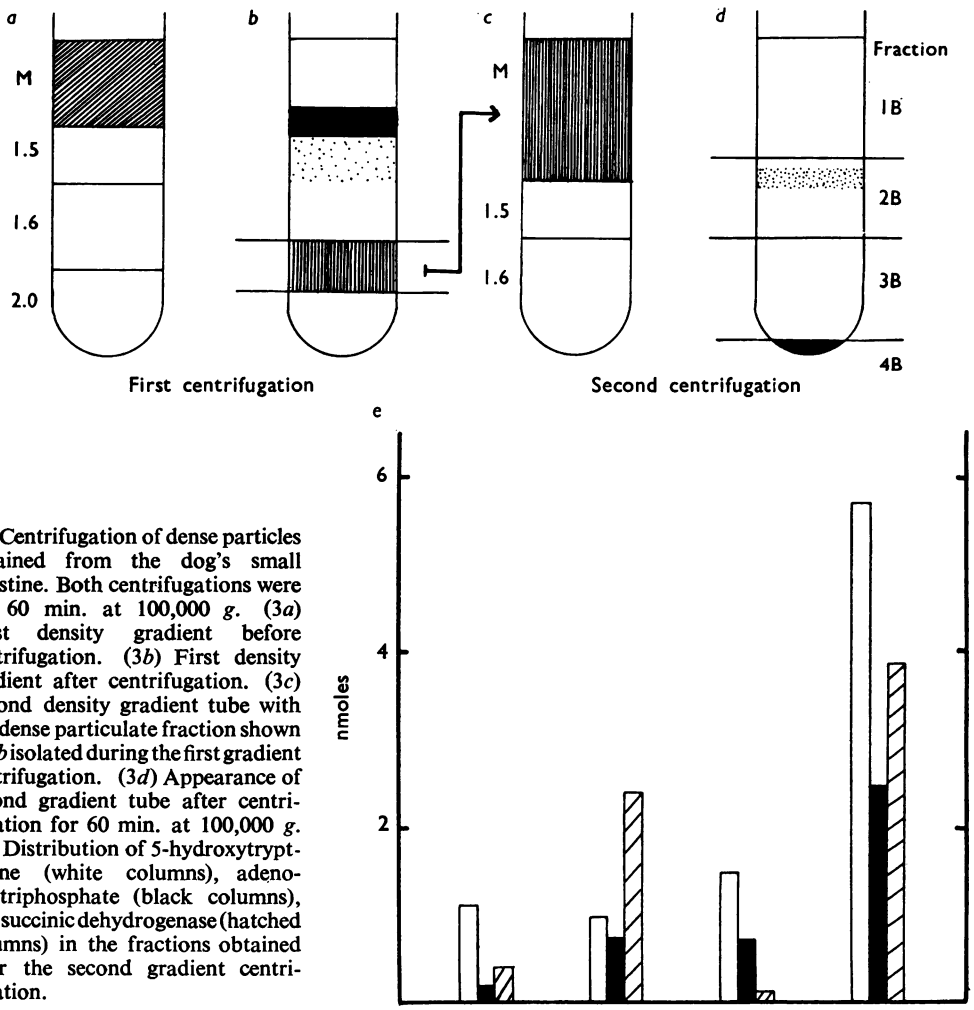

FIG. 3.—Centrifugation of dense particles obtained from the dog's small intestine. Both centrifugations were for 60 min. at 100,000 *g*. (3*a*) First density gradient before centrifugation. (3*b*) First density gradient after centrifugation. (3*c*) Second density gradient tube with the dense particulate fraction shown in 3*b* isolated during the first gradient centrifugation. (3*d*) Appearance of second gradient tube after centrifugation for 60 min. at 100,000 *g*. (3*e*) Distribution of 5-hydroxytryptamine (white columns), adenosinetriphosphate (black columns), and succinic dehydrogenase (hatched columns) in the fractions obtained after the second gradient centrifugation.

centrifugation in 0.3 M sucrose was again followed by two density gradient centrifugations of the resuspended high-speed sediment. The first sucrose gradient (Fig. 3a) was set up with 1.5 M, 1.6 M, and 2.0 M sucrose. The volumes were chosen so as to obtain a separation of the dense layer rich in 5-hydroxytryptamine from the other particulate layers of lower density. Fig. 3b shows that a satisfactory separation of this layer in the lower part of the tube was achieved.

The dense layer obtained in the first density gradient centrifugation was isolated and again diluted with 0.3 M sucrose to a molarity of 1.0 M. This suspension was layered over a second density gradient as shown in Fig. 3c. The gradient did not contain a layer of 2.0 M sucrose at the bottom of the tube; this layer was omitted in order to obtain a sedimentation of particulate material of highest density.

Fig. 3d shows that a sediment was obtained in the second density gradient centrifugation. As in the preceding experiment some opaque material was also present at the top of the gradient.

Fig. 3e shows the composition of tube at the end of the second gradient centrifugation. The sediment, which probably contributed only 1% of the total volume of the tube, contained 63% of the total 5-hydroxytryptamine and also 63% of the total adenosinetriphosphate. The sedimentation of both 5-hydroxytryptamine and adenosinetriphosphate clearly shows that both substances were associated with a particulate fraction. It may be noted that the sediment also contained some succinic dehydrogenase activity.

Molar Ratio, 5-Hydroxytryptamine : Adenosinetriphosphate

Three experiments were carried out in which 5-hydroxytryptamine and adenosinetriphosphate were determined in the fractions obtained after the first density gradient centrifugation. The mean figure for total 5-hydroxytryptamine, as recovered in all these fractions, was 8.7 ± 2.8 nmoles/g. of fresh tissue; the corresponding figure for adenosinetriphosphate was 6.0 ± 2.1 nmoles: this gives a molar ratio of 1.5. The mean 5-hydroxytryptamine content of the dense particle fraction was 4.1 ± 0.9 nmoles, the mean adenosinetriphosphate content 1.6 ± 0.4 nmoles; thus the mean molar ratio was 2.6. This increase in the molar ratio is accounted for by the fact that some adenosinetriphosphate was removed without a concomitant loss of 5-hydroxytryptamine. It seems likely that the adenosinetriphosphate removed was present in mitochondrial elements.

Discussion

The work reported in this paper confirms and extends previous observations, according to which a substantial part of the intestinal 5-hydroxytryptamine is present in a structural element that can be sedimented by high-speed centrifugation of a sucrose homogenate. Density gradient centrifugation was successful in achieving a partial separation of the particle-held 5-hydroxytryptamine from succinic dehydrogenase, but even with the higher degree of purification now achieved some enzymic activity was still found in the fraction that was rich in 5-hydroxytryptamine. Preliminary studies by electron microscopy of the fractions obtained revealed the presence of fragmented mitochondria; it is probable, therefore, that the succinic dehydrogenase is associated with these fragments.

The main interest of the present experiments lies in the fact that the fractions rich in 5-hydroxytryptamine invariably also contained similar amounts of adenosinetriphosphate. In the adrenal medulla, molar ratios of amine:adenosinetriphosphate of about 4 have been reported, and it is of interest that the figure of approximately 3 found in the present experiments is not very different from the ratio in the adrenal medulla. At the present stage of our knowledge the mechanism by which the 5-hydroxytryptamine is held at its site of storage is unknown, but the present experiments show that enough adenosinetriphosphate is present to assign to it a role similar to that which it plays in the storage of the catechol amines in the chromaffin tissue and in the adrenergic neurone.

I am grateful to Dr. H. Blaschko for the hospitality extended to me during my stay in Oxford and to Mrs. Rosemary Bonney for skilful help with the assays.

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THE ANTICHOLINESTERASE ACTIVITY OF SOME ANTIADRENALINE AGENTS

BY

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The contractions of the isolated guinea-pig vas deferens in response to stimulation of the sympathetic hypogastric nerve were potentiated by low concentrations and inhibited by high concentrations of the antiadrenaline agents tolazoline, yohimbine, ergotamine, phenoxybenzamine and piperoxan. Eserine potentiated the contractions of the vas deferens produced by hypogastric nerve stimulation. The cholinesterase activity of an extract of vas deferens was decreased by the antiadrenaline agents. The potentiation of responses to sympathetic stimulation by antiadrenaline drugs, which also possess anticholinesterase activity, can be explained on the basis of a cholinergic sympathetic mechanism.

The effects of antiadrenaline agents on the responses to stimulation of a sympathetic nerve do not always run parallel to their effects on responses induced by noradrenaline. Thus Varagić (1956b) found that tolazoline potentiated the response of the rabbit uterus to sympathetic nerve stimulation, and Huković (1959) reported that phenoxybenzamine increased the response to sympathetic nerve stimulation in the isolated atria of the rabbit.

We have investigated the effects of tolazoline and other antiadrenaline agents on the responses of the isolated vas deferens to sympathetic nerve stimulation, noradrenaline and acetylcholine, and on the cholinesterase activity of the vas deferens.

METHODS

Isolated Innervated Vas Deferens.—The isolated vas deferens with intact hypogastric nerve was prepared by the method of Huković (personal communication). Guinea-pigs weighing about 500 g. were killed by a blow on the head. The abdomen was opened in the midline and the distal colon retracted to one side. The hypogastric nerves were identified and dissected free. The vasa deferentia were cut from their attachments to the epididymis at one end and the urethra at the other and removed, each with its accompanying nerve. The vas deferens was mounted in a 50 ml. organ bath containing McEwen's (1956) solution gassed with 95% oxygen and 5% carbon dioxide and maintained at 29°. The nerve was passed through a stimulating electrode of the type described by Burn and Rand (1960a) consisting of a 1 mm. tube containing 2 adjacent platinum rings. Stimulation was by 2 msec. pulses at a frequency of 10/sec. applied at supramaximal strength (usually 3.0 mA.) for 10 sec. in every 2 min. The time cycle of stimulation was automatically controlled. Contractions were recorded by an isotonic frontal writing lever.

Isolated Vas Deferens.—For experiments in which nerve stimulation was not required the vas deferens was suspended in a 5 ml. bath.

Cholinesterase Activity.—The vasa deferentia from a guinea-pig were ground with Tyrode solution (11 ml.) in a mortar. The whole extract was divided into 2 ml. aliquots to serve as source of enzyme in each reaction mixture. The drugs examined for anticholinesterase activity were mixed with the enzyme 10 min. before the addition of substrate and brought to 32°. Acetylcholine as substrate was added to reach a final concentration of 0.5 μg./ml. in a final volume of reaction mixture of 5 ml. At various time intervals after this, the reaction mixture was agitated and 0.1 ml. samples were withdrawn and assayed for acetylcholine on a strip of guinea-pig ileum suspended in Tyrode solution in a 5 ml. bath. A dose-response curve for acetylcholine was obtained upon the ileum at the beginning and the end of each experiment.

RESULTS

Sympathetic Stimulation.—The contractions of the isolated vas deferens in response to stimulation of the sympathetic hypogastric nerve for 10 sec. at 2 min. intervals maintained a steady amplitude over long periods of time. The vas deferens was contracted by acetylcholine and noradrenaline.

When we attempted to block the response to sympathetic stimulation with the antiadrenaline

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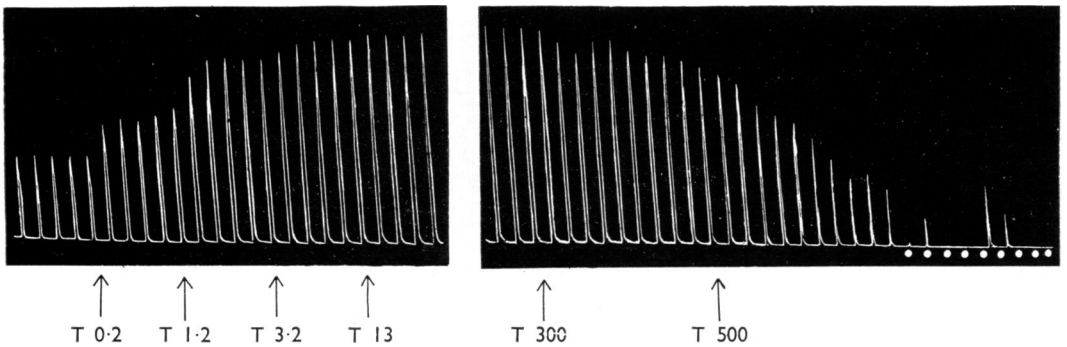

FIG. 1.—Contractions of the isolated guinea-pig vas deferens in response to hypogastric nerve stimulation at 2 min. intervals (white dots indicate stimulation not followed by contractions). At T, tolazoline was added to the bath to produce the concentrations indicated in μg./ml.

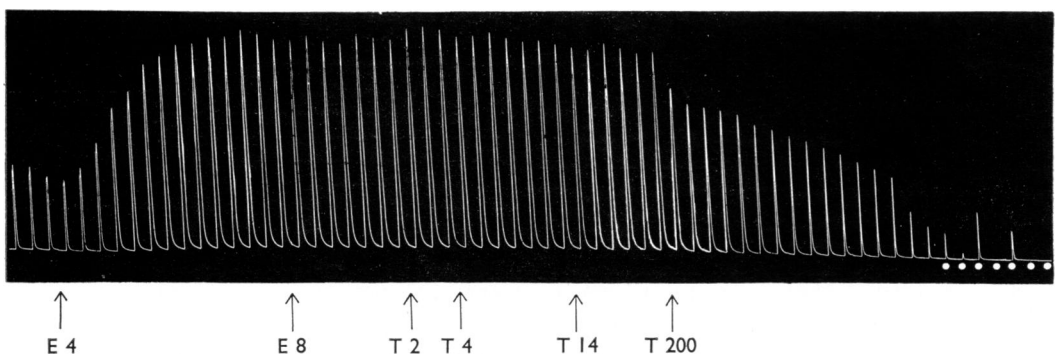

FIG. 2.—As Fig. 1. Eserine was added to the bath at E, and tolazoline at T, to produce the concentrations indicated in μg./ml.

agent tolazoline, it was observed that low doses of tolazoline produced a potentiation of the response (Fig. 1). The concentration of tolazoline required to inhibit the response to nerve stimulation was several hundred times higher than the dose which produced a clear potentiation. Thus in Fig. 1 a potentiation of the response can be seen with 0.2 μg./ml., whereas 500 μg./ml. was required to block.

In other experiments we had found that the response of the vas deferens to sympathetic nerve stimulation was potentiated by eserine. This observation led us to consider that the potentiation of the responses to sympathetic nerve stimulation that we had observed with low doses of tolazoline may be explained if it too possessed an anti-cholinesterase action. In the experiment shown in Fig. 2 the responses of the vas deferens to nerve stimulation were potentiated by eserine in a concentration of 4 μg./ml. An additional 4 μg./ml. when added to the bath had no further

TABLE I

CONCENTRATIONS OF ANTIADRENALINE AGENTS TO POTENTIATE AND TO INHIBIT RESPONSES OF VAS DEFERENS TO HYPOGASTRIC NERVE STIMULATION

	Concentration of Drug Required to Produce		Maximum Potentiation %
	Potentiation (μg./ml.)	Block (μg./ml.)	
Tolazoline	0·1–100	500	280
Phenoxybenzamine	1·0–30	130	140
Yohimbine ..	0·2–1·0	30	200
Ergotamine ..	3·0–30	70	150
Piperoxan	0·2–20	100	150

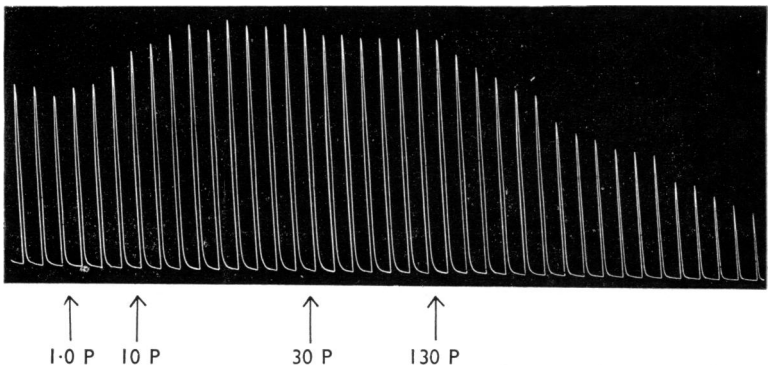

FIG. 3.—As Fig. 1. Phenoxybenzamine was added to the bath at P to produce the concentrations indicated in μg./ml.

effect, thus showing that the potentiation due to inhibition of cholinesterase was complete. Gradually increasing concentrations of tolazoline were next added to the bath without causing any further potentiation of the responses.

The effect of low doses of other antiadrenaline agents was tested on the responses of the vas deferens to hypogastric nerve stimulation. The results obtained with phenoxybenzamine are shown in Fig. 3. The responses were potentiated by a low dose of phenoxybenzamine (1.0 μg./ml.), a higher dose (30 μg./ml.) had no further effect, and finally at a still higher dose (130 μg./ml.) the responses gradually decreased until there was complete block. Similar results were obtained for yohimbine, piperoxan and ergotamine. The findings are summarized in Table I.

Noradrenaline and Acetylcholine.—The contractions of the isolated vas deferens in response to acetylcholine added to the bath were potentiated by tolazoline, but the responses to noradrenaline showed no sign of potentiation. Thus in Fig. 4, 30 μg./ml. of tolazoline inhibited the response to noradrenaline but potentiated the response to

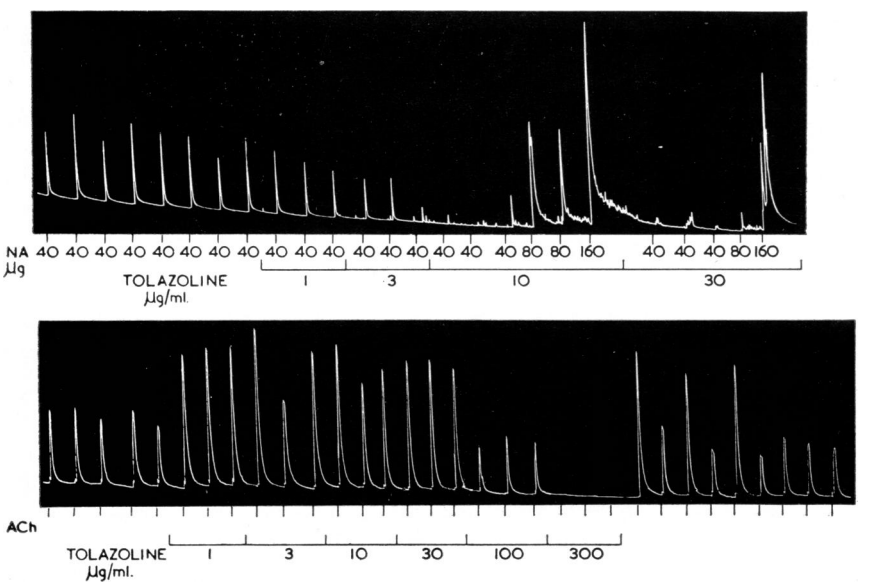

FIG. 4.—Effect of tolazoline on responses of the isolated guinea-pig vas deferens to noradrenaline and acetylcholine. In the upper tracing, the responses to 40 μg. of noradrenaline were abolished by 10 μg./ml. of tolazoline, although higher doses of noradrenaline (80 μg. and 160 μg.) were still effective. In the lower tracing the responses to acetylcholine (5 μg.) were potentiated by 1 to 30 μg./ml. of tolazoline, and inhibited by 300 μg./ml.

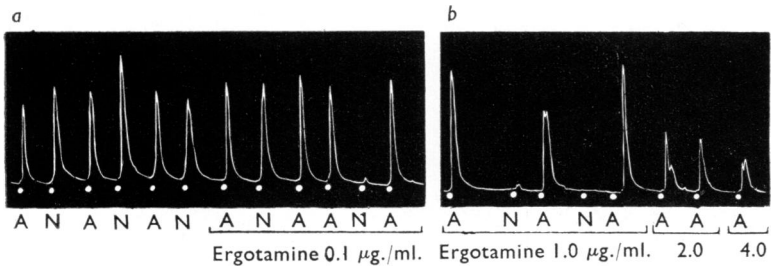

FIG. 5.—Effect of ergotamine on responses of the isolated guinea-pig vas deferens to acetylcholine (A, 0.2 μg./ml.) and noradrenaline (N, 2 μg./ml.).

acetylcholine. The block of noradrenaline by tolazoline was readily overcome by larger doses of noradrenaline.

Ergotamine in concentrations of 0.1 to 1.0 μg./ml. potentiated the responses to acetylcholine as shown in Fig. 5. In higher concentrations (2.0 to 4.0 μg./ml.), ergotamine exhibited an atropine-like action in diminishing the responses to acetylcholine. Similar results were obtained with phenoxybenzamine, piperoxan and yohimbine; low concentrations potentiated the contractions produced by acetylcholine, while higher concentrations reduced them.

We observed no potentiation of the contractions of the vas deferens in response to noradrenaline with ergotamine (Fig. 5), phenoxybenzamine, piperoxan or yohimbine.

Atropine and Eserine.—It is apparent that the antiadrenaline agents which we have investigated possess an atropine-like action in that they depress the response of the vas deferens to acetylcholine.

Our findings with tolazoline had led us to suppose that the potentiation of the responses of the vas deferens to hypogastric nerve stimulation could be attributed to its anticholinesterase activity. Since the antiadrenaline agents potentiated the responses to hypogastric nerve stimulation, but in addition possessed an atropine-like action, we investigated the effect of atropine on the potentiation of the responses to hypogastric nerve stimulation produced by eserine. In Fig. 6 the responses to hypogastric nerve stimulation were reduced in the presence of 0.1 μg./ml. of atropine, which was a sufficient dose to block completely the response to acetylcholine. Eserine (4 μg./ml.) when added to the bath still potentiated the responses to sympathetic nerve stimulation.

Fig. 7 illustrates an experiment in which dibenzyline produced a potentiation of the responses of the vas deferens to nerve stimulation although the dose of phenoxybenzamine used was sufficient to abolish the responses to both acetylcholine and noradrenaline.

Anticholinesterase Activity.—There were grounds for considering that the action of low doses of the antiadrenaline agents in potentiating the responses of the vas deferens to hypogastric nerve stimulation was due to their anticholinesterase activity. We selected a biological assay method for determining residual acetylcholine in the enzyme reaction mixture since it was the only way in which we could test enzyme activity on low concentrations of substrate. Thus, the acetylcholine content of the reaction mixture was 2.3×10^{-6} M; for the manometric determination of anticholinesterase activity concentrations of 1×10^{-2} M have generally been employed.

Tolazoline was the most effective of the drugs tested in inhibiting the cholinesterase of guinea-pig vas deferens. Fig. 8 shows that inhibition was detectable with 1 μg./ml., appreciable with 10 μg./ml., and complete with 500 μg./ml. Ergotamine (100 μg./ml.) produced a 35% inhibition

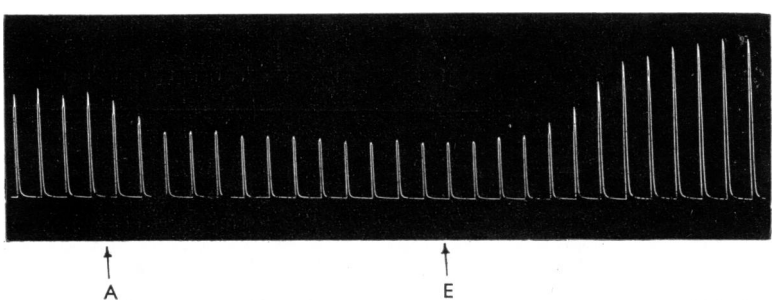

FIG. 6.—As Fig. 1. At A, atropine was added to the bath to produce a concentration of 0.1 μg./ml. At E, eserine was added to produce a concentration of 4 μg./ml.

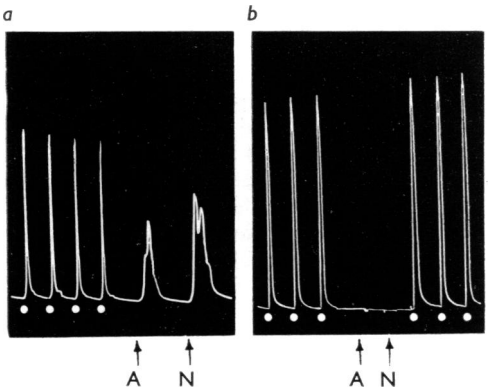

FIG. 7.—Isolated guinea-pig vas deferens. At white dots the hypogastric nerve was stimulated. At A, 10 μg./ml. of acetylcholine, and at N, 10 μg./ml. of noradrenaline was added. Between tracings (*a*) and (*b*) phenoxybenzamine was added to produce a bath concentration of 3 μg./ml.

of cholinesterase activity measured over the first 10 min. of the reaction.

Piperoxan and yohimbine were approximately equiactive; both produced a 30% inhibition of enzyme activity in concentration of 100 μg./ml. Phenoxybenzamine was the least active; 50 μg./ml. producing a 10% inhibition, and 500 μg./ml. a 25% inhibition during the first 10 min. of enzyme action.

DISCUSSION

Jang (1941) attributed the increase in response to sympathetic stimulation produced by ergotamine, piperoxan, and yohimbine to their action in enhancing the response to adrenaline. He found that small doses of these three drugs increased the vasoconstriction in the rabbit ear produced by adrenaline. Other reports occur in the literature of potentiating action towards adrenaline and noradrenaline of antiadrenaline agents when they are used in low doses. Thus Holzbauer and Vogt (1955) reported that dibenamine, phenoxybenzamine, and dihydroergotamine made the rat uterus more sensitive to the inhibitory action of adrenaline, although tolazoline, yohimbine, piperoxan, and phentolamine had no such effect. In order for Jang's suggestion to hold good it would be necessary to show that the antiadrenaline drugs which potentiated the response of the vas deferens to sympathetic stimulation were effective in potentiating the responses to noradrenaline. We have not observed any increase in response to noradrenaline in the presence of antiadrenaline agents.

The potentiation of the responses to sympathetic stimulation produced by low concentrations of antiadrenaline agents can be understood in the light of their anticholinesterase action. There are two ways in which an inhibition of the

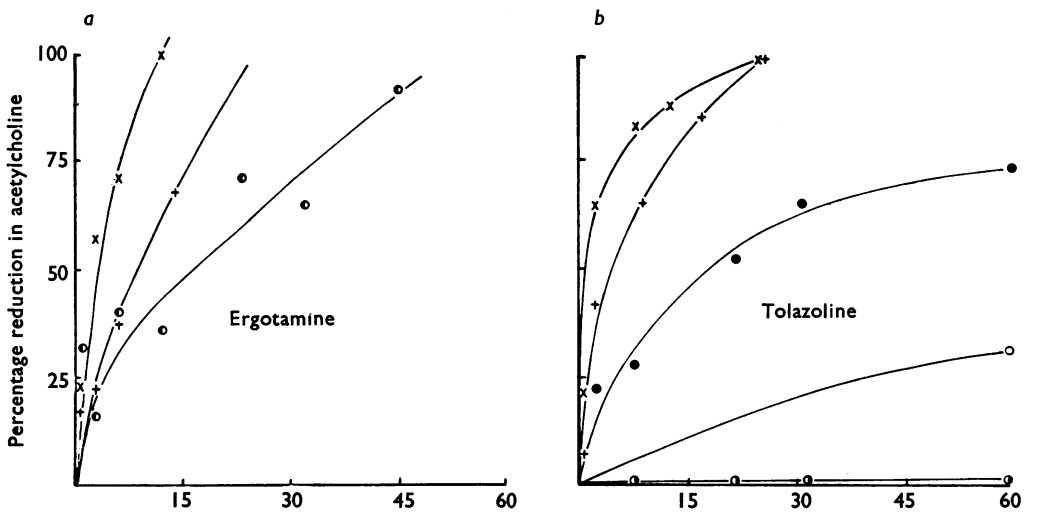

FIG. 8.—The rate of the destruction of acetylcholine by an extract of guinea-pig vas deferens in the presence of various concentrations of ergotamine (*a*) and tolazoline (*b*). X=Control. +=1 μg./ml. ●=10 μg./ml. O=50 μg./ml. ◐=100 μg./ml. ◑=500 μg./ml. Ordinate: % reduction in acetylcholine concentration. Abscissa: time in min.

destruction of acetylcholine from cholinergic sympathetic fibres could increase the responses of the vas deferens to hypogastric nerve stimulation: by increasing the direct action of acetylcholine on effector cells, or its intermediary action in liberating noradrenaline. The mechanism by which acetylcholine and cholinergic sympathetic fibres might liberate noradrenaline has been discussed by Burn and Rand (1959, 1960b). The direct action of acetylcholine on the vas deferens was blocked by atropine, but inhibition of cholinesterase by eserine still led to potentiation of the responses to sympathetic stimulation (Fig. 6) which favours the idea that it is the action of acetylcholine as an intermediary in noradrenaline release that is being potentiated. Thus ergotamine, phenoxybenzamine, piperoxan, and yohimbine, which had atropine-like activity, nevertheless potentiated the responses to sympathetic nerve stimulation.

The potentiation of the responses to sympathetic stimulation by anticholinesterase drugs has been observed in the nictitating membrane (Bacq and Fredericq, 1935a), in the rabbit ear (Burn and Rand, 1960b), in some experiments in the rabbit uterus (Varagić, 1956b) and by us in the guinea-pig vas deferens.

Potentiation of responses to sympathetic stimulation by low concentrations of anti-adrenaline drugs has been observed in isolated rabbit atria with dibenzyline (Huković, 1959), in the isolated rabbit uterus with tolazoline (Varagić, 1956b), in the cat nictitating membrane with piperoxan (Bacq and Fredericq, 1935b) and with yohimbine and ergotoxine (Jang, 1941), and in the rabbit ear with piperoxan (Jang, 1941).

Anticholinesterase activity has been reported previously for ergotamine with horse blood as the source of enzyme (Matthes, 1930), with horse serum (Gautrelet and Scheiner, 1939), with defibrinated cat blood (Brügger, 1938), and with human serum (Thompson, Tickner, and Webster, 1955). Schär-Wüthrich (1943) found that tolazoline (and two other imidazolines) inhibited cholinesterase from human serum and brain. Some actions of tolazoline can be interpreted as due to its inhibition of cholinesterase. For example, tolazoline decreased the rate of isolated rabbit atria and potentiated acetylcholine-induced contraction in the frog rectus and guinea-pig ileum (Gowdey, 1948). The gastro-intestinal stimulating action and the effect on heart rate could be prevented by atropine (Ahlquist, Huggins, and Woodbury, 1947).

Varagić (1956a) found that tolazoline altered the response of the isolated rabbit colon to sympathetic stimulation from a simple inhibition to a biphasic response consisting of an initial contraction followed by relaxation. This observation could not be explained in terms of an antagonism or reversal of the inhibition by tolazoline since Ahlquist *et al.* (1947) showed that tolazoline did not influence the inhibitory action of adrenaline, and Gowdey (1948) found tolazoline reduced but did not reverse the inhibitory action of adrenaline on the rabbit duodenum. Gillespie and Mackenna (1959) found that rabbit colon taken from a reserpine-treated rabbit contracted in response to sympathetic stimulation, and the contraction was abolished by atropine. This can be construed as evidence for cholinergic nerves running in the sympathetic supply to the colon. The action of the acetylcholine liberated from such nerves might be enhanced by an anticholinesterase so that the normal colon would be exposed to the combined effect of acetylcholine and noradrenaline to result in the biphasic response observed by Varagić (1956a). In fact, Varagić observed that eserine only produced a biphasic response in 2 of 11 preparations, but that eserine could enhance the effect of tolazoline. The initial contraction of the colon in response to sympathetic stimulation in the presence of tolazoline was abolished by atropine.

Brown and Gillespie (1957) found that the amount of noradrenaline appearing in the splenic venous blood after stimulating the splenic nerves was increased in the presence of phenoxybenzamine. Huković (1959) suggested that the appearance of a greater amount of transmitter (noradrenaline) might explain both his finding that phenoxybenzamine increased the effect of sympathetic stimulation on the rabbit atria, and Varagić's (1956b) finding that the response of the rabbit uterus to sympathetic stimulation was increased by tolazoline. We believe that an increased amount of transmitter could be released in the presence of an anticholinesterase (such as phenoxybenzamine or tolazoline) if there were a cholinergic process involved in the release of noradrenaline. Such a mechanism has been proposed by Burn and Rand (1959, 1960b) and additional evidence for it has been obtained recently when it was found that hemicholinium produced transmission failure in isolated vas deferens stimulated via its hypogastric sympathetic nerve (Rand and Chang, 1960).

Goodman and Gilman (1956) have pointed out that, "Practically every important class of drug and innumerable compounds of lesser interest have been tested for their effects on cholinesterase activity. The drugs reported to inhibit various cholinesterases outnumber those that are

apparently ineffective. Numerous exorbitant claims have been made that certain agents act principally through cholinesterase inhibition." Nevertheless the use of antiadrenaline agents as tools for investigating mechanisms must be evaluated in the light of our findings that they possess anticholinesterase activity. Thus Burnstock (1958) found that piperoxan increased the tone of longitudinal muscle in the trout stomach. He attributed this to the antagonism of an inhibitory action of adrenaline on the gut and the resultant was an acetylcholine-induced spasm. Our results show that another explanation may be the potentiation of acetylcholine by virtue of the anticholinesterase activity of piperoxan.

Finally, we wish to point out that we have no evidence to suggest that the antagonistic action of antiadrenaline agents is associated with their anticholinesterase activity.

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THE EFFECTS OF CORTICOSTEROIDS AND RELATED COMPOUNDS ON THE HISTAMINE AND 5-HYDROXY-TRYPTAMINE CONTENT OF RAT TISSUES

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Daily intramuscular injections of cortisone, prednisolone, triamcinolone, dexamethasone, fludrocortisone and 2-methylfludrocortisone markedly reduced the histamine and 5-hydroxytryptamine contents of the skin and small intestine of rats; there was an increase, however, in the amine contents of the stomach. The sex hormones were weaker in these effects while deoxycortone and corticotrophin were inactive. When the skin was depleted of half of its 5-hydroxytryptamine by prolonged treatment with polymyxin B, the rate of recovery of this amine was retarded by the glucocorticoids. The glucocorticoid activity of a steroid and its ability to produce changes in the histamine and 5-hydroxytryptamine contents of tissues are related.

A functional relationship between histamine and the adrenal cortex was postulated by Dale in 1920 when he found that after adrenalectomy cats became about five times more sensitive to intravenous injections of histamine. A similar result has since been obtained by other workers using different species. The histamine contents of rat tissues are lowered after treatment with adrenal cortical steroids and raised after adrenalectomy (Schayer, Smiley, and Davis, 1954; Halpern and Briot, 1956). Recently, the 5-hydroxytryptamine contents of tissues have been shown to be similarly altered, and thus the secretion of the adrenal cortex may exert a functional control over the tissue reserves of histamine and 5-hydroxytryptamine (Hicks and West, 1958a and 1958b). The present work was undertaken to test this hypothesis further by using more potent corticosteroids than those used by previous authors.

METHODS

Groups of 4 female albino rats (120 to 180 g.) were used in all experiments. They were fed on No. 41B cubes (Associated London Flour Millers) and allowed drinking water *ad libitum*. The temperature of the room in which the animals were housed was 70±1° F.

Drug Treatment.—Aqueous suspensions of the corticosteroids (10 mg./kg.) were injected daily by the intramuscular route. Cortisone acetate, prednisolone, fludrocortisone, triamcinolone, and deoxycortone acetate were given for 4 and 9 days, whilst dexamethasone and 2-methylfludrocortisone (9α-fluoro-11β,17α, 21-trihydroxy-2α-methylpregn-4-ene-3,20-dione) were given only for 4 days. The formulae of these steroids are shown in Fig. 1. Intramuscular injections of oily solutions of oestradiol monobenzoate, stilboestrol and testosterone propionate (10 mg./kg.) were given on alternate days for 21 days. Long-acting corticotrophin (A.C.T.H., Organon, batch No. 6991) was injected intramuscularly for 9 days in daily doses of 1, 2, and 5 i.u. per rat. Groups of control rats received the corresponding volumes of the suspending fluid, arachis oil, or saline. The animals were killed 24 hr. after the last injection.

Depletion of Tissue Amines.—Repeated intraperitoneal injections of polymyxin B were used to produce maximal depletion of histamine in tissues rich in mast cells (for example, the skin). This treatment also lowers the 5-hydroxytryptamine content of the skin by about 50% (Telford and West, 1960). One group of polymyxin-treated rats was killed 24 hr. after the last injection, while other polymyxin-treated groups received daily doses of either saline or cortisone, prednisolone or deoxycortone (10 mg./kg.) for 9 days before being killed.

Extraction of Tissues and Assay Procedures for Histamine and 5-Hydroxytryptamine

The tissues studied in detail were abdominal skin, ears, dorsal skin of the hind feet, jejunum and pyloric stomach. On occasion, the fundic stomach duodenum, ileum, colon and lung were also used, and in some experiments with prednisolone the whole animal was extracted. Samples of the tissues from

Corticosteroid	Abbrev.	Formula					
DEOXYCORTONE	D	$=H_2$	$=H_2$	$-H$	$=H_2$	$-H$	$=H_2$
CORTISONE	C	$=H_2$	$=H_2$	$-H$	$=O$	$-OH$	$=H_2$
PREDNISOLONE	P	$-H$ double bond	$-H$	$-H$	$-H$ $-OH$	$-OH$	$=H_2$
TRIAMCINOLONE	T	$-H$ double bond	$-H$	$-F$	$-H$ $-OH$	$-OH$	$-H$ $-OH$
DEXAMETHASONE	Dx	$-H$ double bond	$-H$	$-F$	$-H$ $-OH$	$-OH$	$-H$ $-CH_3$
FLUDROCORTISONE	Fc	$=H_2$	$=H_2$	$-F$	$-H$ $-OH$	$-OH$	$=H_2$
2-METHYLFLUDROCORTISONE	MFc	$-CH_3$ $-H$	$=H_2$	$-F$	$-H$ $-OH$	$-OH$	$=H_2$

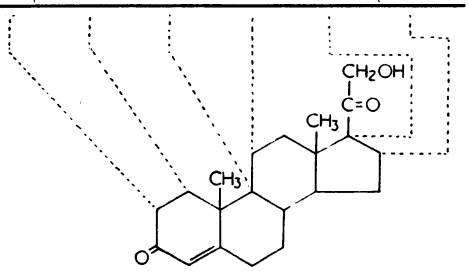

FIG. 1.—Formulae of the steroids used in the present study.

the 4 rats in each group were pooled, and either extracted with trichloracetic acid and assayed on the isolated guinea-pig ileum for histamine or extracted with acetone and assayed on the isolated rat uterus for 5-hydroxytryptamine. The methods have been described in detail elsewhere (Parratt and West, 1957). Each value of histamine and 5-hydroxytryptamine in this paper refers to the base and represents the mean of the results obtained from at least 3 separate experiments. There was considerable variation in the amounts of extractable amine from many tissues. Statistical analysis showed that values differing from the means by more than 25% were significant ($P=0.95$). The values quoted for rat skin in Table III and Figs. 2, 3, 4, 11, and 12 are the means of the 3 values for abdominal skin, ears and dorsal skin of the feet.

The steroids themselves may be extracted by the procedures used. However, concentrations of 10^{-5} of each steroid did not modify the responses of the standard amines on the isolated biological preparations.

Histological Examination. — Fresh tissue spreads from subcutaneous connective tissue, mesentery and

TABLE I

THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT (μG./G.) OF TISSUES OF CONTROL RATS

Tissue	Histamine	5-Hydroxytryptamine
Abdominal skin ..	30·1	1·9
Skin of the feet ..	66·2	1·5
Ears	37·9	1·4
Fundic stomach ..	12·6	1·8
Pyloric stomach ..	31·1	2·1
Duodenum ..	25·2	5·6
Jejunum	25·1	3·2
Ileum	23·8	5·5
Colon	17·6	3·8
Lung	8·0	3·9
Whole rat (μg.) ..	1,194·6	24·3

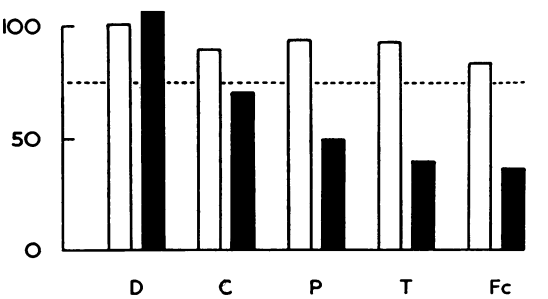

FIG. 2.—Histograms to show the effects of corticosteroids (10 mg./kg. for 4 days) on the histamine (□) and 5-hydroxytryptamine (■) contents of rat skin. All values are expressed as percentages of the control values. Values under 75% denote a loss of amine. Abbreviations of the names of the corticosteroids are as shown in Fig. 1. Note that the more active glucocorticoids depleted the 5-hydroxytryptamine but not the histamine.

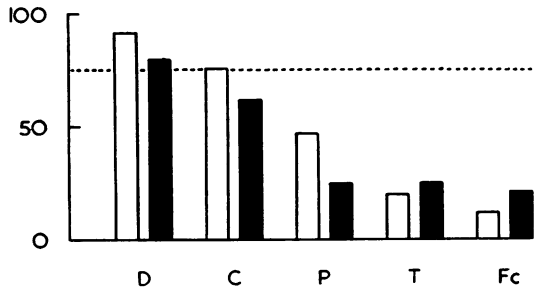

FIG. 3.—As Fig. 2, but corticosteroids were administered for 9 days. Note that the glucocorticoids depleted the skin of both 5-hydroxytryptamine and histamine.

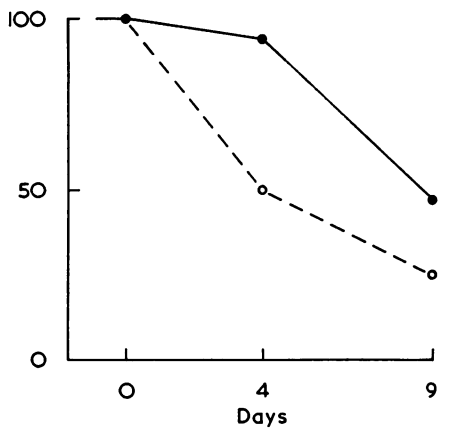

FIG. 4.—The effect of treating rats with prednisolone (10 mg./kg. daily for 4 and 9 days) on the histamine (●——●) and 5-hydroxytryptamine (O - - - O) contents of rat skin. All values are expressed as percentages of the control values. Note that 5-hydroxytryptamine was depleted before histamine.

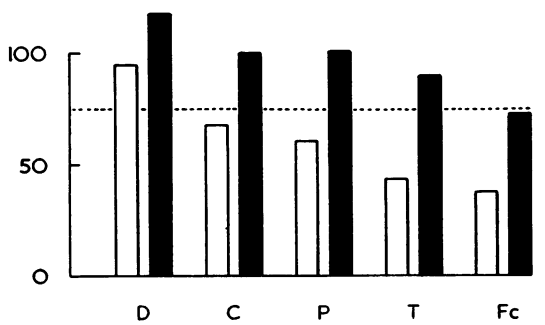

FIG. 5.—Histograms to show the effects of corticosteroids (10 mg./kg. for 4 days) on the histamine (□) and 5-hydroxytryptamine (■) contents of rat jejunum. All values are expressed as percentages of the control values. Values under 75% denote a loss of amine. Abbreviations as in Fig. 1. Note that the more active glucocorticoids depleted the histamine but not the 5-hydroxytryptamine.

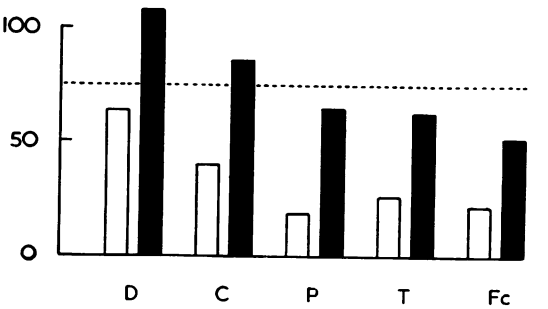

FIG. 6.—As Fig. 5, but corticosteroids were administered for 9 days. Note that the jejunum was depleted of both amines.

pleura, were fixed in dehydrated ethanol. They were then stained with toluidine blue (0.1% w/v aqueous), washed with 50% (v/v) ethanol, and taken through xylene before mounting.

RESULTS

The Histamine and 5-Hydroxytryptamine Content of Rat Tissues

The histamine and 5-hydroxytryptamine content of tissues which are rich sources of these amines in the rat is shown in Table I. The skin generally contains more histamine but less 5-hydroxytryptamine than the gut. It should be noted that the histamine content of the whole animal is about 50 times that of 5-hydroxytryptamine.

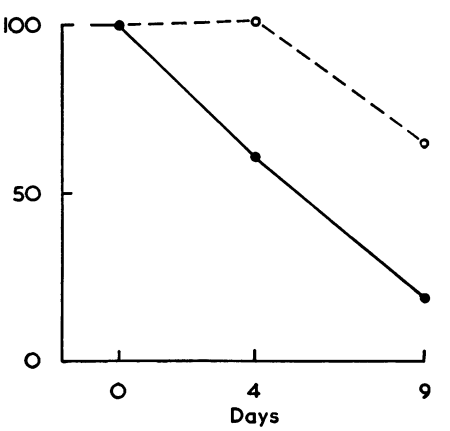

FIG. 7.—The effects of treating rats with prednisolone (10 mg./kg. daily for 4 or 9 days) on the histamine (●——●) and 5-hydroxytryptamine (O - - - O) contents of rat jejunum. All values are expressed as percentages of the control values. Note that histamine was depleted before 5-hydroxytryptamine.

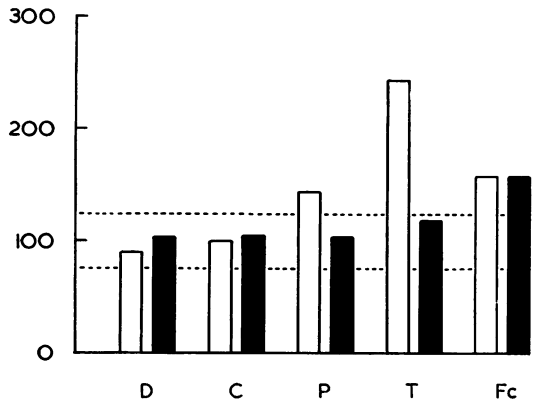

FIG. 8.—Histograms to show the effects of corticosteroids (10 mg./kg. for 4 days) on the histamine (□) and 5-hydroxytryptamine (■) contents of the pyloric stomach of rats. All values are expressed as percentages of the control values. Values over 125% denote an increase in amine content. Abbreviations as in Fig. 1. Note the increase in both amines, particularly the histamine.

The Effects of Some Corticosteroids on the Histamine and 5-Hydroxytryptamine Contents of Rat Tissues

Skin.—Treatment with the corticosteroids for 4 days produced a loss of 5-hydroxytryptamine, but not of histamine, from the skin. The most active steroids were those with the most potent glucocorticoid activity, deoxycortone (a mineralocorticoid) being ineffective (Fig. 2). When treatment was extended to 9 days, not only was there a further reduction in 5-hydroxytryptamine but the skin was also depleted of its histamine. For example, only 12% of the histamine and 21% of the 5-hydroxytryptamine remained in the skin after treatment with fludrocortisone (Fig. 3). With prednisolone the loss of skin 5-hydroxytryptamine preceded that of histamine (Fig. 4).

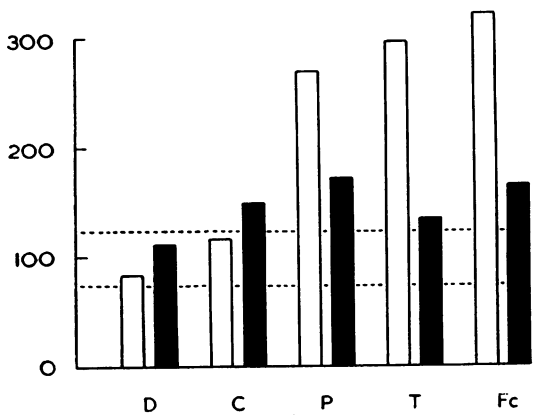

FIG. 9.—As Fig. 8, but corticosteroids were administered for 9 days.

Jejunum.—In this tissue, treatment with the corticosteroids for 4 days produced a loss of histamine but not of 5-hydroxytryptamine. As in the skin, the most active steroids were those possessing most potent glucocorticoid activity (Fig. 5). Deoxycortone was inactive. Extending the treatment to 9 days resulted in a further

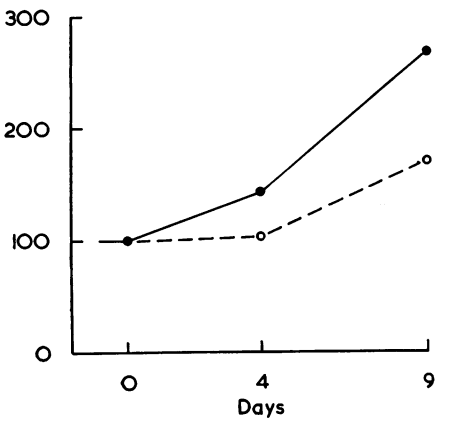

FIG. 10.—The effects of treating rats with prednisolone (10 mg./kg. daily for 4 or 9 days) on the histamine (●——●) and 5-hydroxytryptamine (O - - - O) contents of rat pyloric stomach. All values are expressed as percentages of the control values. Note the increase in both amines, particularly the histamine.

TABLE II

THE EFFECTS OF 9 DAILY DOSES OF PREDNISOLONE (10 MG./KG.) ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

All values are expressed as percentages of the control values.

Tissue	Histamine	5-Hydroxytryptamine
Abdominal skin ..	55	25
Skin of the feet ..	41	28
Ears	46	23
Fundic stomach ..	287	129
Pyloric stomach ..	270	173
Duodenum ..	19	57
Jejunum	19	65
Ileum	16	65
Colon	45	64
Lung	104	66
Whole rat	59	43

TABLE III

THE EFFECTS OF 4 DAILY DOSES OF DEXAMETHASONE, FLUDROCORTISONE AND 2-METHYLFLUDROCORTISONE (10 MG./KG.) ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

All values are expressed as percentages of the control values.

Tissue	Dexamethasone		Fludrocortisone		2-Methylfludrocortisone	
	Histamine	5-Hydroxytryptamine	Histamine	5-Hydroxytryptamine	Histamine	5-Hydroxytryptamine
Skin	85	44	84	37	111	61
Jejunum ..	37	59	38	73	38	99
Pyloric stomach	131	102	158	158	197	90

reduction in the histamine levels and a considerable loss of 5-hydroxytryptamine. For example, only 22% of the histamine and 52% of the 5-hydroxytryptamine remained in the jejunum after treatment with fludrocortisone (Fig. 6). With prednisolone, the loss of histamine in the jejunum preceded that of 5-hydroxytryptamine (Fig. 7). This alteration in the amine content was the reverse of that occurring in the skin.

Pyloric Stomach.—In striking contrast to the effects in the skin and jejunum, the histamine content of the pyloric stomach was markedly raised by treatment with the more potent corticosteroids (Figs. 8 and 9). For example, the histamine content of the pyloric stomach after 9 injections of fludrocortisone was more than 3 times the control value and the 5-hydroxytryptamine content was also raised but to a lesser degree. Deoxycortone was ineffective. The results with prednisolone are plotted graphically in Fig. 10.

Other Tissues.—The effect of prednisolone on the histamine and 5-hydroxytryptamine contents of other rat tissues is shown in Table II. The increase in the histamine content of the fundic stomach was similar to that found in the pyloric stomach, and the decrease in the histamine content of the duodenum and ileum was comparable to that found in the jejunum. The histamine content of the lung, however, was unaffected. The 5-hydroxytryptamine contents of the intestinal tissues and lung were reduced to about 60%.

The Whole Animal. — After 9 doses of prednisolone, both the histamine and the 5-hydroxytryptamine contents of the whole animal were reduced by about 50% (Table II).

The Effects of Dexamethasone and 2-Methylfludrocortisone on the Histamine and 5-Hydroxytryptamine Contents of Rat Tissues

Dexamethasone and 2-methylfludrocortisone produced changes in the tissue amines which closely followed those found after other glucocorticoids (Table III). Thus, the 5-hydroxytryptamine in the skin and the histamine in the jejunum were reduced, and the histamine in the pyloric stomach was slightly increased.

The Effects of Corticosteroids on Tissue Mast Cells

Corticosteroids produced changes in the tissue mast cells of the subcutaneous connective tissue which closely followed the release of histamine from the skin. Marked degranulation or disruption of many of the cells occurred after treatment with the more active compounds. In the mesentery, the most prominent feature was extensive swelling of the mast cells in the "windows," the size of more than one-third being about twice that of mast cells from control animals. The pleural mast

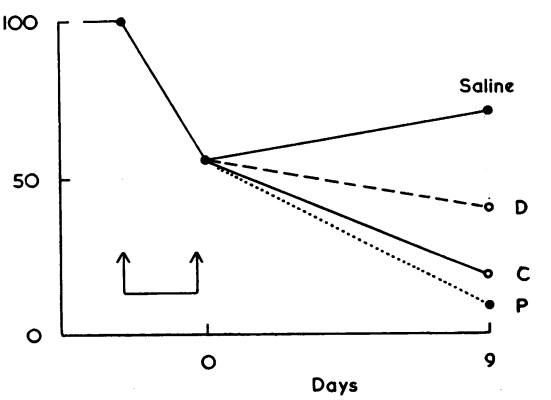

FIG. 11.—The effects of corticosteroids (10 mg./kg. for 9 days) on the recovery rate of skin 5-hydroxytryptamine, after depletion by repeated doses of polymyxin B (between the two arrows). All values are expressed as percentages of the control values. Note that the corticosteroids not only retarded the recovery of skin 5-hydroxytryptamine but lowered the value still further.

TABLE IV

THE EFFECTS OF SEX HORMONES (10 MG./KG. ON ALTERNATE DAYS FOR 21 DAYS) ON THE HISTAMINE AND 5-HYDROXYTRYPTAMINE CONTENT OF RAT TISSUES

All values are expressed as percentages of the control values.

Tissue	Oestradiol		Stilboestrol		Testosterone	
	Hist-amine	5-Hydroxy-tryptamine	Hist-amine	5-Hydroxy-tryptamine	Hist-amine	5-Hydroxy-tryptamine
Abdominal skin	91	30	94	40	93	56
Skin of the feet	93	38	104	48	85	62
Ears	65	29	113	21	91	46
Jejunum ..	110	57	87	72	78	54
Pyloric stomach	129	97	172	99	300	189

cells, however, showed little or no change after prednisolone treatment, and there was no histamine release from the lungs.

The Effect of Corticosteroids on Adrenal Weight and Body Weight

Treatment with the more active glucocorticoids for 9 days resulted in a steady loss of body weight of the animals. The loss with prednisolone, for example, was 18%, with fludrocortisone it was 21%, and with triamcinolone it was 26%. There was no change in body weight after cortisone treatment, whilst rats receiving deoxycortone gained weight at the same rate as did the control animals. The total adrenal weights, expressed as percentages of body weight, also decreased under treatment with the more active glucocorticoids.

The Effects of Corticotrophin on the Histamine and 5-Hydroxtryptamine Contents of Rat Tissues

Corticotrophin in the doses used increased the weight of the adrenal glands by over 300% but did not change either the histamine content or the 5-hydroxytryptamine content of any of the rat tissues studied.

The Effects of Sex Hormones on the Histamine and 5-Hydroxytryptamine Contents of Rat Tissues

Treatment with a natural female sex hormone (oestradiol), a synthetic female sex hormone (stilboestrol), and a male sex hormone (testosterone) lowered the skin 5-hydroxytryptamine without markedly altering the skin histamine. The changes in the amine contents of the jejunum were small, but there were increases in the histamine content of the pyloric stomach (Table IV). These changes are similar to those found after short-term treatment with the glucocorticoids.

The Effect of Corticosteroids on the Histamine and 5-Hydroxytryptamine Contents of Tissues of Rats Pre-treated with Polymyxin B

The rate of recovery of skin histamine after depletion by repeated doses of polymxin B was

TABLE V

COMPARISON OF THE EFFECTIVENESS OF CORTICOSTEROIDS IN ALLERGY AND INFLAMMATION WITH THEIR GLUCOCORTICOID AND MINERALOCORTICOID ACTIVITIES, AND WITH THEIR EFFECTS ON HISTAMINE AND 5-HYDROXYTRYPTAMINE METABOLISM

All values are expressed on a relative scale from 0 to +++.

Corticosteroid	Allergy and Inflammation	Glucocorticoid Activity	Mineralocorticoid Activity	Histamine and 5-Hydroxytryptamine Metabolism
Cortisone	+	+	0	+
Prednisolone	++	++	0	++
Fludrocortisone	++	+++	+	+++
Triamcinolone	++	+++	0	+++
Dexamethasone	++	+++	0	+++
2-Methylfludrocortisone ..	++	+++	+++	+++
Deoxycortone	0	0	+	0

not significantly altered by treatment with prednisolone, deoxycortone or cortisone. The rate of recovery of skin 5-hydroxytryptamine, however, was retarded not only by treatment with prednisolone or with cortisone but also by treatment with deoxycortone. Polymyxin reduced the skin 5-hydroxytryptamine by about 50%, and repeated doses of the corticosteroids lowered the values even further (Fig. 11).

Discussion

The pronounced changes occurring in the histamine and 5-hydroxytryptamine contents of rat tissues after injections of synthetic corticosteroids support the hypothesis of Hicks and West (1958a) that the tissue reserves of these amines may in part be regulated by the secretion of the adrenal cortex. The adrenal steroids most active in producing changes in the amine contents of tissues are those possessing greatest glucocorticoid activity, and no correlation has been found between the mineralocorticoid activity of corticosteroids and their effects on tissue amine levels (see Table V). The most potent glucocorticoids used in the present experiments, for example, are triamcinolone, dexamethasone, fludrocortisone and 2-methylfludrocortisone, and these are the most potent depletors of histamine and 5-hydroxytryptamine from the skin and small intestine. Prednisolone is weaker in these respects, yet it is more active than cortisone; deoxycortone, a mineralocorticoid, is inactive. 2-Methylfludrocortisone, which possesses both marked mineralocorticoid and marked glucocorticoid activity, is no more active in producing changes in the amine contents of tissues than other synthetic steroids of similar glucocorticoid potency.

Adrenal cortical steroids are now widely used in the treatment of allergic and inflammatory states. The steroids most effective in these conditions are those which influence the metabolism of carbohydrates. The present results show that the relative effectiveness of the adrenal cortical steroids by injection in allergic states, or by topical application in skin diseases, parallels not only their effectiveness in regulating carbohydrate metabolism but also in reducing the histamine and 5-hydroxytryptamine contents of tissues of the rat such as the skin and small intestine (Table V). The most potent analogues of cortisone have produced depletion of histamine in several tissues which is as great as that produced by the most potent of the chemical histamine liberators. As histamine is one of the mediators of the allergic and inflammatory response, the therapeutic effects of the adrenal cortical steroids in these conditions may be explained in part by their action on histamine metabolism whereby the tissue reserves of this amine are lowered.

The doses of glucocorticoids used clinically in the treatment of allergic diseases are unphysiological, as also are the doses used in the present experiments. Yet the action of any one glucocorticoid in producing depletion of the tissue amines is not always proportional to the dose given. Doses of 0.5 and 1 mg./kg., for example, have been found to produce changes in the histamine and 5-hydroxytryptamine contents of the tissues which are similar to those found after 10 mg./kg., and increasing the dose to 50 mg./kg. (Hicks and West, 1958a) also does not increase the effects.

The tissues chosen for study are all rich sources of histamine and 5-hydroxytryptamine in the rat, but the distribution of the enzymes responsible for the formation and destruction of these amines varies from tissue to tissue. For example, the pyloric stomach is the most potent source of histidine decarboxylase whilst the jejunum is one of the richest sources of histaminase; the skin, however, contains little of either enzyme. Schayer (1956), using tracer techniques, has reported that cortisone increases the histamine-binding capacity of the pyloric stomach, but reduces the histidine decarboxylase activity in the lung and strongly decreases the rate of binding of new histamine in the skin. Recently, Schayer (1960) has postulated that there are at least two types of histidine decarboxylase, one producing histamine which is bound in mast cells and one producing free histamine. Cortisone and the more active adrenal cortical steroids used in the present experiments may influence one of the types preferentially. In fact, the glucocorticoids may stimulate the histidine decarboxylase activity of the pyloric stomach so that the histamine content of this tissue is increased, but reduce the formation of histamine in other tissues.

The effects of adrenalectomy on the histidine decarboxylase and histamine-binding activities of rat tissues are the reverse of those obtained after injections of cortisone (Schayer, 1956). The decrease in the histamine-binding activity of the pyloric stomach, however, does not involve a decrease in histidine decarboxylase activity (Schayer, 1957). Earlier work by Karady, Rose, and Browne (1940) had shown that adrenalectomy in rats results in a loss of histamine activity in the lung and in the small intestine. Thus, the action of adrenal cortical steroids on histamine metabolism appears to involve at least three systems: (1) the formation of histamine as a result of

histidine decarboxylase activity, (2) the binding of histamine with other tissue components, and (3) the inactivation of histamine as a result of histaminase activity. In a similar way, the steroids may modify the formation, binding and inactivation of 5-hydroxytryptamine, since the changes in the tissue levels of this amine are comparable with those of histamine. Studies are now being made on the action of adrenal cortical steroids on the enzymes responsible for the formation and inactivation of both of these amines.

Changes in the appearance of the mast cells occurring during the treatment with glucocorticoids resemble those found when histamine liberators are given. Asboe-Hansen (1952) using cortisone obtained similar results. This author is of the opinion that mast cells are the source of hyaluronic acid in the connective tissue ground substance, and he has suggested that the damaging effect of glucocorticoids on the tissue mast cells may partly explain their clinical value in connective tissue diseases, where the hyaluronic acid content is increased. Several authors (see Asboe-Hansen, 1958) have reported that treatment with corticotrophin likewise reduces the number of mast cells, yet in the present work there were no major changes in the size, shape or number of these cells, nor were there any alterations in the histamine and 5-hydroxytryptamine contents of the tissues. Schayer (1956) also failed to detect any changes in the histidine decarboxylase or histamine-binding activities of various rat tissues after treatment with corticotrophin. The sex hormones, which possess weak adrenal cortical-like activity, similarly did not alter the mast cell population.

Long-term treatment for 9 days with prednisolone, fludrocortisone, and triamcinolone resulted in gastric ulcers in several rats, and also caused petechial haemorrhages in the jejunum and ileum. Histamine is an effective stimulus of gastric secretion of high acidity, and it is thus possible that the increase in the histamine content of the pyloric stomach after treatment with glucocorticoids is the cause of the gastric haemorrhage. Clinically, an important side-effect of glucocorticoid therapy is irritation of the gastric mucosa.

We wish to thank Dr. R. P. Edkins of Organon Laboratories for generous supplies of cortisone acetate and prednisolone. 2-Methylfludrocortisone was kindly supplied by Upjohn of England.

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THE ANATOMICAL BASIS OF THE STRAUB PHENOMENON

BY

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(RECEIVED MAY 31, 1960)

The administration of morphine was followed in white mice by a typical Straub reaction which consisted of the tail becoming rigid and erected across the back of the animal in an S-shaped curve. This reaction was accompanied by restlessness, excitability, extension rigidity of the hindlimbs, forcible viodance of faeces and prominence of the perineum. The Straub reaction was abolished by general anaesthesia with pentobarbitone or ether, by administration of tubocurarine, by bilateral section of the muscles causing extension to the tail, and by the removal of the circulation to the lower extremity. The reaction was modified by unilateral section of the extensor muscles of the tail. Section of the spinal cord, decortication, division of the anal sphincter and perineal floor, or ablation of the pelvic splanchnic nerves did not suppress the appearance of the Straub response. It was concluded that the phenomenon described by Straub (1911) was produced mainly by the action of the sacro-coccygeus dorsalis muscle, and that it was also necessary that the lumbo-sacral cord with its peripheral nervous outflow should be intact and that these functioning units should have an adequate circulation.

Straub (1911) described the sensitive biological reaction for morphine which bears his name. He stated that " when white mice are injected with a small quantity of morphine under the skin of the back, their tails go into a condition of catatonic rigidity which is manifested in such a way that, during the strongest dorsiflexion, the tail is in a position almost parallel to the vertebral column." Subsequent investigators have confirmed and extended these observations. Herrmann (1912) called attention to restlessness, reflex excitability, extension paralysis of the hindquarters, lordosis of the vertebral column and an S-shaped curve of the tail. Van Leersum (1918) reported that defaecation occurred soon after the injection of morphine and that the perineum became prominent. The explanations for the phenomenon found in current textbooks of pharmacology show some disregard for anatomical and functional considerations, particularly in connexion with the skeletal and visceral musculature involved. Gaddum (1955) states that " morphine has a peculiar effect on mice, making them hold their tails erect owing to spasm of the anal and vesical sphincters," and similar explanations are given by Grollman (1958) and Sollman (1957). These explanations appear to stem from the work of Macht (1920), who confused skeletal with smooth muscle activity, and who credited Van Leersum (1918) with showing that " the stiffening of the tail was really due to spasm of the sphincters." The present experiments were carried out in order to investigate the anatomical basis for the Straub and associated reactions which take place in white mice after the administration of morphine.

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METHOD

Thirty-six healthy male and female white mice (T.O. Swiss Strain) of 25 to 30 g. body weight were divided into groups each of 3 animals. Operative procedures where necessary were carried out under sterile surgical conditions using ether anaesthesia. Prior to each observation, the mouse was injected subcutaneously with a solution of morphine hydrochloride at a dose equivalent to 100 mg./kg. of body weight. The observations were continued for a period of 1 hr. following each injection.

RESULTS

Group 1

No treatment and no surgical interference was given to these animals. A typical Straub reaction was always obtained (Fig. 1). Within 30 sec.

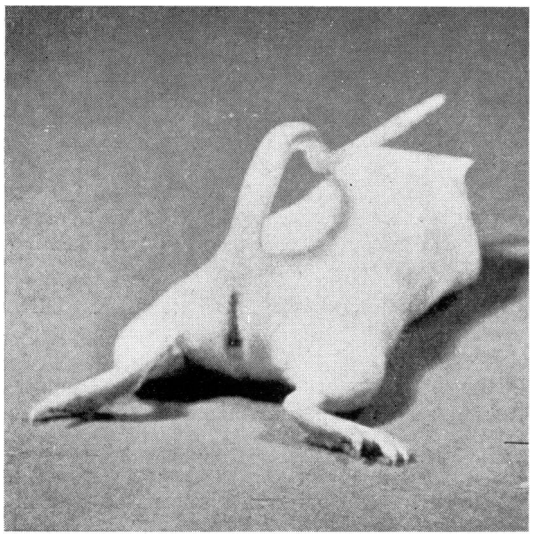

FIG. 1.—The appearance of a white mouse following an injection of morphine. The erection, curvature and disposition of the tail was described by Straub. Other prominent features shown here are the extension paralysis of the hindlimbs, lordosis of the vertebral column, and the prominence of the perineum.

FIG. 2.—Tensing of perineum and voiding of faeces immediately following injection of morphine.

of injection, the animal became restless; between 2 and 5 min., the tail stiffened and usually took up an S-shaped curvature, and the hindlimbs showed extension rigidity, the animal looking as if it were "stepping on tip-toe." Faeces were voided, the anus protruded slightly, and the perineum became tense (Fig. 2).

Group 2

Before the injection of morphine, each animal was anaesthetized by an intraperitoneal injection of 32 mg./kg. of body weight of sodium pentobarbitone (Nembutal). No Straub reaction occurred under general anaesthesia. If the animal regained consciousness within 10 to 15 min. of the morphine injection, the phenomenon developed. If the period required for the return of consciousness was longer the response was not seen.

Group 3

These animals were anaesthetized with ether before the administration of morphine. No responses were observed while the animals were unconscious. However, a Straub phenomenon gradually appeared as consciousness returned.

Conversely, animals exhibiting the Straub reaction lost the tail rigidity and other features of the phenomenon as soon as deep general anaesthesia was attained.

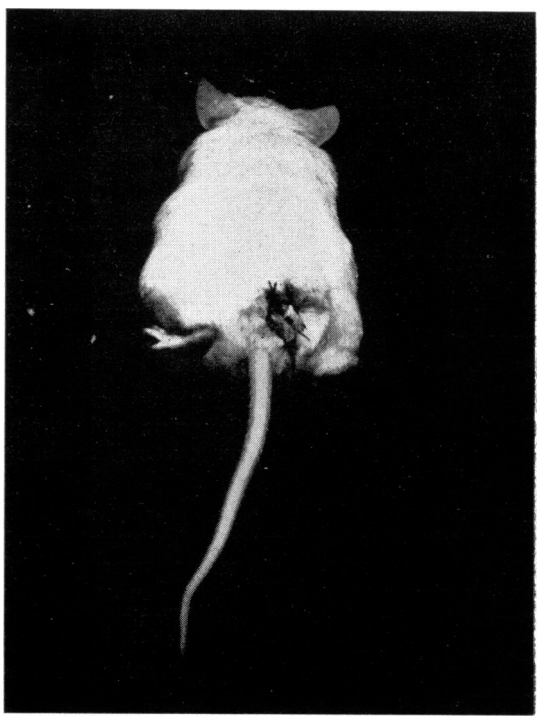

FIG. 3.—Bilateral cutting of the sacro-coccygeus dorsalis muscle has prevented the erection of the tail in the typical Straub reaction.

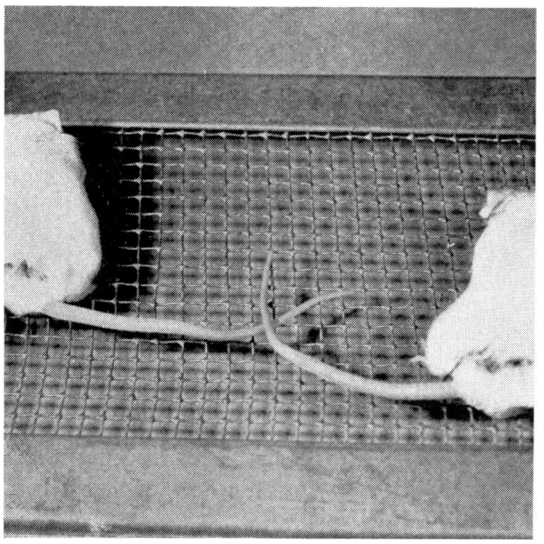

FIG. 4.—After unilateral cutting of the sacro-coccygeus dorsalis muscle, administration of morphine was followed by a deviation of the tail to the side of the intact muscle.

Group 4

Following either subcutaneous or intramuscular injection of tubocurarine (0.0075 mg./animal), the Straub phenomenon could not be elicited. Treatment of an animal showing the Straub reaction with tubocurarine abolished the response.

Group 5

The extensor muscles and tendons to the tail on both sides were sectioned at their attachment to the vertebral column. The stiffening and S-shaped curving of the tail was abolished (Fig. 3), although the other features of the Straub reaction remained.

Group 6

The extensor muscles serving only one side of the tail were sectioned. Stiffening and curving of the tail occurred towards the side of the intact muscles (Fig. 4).

Group 7

The spinal cord was cut across at various levels at the lower thoracic and lumbar segments. Following morphine administration the animal became excited and a moderate stiffening and curling of the tail took place. The hindlimbs which were paralysed went into fuller extension than normal.

Group 8

Decortication did not prevent morphine causing the Straub reaction. The animals became markedly excited and an increased extensor rigidity occurred. Extension paralysis was more prominent than in the intact animal, and was followed by spasms and convulsions.

Group 9

The anal sphincter was divided by cuts along the long axis. Morphine administration was followed by a Straub reaction, but without forcible voidance of faeces or protrusion of the anus.

Group 10

The muscles associated with the perineal floor were cut on both sides of the pelvic outlet. Following morphine, a Straub reaction developed, but there was neither protrusion of the anus nor prominence of the perineum.

Group 11

The pelvic splanchnic nerves were destroyed by the clearance of fascia in the pelvic floor and posterior abdominal wall. When morphine was given, a Straub reaction associated with anal and perineal responses occurred.

Group 12

The abdominal aorta and inferior vena cava were ligated just above the bifurcation of the common iliac vessels. This produced immediate cyanosis of the tail and lower extremities, followed by local paralysis. The Straub reaction could not then be elicited.

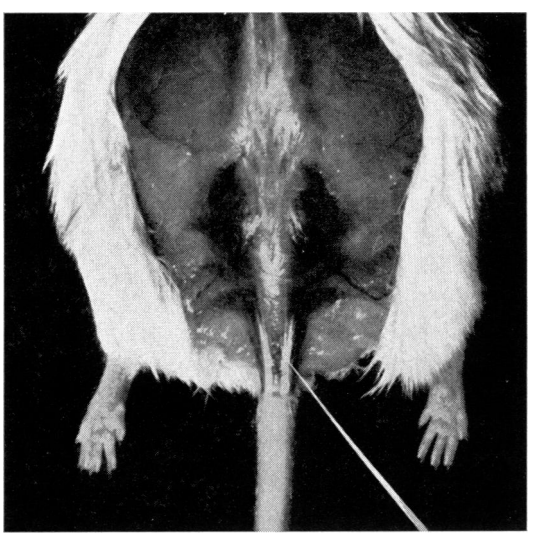

FIG. 5.—Dissection to show the sacro-coccygeus dorsalis muscle which is responsible for elevation and rigidity of the tail. The pointer indicates the tendons leading from this muscle itself which appears as the darkened mass on each side of the vertebral column.

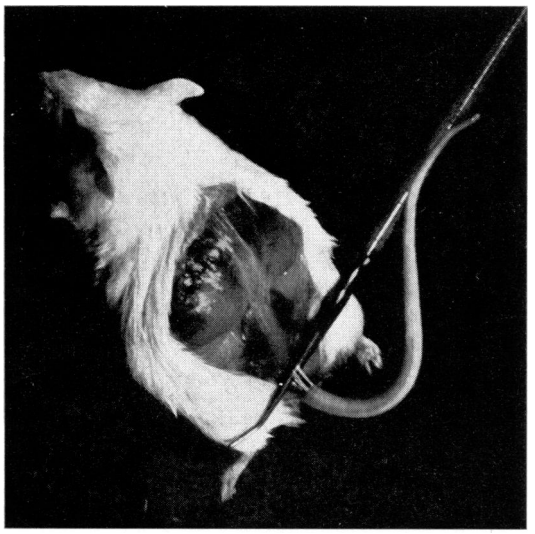

FIG. 6.—Tension on the tendons of the sacro-coccygeus dorsalis muscle has caused the tail to assume a position closely similar to that seen after morphine administration.

DISCUSSION

These experiments provide evidence that the phenomenon described classically by Straub (1911) is dependent upon two primary anatomical units. One neural, composed of segments of the lumbo-sacral cord, the lower motor neurone outflow and functioning motor end-plates. The other musculo-skeletal, consisting of intact musculature to the tail with tendinous attachments to a multi-articulated skeleton.

The muscle specifically responsible for the rigidity, dorsi-extension, and S-shaped curving of the tail was the sacro-coccygeus dorsalis muscle (Fig. 5). Contraction of this muscle gave rise to the typical appearance of the tail following morphine administration (Fig. 6).

Our experiments showed that the constriction of the anal and vesicle sphincters does not appear to be the cause of the Straub phenomenon. However, anal and perineal responses did occur after injection of morphine into white mice, but formed only part of a much wider pattern of muscular activity.

These findings are in agreement with those of Heinekamp (1923), who concluded that "the Straub biologic test is due to direct stimulation of the spinal cord." Furthermore, from electro-encephalographic studies, Leimdorfer (1948) found evidence that the elevation of the tail was a reflex phenomenon originating in the spinal cord. He suggested that it was likely that morphine facilitated an increased activity along the cerebro-spinal pathways.

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BIOLOGICAL ACTIVITY OF SYNTHETIC POLYPEPTIDES WITH BRADYKININ-LIKE PROPERTIES

BY

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The biological activity of synthetic polypeptides containing the amino acids of natural pure trypsin-bradykinin and snake-venom-bradykinin has been investigated. A series of tests for bradykinin-like activity in stimulating plain muscle, depressing the blood pressure and increasing capillary permeability was used on various species. A nonapeptide with the following structure: H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH elicited qualitatively and quantitatively the effects of the pure natural bradykinins. An octapeptide with the following structure: H-L-Arg-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH also exerted bradykinin-like effects but was 50 to 100 times less active than the nonapeptide. Three other octapeptides and a heptapeptide were without any significant effect. Further work will demonstrate if the nonapeptide A is synthetic bradykinin or a peptide with bradykinin-like activity.

Note added since submission of this paper: The data from this investigation were personally precommunicated to Elliott, Lewis, and Horton, who have since found that the structure of pure trypsin-bradykinin is identical with the structure of the nonapeptide A. Therefore, this synthetic nonapeptide is in fact synthetic bradykinin.

Since the discovery of bradykinin, its polypeptide nature has been suggested and amply verified (Rocha e Silva, Beraldo and Rosenfeld, 1949; Rocha e Silva, 1955, 1960). The amino acid composition of bradykinin prepared by the action of trypsin on the fraction of ox plasma precipitating between 35 and 45% of saturation by ammonium sulphate (trypsin-bradykinin) was recently studied by Elliott, Lewis, and Horton (1960a) following its isolation; according to these investigators the molecule of trypsin-bradykinin contains two moles each of arginine, phenylalanine, and proline, and one mole each of glycine and serine. Recently Elliott, Lewis, and Horton (1960b) put forward an octapeptide structure for bradykinin with the amino acid sequence C in Table I.

TABLE I
STRUCTURE OF POLYPEPTIDES A TO F

H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH	A
H-L-Arg-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH	B
H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Phe-L-Arg-OH	C
H-L-Arg-L-Pro-L-Pro-L-Phe-Gly-L-Ser-L-Phe-L-Arg-OH	D
H-L-Arg-L-Pro-Gly-L-Pro-L-Phe-L-Ser-L-Phe-L-Arg-OH	E
H-L-Arg-L-Pro-Gly-L-Phe-L-Ser-L-Phe-L-Arg-OH	F

On this basis, Boissonnas, Guttmann, and Jaquenoud (1960a, b) have synthesized this octapeptide and five other related polypeptides (Table I). Whereas the polypeptide having the structure proposed for bradykinin (C in Table I) showed practically no bradykinin-like activity, another octapeptide with the same amino acids but in a different sequence (B in Table I) was active (Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer, 1960). Still more activity was exhibited by a nonapeptide: H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH containing three proline residues instead of two (A of Table I, Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer, 1960; Boissonnas, Guttman, and Jaquenoud, 1960a).

The investigations described below are mainly concerned with a description of the biological activity of this potent nonapeptide and other related peptides synthesized by Boissonnas, Guttmann, and Jaquenoud (1960a, b). A brief description of some of the findings has already been

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published (Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer, 1960).

The new peptides were tested on a number of biological preparations known to be sensitive to bradykinin (Rocha e Silva, 1952, 1955, 1960; Holdstock, Mathias, and Schachter, 1957; Hamberg, 1959; Collier, Holgate, Schachter, and Shorley, 1959; Elliott, Horton, and Lewis, 1960).

METHODS

Materials.—The polypeptides A to F (as hydrochlorides) in an analytically pure state were kept in stock solutions in water containing 1 mg. or 0.1 mg. of free peptide per ml. in a refrigerator at +4°. Saline was used for further dilutions.

In some experiments a comparison was made with a preparation of natural trypsin-bradykinin prepared by Dr. J. F. Pechère from bovine plasma by incubation with trypsin and partly purified by a modification of the method of Hamberg and Deutsch (1958); in other experiments acetylcholine chloride, histamine dihydrochloride, adrenaline hydrochloride, and pure synthetic oxytocin were used for comparison. Atropine sulphate and thenalidine tartrate were sometimes employed to differentiate effects due to bradykinin from those elicited by acetylcholine and histamine. The doses refer to the salts.

Isolated Smooth Muscles.—Preparations of ileum and seminal vesicle of the guinea-pig and of rabbit duodenum were suspended in Tyrode solution at 37°.

Preparations of rat uterus were suspended in de Jalon solution at 30°. On the day preceding the experiment the rats were treated with 0.2 mg. stilboestrol subcutaneously.

The organ baths for the guinea-pig ileum and the rat uterus contained 10 ml. solution; those for the other organs contained 50 ml.

Smooth Muscles in vivo.—To record bronchoconstrictor action the overflow method of Konzett and Rössler (1940) with positive-pressure ventilation was used on guinea-pigs anaesthetized with urethane (1.2 g./kg. intraperitoneally) and on spinal cats.

Arterial Blood Pressure.—For anaesthesia a mixture of chloralose (0.05 g./kg.) and urethane (0.4 g./kg.) was given subcutaneously in cats and intravenously in dogs; rabbits received 1.6 g./kg. urethane subcutaneously; rats were injected with 2.0 g./kg. urethane subcutaneously; guinea-pigs were given 1.2 g./kg. urethane intraperitoneally; roosters were anaesthetized with 0.2 g./kg. phenobarbitone sodium intramuscularly.

Injections were made into the femoral, jugular, or brachial vein; arterial blood pressure was recorded from a carotid, femoral, or sciatic artery by means of a mercury manometer.

In some experiments on the cats the renal volume (left kidney) was recorded plethysmographically by means of a Roy oncometer.

In some cats and dogs the respiration was recorded concomitantly with the blood pressure either by means of a Gildemeister (1922) apparatus (gas meter) or simply by a rubber cuff lying around the lower thorax and connected to a Marey tambour.

Capillary Permeability.—Guinea-pigs were given 0.2 ml. of a 2% solution of Evans blue into the saphenous vein; 1 hr. later, intradermal injections of the test substances (0.1 ml.) were made on the back at positions approximately 10 mm. from the midline. To estimate the effect of various substances and doses, the animals were killed and depilated 1 hr. after injection, and a comparison was made of the intensity of the blue colouring around the injection site.

RESULTS

Effects on Smooth Muscle

In vitro *Preparations.*—The nonapeptide A produced a delayed slow contraction of all smooth muscle structures so far investigated. The *guinea-pig ileum* seemed the most suitable for an assay, since it was fairly sensitive and graded responses were available over a reasonable range of concentrations. Nonapeptide A was effective when given in doses of 1 ng./ml. or more. Fig. 1 shows the slow contraction of the guinea-pig ileum elicited by nonapeptide A as compared with the quick contraction produced by histamine

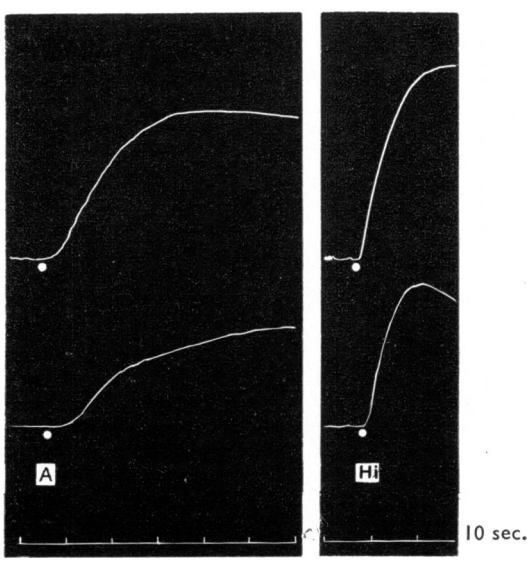

FIG. 1.—Isolated guinea-pig ileum; 10 ml. bath. Responses of two pieces of ileum to 16 ng./ml. of nonapeptide A (A) and histamine (Hi); drum speed 78 mm./min. Note the difference between the delayed contractions after nonapeptide A and the quick contractions after histamine. Time: 10 sec.

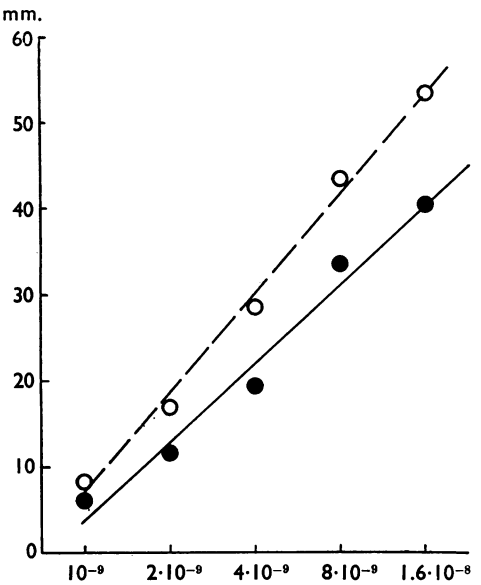

FIG. 2.—Dose-response curve for the nonapeptide A ●——● and histamine O——O on the isolated guinea-pig ileum. Each point represents the mean of 13 to 21 experiments.

(submaximal doses of both compounds). Dose-response curves of nonapeptide A and (for comparison) of histamine under the same conditions are depicted in Fig. 2. The curves lie close together, and their slope differs only slightly. In both instances there is a straight-line relationship between the log dose and the response. Nonapeptide A was nearly as active as histamine (weight for weight).

On the guinea-pig ileum, octapeptide B gave the same type of response as nonapeptide A, but was about 100 times less active.

The peptides C, D, E in doses up to 10 μg./ml. and the peptide F up to 2.5 μg./ml. were without effect on the guinea-pig ileum.

Atropine 10 ng./ml. and an antihistamine (thenalidine) 20 ng./ml. added to the bath for 5 min. completely abolished the effects of submaximal doses of acetylcholine or histamine respectively and reduced the effect of the peptides A and B by about 40%. The same observation was made with the partly purified bradykinin preparation. These inhibitory effects of atropine and of the antihistamine are not specific, since a reduction of the same magnitude was also seen when histamine was given after atropine or acetylcholine was added after the antihistamine.

However, the effect of the peptides A and B was completely abolished when they were boiled with concentrated hydrochloric acid. Also, incubation with undiluted guinea-pig serum 0.6 μg. of A or 60 μg. of B in 0.5 ml. saline + 0.1 ml. serum for 30 sec. for A and 15 min. for B abolished the effects of these peptides on the guinea-pig ileum.

The *duodenum of the rabbit* was also sensitive to nonapeptide A, although the response varied from preparation to preparation to a greater extent than in the case of the guinea-pig ileum. Nonapeptide A was effective in doses of 1 ng./ml. Fig. 3 shows—following an initial relaxation—the slow contraction after nonapeptide A and the quick contraction after acetylcholine. Small

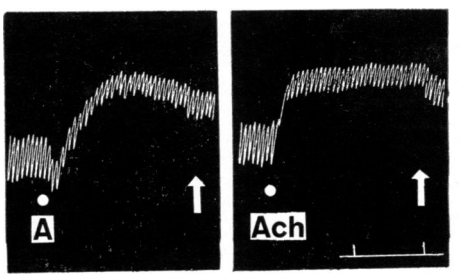

FIG. 3.—Isolated rabbit duodenum; 50 ml. bath. Responses to 2.5 ng./ml. nonapeptide A (A) and to 5 ng./ml. acetylcholine (Ach). Drum speed 14 mm./min. At the arrows: washing out. Note the slow contraction after nonapeptide A and the quick contraction after acetylcholine. Time: 1 min.

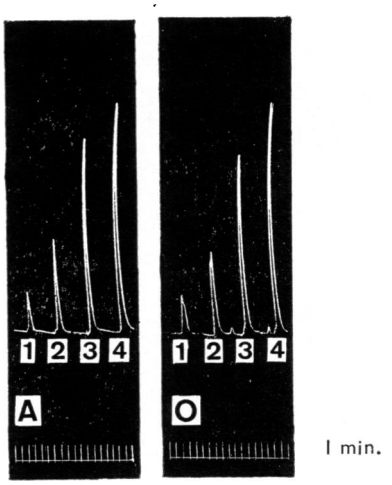

FIG. 4.—Isolated rat uterus; 10 ml. bath. Contractions after increasing doses of nonapeptide A (A) and pure synthetic oxytocin (O). Doses of A: 1= 0.1 ng./ml.; 2=0.13 ng./ml.; 3=0.2 ng./ml.; 4= 0.33 ng./ml. Doses of O: 1=0.044 ng./ml.; 2= 0.066 ng./ml.; 3=0.088 ng./ml.; 4=0.11 ng./ml. Time: 1 min.

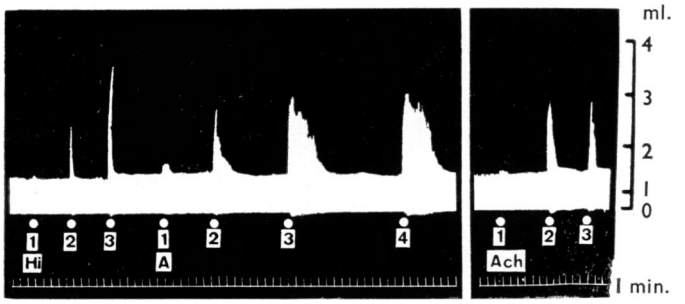

FIG. 5.—Reduction of tidal air induced by nonapeptide A (A), histamine (Hi), and acetylcholine (Ach) in the guinea-pig (0.5 kg.). Doses of Hi: 1=2 μg./kg.; 2=4 μg./kg.; 3=8 μg./kg. Doses of A: 1=0.1 μg./kg.; 2=0.2 μg./kg.; 3=0.4 μg./kg.; 4=0.8 μg./kg. Doses of Ach: 1=10 μg./kg.; 2=20 μg./kg.; 3=40 μg./kg. Time: 1 min.

doses of both compounds were used. Owing to the spontaneous pendulum movements, quantitative determination is not as easy on the rabbit duodenum as on the guinea-pig ileum. A bracketing procedure in which nonapeptide A and acetylcholine were given alternately to pieces of the duodenum showed that the activity of nonapeptide A was similar to that of acetylcholine (weight for weight): sometimes it was more active and sometimes it was less active. No exact quantitative comparison of both compounds was made.

The most sensitive preparation so far known is the *rat uterus*, which responded to doses of 0.03 ng./ml. nonapeptide A. However, the dose-response relationship on the rat uterus is occasionally not as good over a wide range of doses as is that on the guinea-pig ileum. In Fig. 4 the effects of increasing doses of nonapeptide A and of oxytocin are shown. Oxytocin was, on a weight basis, usually more active than nonapeptide A (Fig. 4). Dose-response curves of nonapeptide A and of oxytocin showed a straight-line relationship between the log dose and the response. However, the line belonging to oxytocin is steeper.

Octapeptide B also elicited contractions of the rat uterus. Comparable effects were observed after doses which were 70 times greater than those of nonapeptide A.

The peptides C, D, and E were without effect on the rat uterus in doses up to 10 μg./ml. The peptide F was not tested on the rat uterus.

The preparation least sensitive to nonapeptide A was the *seminal vesicle of the guinea-pig*. 0.5 μg./ml. was needed to elicit a contraction. In this respect nonapeptide A was about as active as adrenaline (weight for weight).

In vivo *Preparations*.—Nonapeptide A produced a decrease in tidal air in anaesthetized guinea-pigs (6 experiments) and spinal cats (4 experiments). This effect on the distensibility of the lungs is in all probability due to bronchoconstriction (Konzett, 1956).

The guinea-pig lungs were rather sensitive; an effect was noted with doses as small as 0.2 μg./kg. (see Fig. 5). Increasing the dose of nonapeptide A enhanced either the duration or the degree of the effect.

Under comparable conditions histamine was (weight for weight) approximately 20 times less active than nonapeptide A and acetylcholine was 100 times less active (Fig. 5). In contrast to nonapeptide A, even high doses of histamine and acetylcholine did not produce a long-lasting effect.

Octapeptide B was also active in the guinea-pig preparation. The doses needed were about 50 times greater than that of nonapeptide A.

In spinal cats the doses of nonapeptide A required to decrease the tidal air were higher than these in guinea-pigs. Less than 10 μg./kg. was usually inactive. Fig. 6 shows the effect of increasing doses (12.8 to 102.4 μg./kg.) of nonapeptide A in a sensitive preparation. The effect of 1 μg./kg. histamine is also shown to illustrate

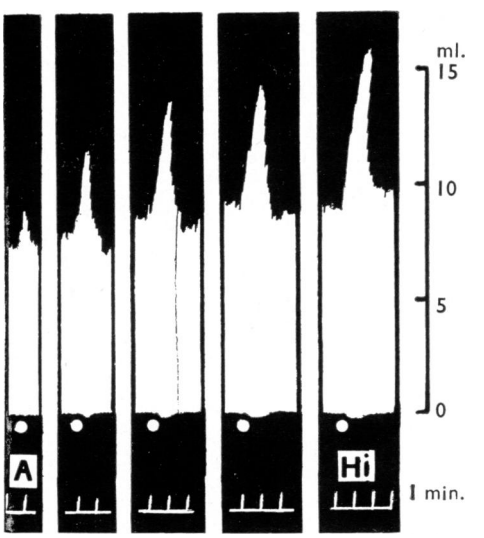

FIG. 6.—Reduction of tidal air induced by nonapeptide A (A) and histamine (Hi) in the spinal cat (2.6 kg.). Doses of A (from left to right): 12.8 μg./kg., 25.6 μg./kg., 51.2 μg./kg., and 102.4 μg./kg.; dose of histamine: 1 μg./kg. Time: 1 min.

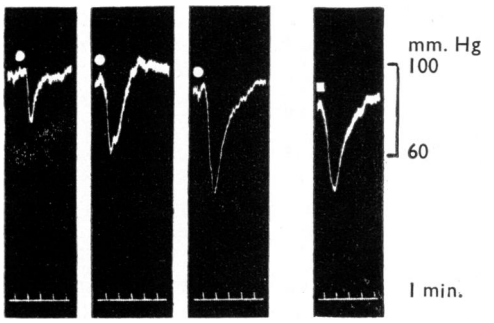

FIG. 7.—Effect of nonapeptide A (●) and of acetylcholine (■) on the blood pressure of the rabbit (3.7 kg.). Doses of nonapeptide A (from left to right): 0.05 μg./kg., 0.1 μg./kg., and 0.2 μg./kg.; dose of acetylcholine: 0.2 μg./kg. Time: 1 min.

the different sensitivity of the bronchial muscle of the spinal cat to the two compounds. In contrast to the guinea-pig, histamine was here more active (approximately 100 times) than nonapeptide A.

Effects on the Arterial Blood Pressure of Different Species

Nonapeptide A lowered the arterial blood pressure of all mammals so far investigated, namely, cat (13 experiments), dog (6 experiments), rabbit (4 experiments), rat (4 experiments), and guinea-pig (6 experiments). In higher doses it even reduced the blood pressure of the chicken (4 experiments).

A comparison of the smallest doses needed to elicit a definite fall in blood pressure in the different species showed the rabbit to be the most sensitive animal towards nonapeptide A (see Fig. 7); 0.05 μg./kg. elicited a barely detectable fall in blood pressure (in 3 of 4 experiments). In the dog, rat, and guinea-pig blood pressure fell after doses around 0.2 μg./kg.; and in the cat (spinal and anaesthetized) doses of 0.5 μg./kg. and more were usually needed.

The depressor effect of threshold doses of nonapeptide A was very short-lasting. Increasing the doses produced greater responses of longer duration. When given at intervals of 15 min. or more, the depressor action of small and medium doses could easily be reproduced in all species.

Fig. 7 gives an example of the blood pressure response to increasing doses of nonapeptide A in the rabbit. The effect of acetylcholine is shown for comparison. In the rabbit (Fig. 7) acetylcholine (on a weight for weight basis) exerts a weaker depressor effect than nonapeptide A.

In dogs, guinea-pigs, and rats there was also a good dose-response relationship between log-dose of nonapeptide A and the fall of blood pressure. Nonapeptide A was more active than acetylcholine on a weight for weight basis in dogs (4 out of 5 experiments) and more active than histamine (6 experiments) in the guinea-pig.

In the anaesthetized cat the effect of relatively high doses of nonapeptide A is shown in Fig. 8. Initially renal volume closely follows the change in blood pressure, but after large doses it increases before the blood pressure has reached the original level. The increase in renal volume seems to

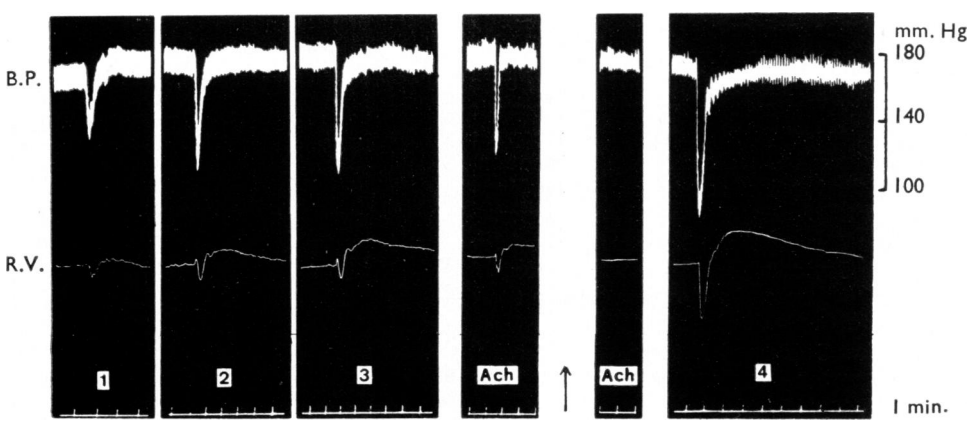

FIG. 8.—Effect of nonapeptide A (1, 2, 3, 4) and of acetylcholine (Ach) on the blood pressure (B.P.) and the renal volume (R.V.) of the cat (2.8 kg.). At the arrow 2 mg. atropine was given. Doses of nonapeptide A: 1=2.5 μg./kg.; 2=7 μg./kg.; 3=14 μg./kg.; 4=35 μg./kg.; dose of acetylcholine: Ach=0.23 μg./kg. Time: 1 min. Note: Atropine completely abolished the effect of acetylcholine but did not interfere with that of nonapeptide A.

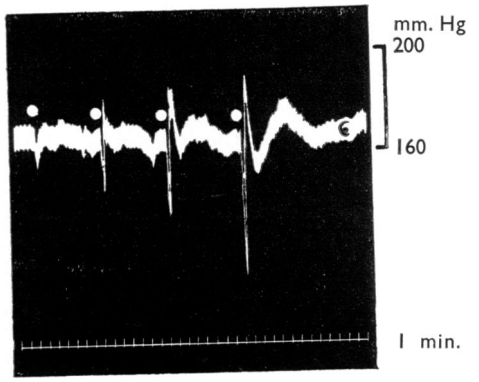

FIG. 9.—Effect of nonapeptide A on the blood pressure of the rooster (1.3 kg.). Doses (from left to right): 37 μg./kg., 74 μg./kg., 148 μg./kg., 296 μg./kg. Time: 1 min.

depend on the dose of nonapeptide A. Fig. 8 also illustrates that atropine does not interfere with the depressor action of nonapeptide A but abolishes the effect of acetylcholine. In the dog also atropine does not influence the depressor effect of nonapeptide A.

During the fall of blood pressure elicited by small and medium doses (0.4 to 3.0 μg./kg.) of nonapeptide A the heart rate rose in the dog but remained nearly unchanged in the rabbit and the cat. At the same time, respiratory rate and volume increased in the dog but not in the cat.

The highest single doses of nonapeptide A given in the course of an experiment were 12.8 μg./kg. in the dog and 300 μg./kg. in the cat. They were not lethal.

In the chicken much higher doses were required to produce a fall of blood pressure. The threshold doses in the rooster were about 40 to 80 μg./kg. (see Fig. 9). There was a fairly good dose-response relationship. Comparable depressor effects on the chicken blood pressure were achieved with approximately 1,000 times smaller doses of acetylcholine.

The fall of blood pressure in the rooster was sometimes followed by a secondary rise to above the original level (see Fig. 9); both the initial fall and the secondary rise of blood pressure were of short duration.

Effects on Capillary Permeability

After intradermal injection nonapeptide A increased the capillary permeability in the skin of guinea-pigs pretreated with Evans blue in 23 experiments (Fig. 10). Doses as small as 1 ng./

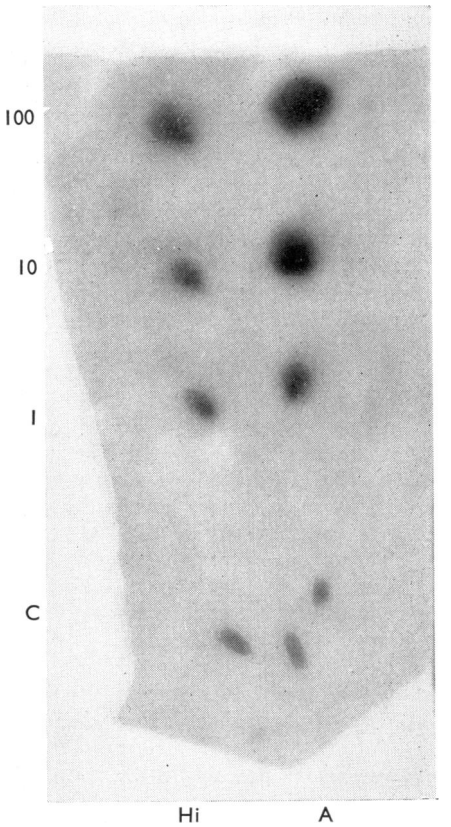

FIG. 10.—Effect of intradermal injections of nonapeptide A and histamine in increasing capillary permeability to circulating Evans blue in the guinea-pig. Dye accumulates at intradermal injection sites. A, nonapeptide A; Hi, histamine; C, control injections with the same volume of saline. Numerals represent the injected doses in ng./0.1 ml. Photo by transmitted light. Note: A comparison of identical doses shows that the extent and density of the "blue" areas are greater after nonapeptide A than after histamine.

0.1 ml. had a definite effect (13 of 16 experiments). The action of histamine under the same conditions in the same animals was about 10 times weaker than that of nonapeptide A.

DISCUSSION

The above-mentioned experiments demonstrate that nonapeptide A (Table I) exhibits actions of bradykinin from natural sources prepared either by the action of trypsin (=trypsin-bradykinin) or by the action of snake-venom (=snake-venom-bradykinin). These actions—slow contraction of the smooth muscle, depression of arterial blood

pressure, and increase in capillary permeability—are inherent in this synthetic nonapeptide A as well as in trypsin-bradykinin and snake-venom-bradykinin. Therefore, the *qualitative* comparison so far shows no discrepancy between the effect of natural bradykinin and that of the synthetic nonapeptide A.

Bradykinin and nonapeptide A react in an identical manner to various procedures. Boiling with concentrated hydrochloric acid destroys snake-venom-bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and nonapeptide A; incubation with guinea-pig serum abolishes the action of trypsin-bradykinin (Rocha e Silva, 1955) and of nonapeptide A. Antihistamines and/or atropine exhibit no specific antagonism to snake-venom-bradykinin and trypsin-bradykinin (Rocha e Silva, Beraldo and Rosenfeld, 1949) and to nonapeptide A. The enzyme chymotrypsin splits arginine from pure trypsin-bradykinin (Elliott, Lewis, and Horton, 1960b) and from nonapeptide A (Boissonnas, Guttmann, and Jaquenoud, 1960a). Further enzymatic treatment by carboxypeptidase splits phenylalanine from the residue of trypsin-bradykinin (Elliott, Lewis, and Horton, 1960b) and from the octapeptide formed from nonapeptide A (Boissonnas, Guttmann, and Jaquenoud, 1960a).

There are also *quantitative* data indicative of a close similarity between bradykinin and nonapeptide A. The rat uterus is 25 to 30 times more sensitive to bradykinin than the guinea-pig ileum (Andrade and Rocha e Silva, 1956). The same relationship was observed for the sensitivity of both organs towards nonapeptide A. Elliott, Horton, and Lewis (1960) found 0.2 ng./ml. of pure natural trypsin-bradykinin was required to contract the rat uterus; this accords with the dose of 0.1 ng./ml. of the synthetic nonapeptide necessary to contract the uterus. The dose of 400 ng./kg. of pure natural trypsin-bradykinin required to produce a depressor response in cats which is mentioned by Elliott, Horton, and Lewis (1960) is in close agreement with the smallest dose of approximately 500 ng./kg. of the synthetic nonapeptide A needed to elicit a blood pressure fall in cats. And finally, the threshold dose of pure natural snake-venom-bradykinin (1 ng./ml.) for contraction of the isolated guinea-pig ileum (Zuber and Jaques, 1960) is identical with the threshold dose of nonapeptide A in this organ.

All these qualitative criteria and the quantitative data available for pure bradykinins reveal a very close similarity between nonapeptide A and natural bradykinin.

As far as we can see there is only one discrepancy between a property of purified snake-venom-bradykinin that has been reported and the action of nonapeptide A, namely, the effect on the blood pressure of the fowl. Rocha e Silva (1960) states that bradykinin was entirely without effect on the blood pressure of the fowl. This is based on experiments in which not pure bradykinin but only partially purified bradykinin was used. On giving high doses of nonapeptide A we observed a definite fall of the arterial blood pressure of the rooster (see Fig. 9); but on a weight basis nonapeptide A was more than 10,000 times less active than oxytocin. The doses of bradykinin required to produce a fall in blood pressure of the chicken were approximately 100 to 200 times higher than the doses required for the same effect in mammals. Rocha e Silva probably did not give sufficiently high doses of partly purified bradykinin to the fowl to elicit a blood pressure fall. His statement (1960) that oxytocin is without effect on the mammalian pressure also does not accord with other observations (du Vigneaud, 1955; Van Dyke, Adamsons and Engel, 1955; Gyermek and Fekete, 1955; Konzett, Berde, and Cerletti, 1956), possibly for the same reason. Therefore, the discrepancy between the reported failure to produce a blood pressure fall with bradykinin in the fowl and our observation that nonapeptide A in very high doses is also depressor in the chicken does not necessarily constitute a difference.

Taking all the above-mentioned facts into consideration we come to the conclusion that nonapeptide A is identical with or at least very closely related to bradykinin.

To give an idea of the general biological activity of nonapeptide A, a comparison may be made with the very potent naturally occurring amines histamine and acetylcholine. Weight for weight, nonapeptide A is more active than histamine in reducing tidal air in guinea-pigs and in causing a fall of blood pressure on the rabbit, guinea-pig, and often on the dog. On a molar basis nonapeptide A is also more active than histamine on the guinea-pig ileum and more active than acetylcholine on the rabbit duodenum.

A comparison of nonapeptide A with oxytocin shows that on the isolated rat uterus nonapeptide A is only 1.5 to 3 times less active than oxytocin (weight for weight and on a molar basis). On the blood pressure of the chicken, however, nonapeptide A is more than 10,000 times less active than oxytocin (weight for weight and on a molar basis).

The synthetic octapeptide (C in Table I) with the sequence of amino acids reported by Elliott, Lewis, and Horton (1960b) to occur in trypsin-bradykinin is without effect even in high concentrations (10 μg./ml. on the guinea-pig ileum and the rat uterus). This is confirmed by a preliminary report of Schwyzer, Rittel, Sieber, Kappeler, and Zuber (1960), who also synthesized this octapeptide and found it to be without effect on the isolated guinea-pig ileum in doses up to 10 μg./ml.

It is, however, remarkable that recently after isolation of a bradykinin resulting from the action of snake-venom on bovine plasma, Zuber and Jaques (1960) have stated that this snake-venom-bradykinin in an essentially pure form contains the five amino acids arginine, phenylalanine, proline, glycine, and serine in the ratio of 2:2:2:1:1. It therefore corresponds to the amino acid composition found for trypsin-bradykinin by Elliott, Lewis, and Horton (1960a). As yet, Zuber and Jaques (1960) have not published the whole sequence of the amino acids.

The fact that an octapeptide with these above-mentioned amino acids can exhibit bradykinin-like properties on the isolated guinea-pig ileum, on the rat uterus, and on the tidal air of guinea-pigs is demonstrated in the example of the synthetic octapeptide B (in Table I). As in the case of bradykinin and of nonapeptide A the rat uterus was more sensitive (approximately 20 times) than the guinea-pig ileum to octapeptide B. Boiling with concentrated hydrochloric acid or incubating with guinea-pig serum abolishes the action of octapeptide B on the isolated guinea-pig ileum. This octapeptide B, however, is markedly less potent (50 to 100 times) in various tests (isolated guinea-pig ileum, isolated rat uterus, tidal air of anaesthetized guinea-pigs) than pure trypsin-bradykinin (Elliott, Horton, and Lewis, 1960) and pure snake-venom-bradykinin (Zuber and Jaques, 1960). It is thus definitely not identical with them.

The other peptides synthesized which contain the above-mentioned amino acids, namely, the octapeptides D and E (Table I) and the heptapeptide F, were without effect on the isolated guinea-pig ileum even in high doses (10 μg./ml. for D and E and 2.5 μg./ml. for F). The octapeptides D and E had in the same doses also no effect on the isolated rat uterus, whereas the heptapeptide F was not investigated on this organ.

The above-mentioned results with the various peptides containing the amino acids of natural bradykinin necessitate a re-examination of the structure of natural bradykinin. The present somewhat complicated situation in the bradykinin field may then be clarified.

Addendum after submission of this paper: In view of the previously reported biological actions of the synthetic nonapeptide A, Elliott, Lewis, and Horton (1960c) have reinvestigated the structure of natural trypsin-bradykinin, and, after new degradation studies, have presented evidence that natural bradykinin is a nonapeptide having the structure of nonapeptide A. Therefore this synthetic nonapeptide is in fact synthetic bradykinin.

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A STUDY OF POTENTIAL HISTIDINE DECARBOXYLASE INHIBITORS

BY

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A series of compounds has been examined for ability to inhibit histidine decarboxylase. Histidine analogues having substituents in the imidazole ring showed a wide variation in potency, but these were all much less active than α-methyldopa [β-(3,4-dihydroxyphenyl)-α-methylalanine], the most potent known inhibitor of histidine decarboxylase. Some tentative conclusions are drawn regarding the relationship between chemical structure and inhibitory activity.

Numerous substances have been tested for their ability to inhibit the enzyme histidine decarboxylase (Werle and Heitzer, 1938; Werle, 1940, 1942; Martin, Graff, Brendel, and Beiler, 1949; Werle and Koch, 1949; Werle, Schauer, and Hartung, 1955; Waton, 1956; Udenfriend, Lovenberg, and Weissbach, 1960). Although several potent inhibitors were found in the course of these studies, none was sufficiently specific to inhibit histidine decarboxylase without simultaneously inhibiting other enzymes. In particular, compounds which act by influencing the availability of the co-decarboxylase pyridoxal 5'-phosphate will inhibit not only histidine decarboxylase but all amino-acid decarboxylases and the transaminases, for which pyridoxal phosphate is also the co-enzyme.

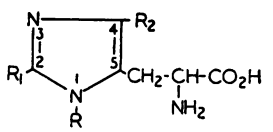
A series of compounds, some of them structural analogues of histidine, has been studied in an attempt to clarify the nature of the active centre of the enzyme, and to determine whether they can inhibit histidine decarboxylase specifically by competition with the normal substrate.

A specific, competitive inhibitor of histidine decarboxylase would be useful as a pharmacological tool. In view of the recent work of Kahlson and co-workers (see Kahlson, 1960) and by Riley and co-workers (Mackay, Marshall, and Riley, 1960), which indicates that histidine decarboxylase may play some part in normal and abnormal growth processes, such a compound might have therapeutic applications.

METHODS

Synthesis of Compounds

Histidine Analogues.—These corresponded to the general formula I. Where substituents could not be introduced directly into the imidazole ring of histidine, the method of Jones and McLaughlin (1949) was employed, the starting material in each case being the correspondingly substituted hydroxymethylimidazole.



I

The position of the iodine atom in L-monoiodohistidine is unknown (Brunings, 1947).

In naming the imidazole derivatives it should be noted that, except in cases where one of the nitrogen atoms in the imidazole ring carries a substituent, positions 4 and 5 are equivalent owing to tautomerism.

5-Hydroxymethyl-2-methylimidazole Hydrochloride.—This was prepared by reacting dihydroxyacetone, acetaldehyde, ammonia, and basic copper carbonate, under the conditions described by Totter and Darby (1944) for the preparation of 5-hydroxymethylimidazole hydrochloride. The compound was isolated as the picrate, and converted to the hydrochloride in the usual way.

4-Hydroxymethyl-1-methylimidazole Hydrochloride.—This was prepared as the picrate by the method of Ruoff and Scott (1950), and converted to the hydrochloride.

In Table I are given the melting points and analyses of new compounds prepared in the course of these studies. The following compounds were prepared as described in the literature: 4-hydroxymethyl-1-methylimidazole picrate (Ruoff and Scott, 1950); 4-methylimidazole (Bernhauer, 1929); 5-hydroxymethyl-4-methylimidazole hydrochloride (Ewins, 1911); and 4-methyl-5-nitroimidazole (Allsebrook, Gulland, and

TABLE I

PROPERTIES OF COMPOUNDS PREPARED. ALL AMINO-ACIDS ARE DL-FORMS

Compound	Molecular Formula	Analyses								m.p.°
		Calculated %				Found %				
		C	H	N	Cl	C	H	N	Cl	
5-Hydroxymethyl-2-methyl-imidazole picrate ..										172–3
5-Hydroxymethyl-2-methyl-imidazole hydrochloride	$C_5H_9ON_2Cl$	40·6	6·1	18·9	—	40·4	5·7	18·8	—	151–2
5-Chloromethyl-2-methyl-imidazole hydrochloride	$C_5H_8N_2Cl_2$	—	—	—	42·2	—	—	—	41·4	149–150
β-(2-Methylimidazol-5-yl)-alanine dihydrochloride (2-methylhistidine) ..	$C_7H_{13}O_2N_3Cl_2$	34·9	5·3	17·4	—	34·6	5·5	16·8	—	257–9 (dec.)
4-Hydroxymethyl-1-methyl-imidazole hydrochloride	$C_5H_9ON_2Cl$	40·6	6·1	18·9	—	39·9	6·1	19·6	—	124–5
4-Chloromethyl-1-methyl-imidazole hydrochloride	$C_5H_8N_2Cl_2$									153–4
β-(1-Methylimidazol-4-yl)-alanine dihydrochloride	$C_7H_{13}O_2N_3Cl_2$	34·9	5·3	17·4	—	35·3	5·6	17·6	—	251–3 (dec.)
β-(4-Methylimidazol-5-yl)-alanine monohydrochloride monohydrate (4-methyl-histidine)	$C_7H_{14}O_3N_3Cl$	37·7	6·3	18·8	15·7	38·5	6·5	17·5	15·1	168–170 (dec.)

Story, 1942). Where melting points were in agreement with those already published, analyses were not carried out.

Histidine.—It was necessary to prepare L-histidine zwitterion from commercial L-histidine monohydrochloride monohydrate to eliminate traces of histamine present. The hydrochloric acid was removed by adding excess silver carbonate to a warm concentrated aqueous solution of the monohydrochloride. After filtration, silver ions were removed from the filtrate by passing in hydrogen sulphide. Filtration and subsequent concentration of the filtrate, under reduced pressure, gave a saturated solution of L-histidine zwitterion from which the latter was precipitated by addition of dehydrated alcohol.

Enzyme Studies

Preparation of Histidine Decarboxylase.—Guinea-pig kidneys were used as the source of mammalian histidine decarboxylase. They were pulped with purified sand, approximately 3 ml. of saline was then added/g. of tissue, and the mixture was ground once more. The fluid was centrifuged at 15,000 *g* for 20 min., and the supernatant was used as a crude enzyme extract.

Incubation Experiments.—The incubation medium for the uninhibited reaction was prepared by pipetting into a centrifuge tube phosphate buffer of *p*H 8.0 (isotonic with blood), 2.0 ml.; 0.9% sodium chloride solution, 0.9 ml.; L-histidine zwitterion solution (25.0 mg./ml. saline), 1.0 ml.; and 3×10^{-3} M aminoguanidine bicarbonate in saline, 0.1 ml. After the tubes and their contents had been left in the thermostat for about 15 min., 1.0 ml. of enzyme extract was added, the tubes were stoppered, inverted to mix the solutions, and the time was noted. The final volume in each tube was 5.0 ml. so that the final concentrations of L-histidine and aminoguanidine were respectively 32.2×10^{-3} M and 6×10^{-5} M. Aminoguanidine in the concentration used has been shown by Waton (1956) to inhibit completely any histaminase present; it was added as a routine precaution, though the histaminase activity of guinea-pig kidney extracts is very low. In order to measure the rate of the reaction in presence of decarboxylase inhibitor, the latter was added either in solid form or as a neutralized solution in saline, with the volume of pure saline added to the incubation medium correspondingly reduced. Inhibition was therefore studied at constant *p*H, temperature and substrate concentration. Since the enzyme concentration was constant only for a given extract at a given time, simultaneous studies on the inhibited and uninhibited reaction had to be carried out using the same extract.

The reaction was stopped by adding 0.20 ml. of 2 N hydrochloric acid to the centrifuge tubes to make the media just acid, and boiling for 1 min. to coagulate protein. The latter was centrifuged down, and the

solutions were stored overnight at 4°. The histamine produced was estimated by the 4-point method on atropinized guinea-pig ileum at 34°, using an automatic assay apparatus. Control experiments were carried out to detect any histamine either in the reagents, or produced by interaction of enzyme with the inhibitors. The effect of inhibitors on the assay of histamine was checked, and the stability of histamine under the conditions used was also studied. No serious complications were encountered.

Estimation of Inhibitor Potency.—An approximate test of the inhibitor potency was made by simply incubating the inhibitor with L-histidine and enzyme extract at 36° for about 3 hr. Then an approximate estimate was made of the molar concentration of inhibitor required to reduce the velocity of the uninhibited reaction by 50% (C_{50}). Two concentrations of inhibitor, calculated to produce about 30% and 70% inhibition, were then chosen for more detailed study. The incubation media were prepared as described, the volumes in each tube being increased to 25 ml., so that samples (7 ml.) could be removed at known times after the addition of the enzyme. The samples were made just acid, boiled, centrifuged, and stored at 4° until assayed.

Results

Graphs were prepared in which the concentration of histamine in the incubation mixtures was plotted against time. From each graph the rate of production of histamine at the beginning of the reaction (initial velocity) was determined.

The variation of the initial velocity with substrate concentration, at constant enzyme concentration, was determined in a preliminary experiment. The results (Fig. 1) show that the substrate concentration used was well below the level which would saturate the enzyme.

From a plot of the reciprocal of the initial velocity against the concentration of inhibitor in the medium the C_{50} value was obtained as shown in Fig. 2, which represents an ideal case. This inhibitor concentration is related to the inhibitor-enzyme equilibrium constant, the exact relationship depending on whether the inhibition is competitive or non-competitive (Lineweaver and Burk, 1934). When the type of inhibition is not known with certainty, as in the present examples, the C_{50} value can still be used as an index of the inhibitory power of the compound under test. The reproducibility of the C_{50} determination was tested with D-histidine using 3 different guinea-pig extracts; the error in C_{50} obtained in this way may be as much as 20%. The C_{50} values for the compounds tested are given in Table II. It should be noted that they depend on the substrate concentration and *p*H.

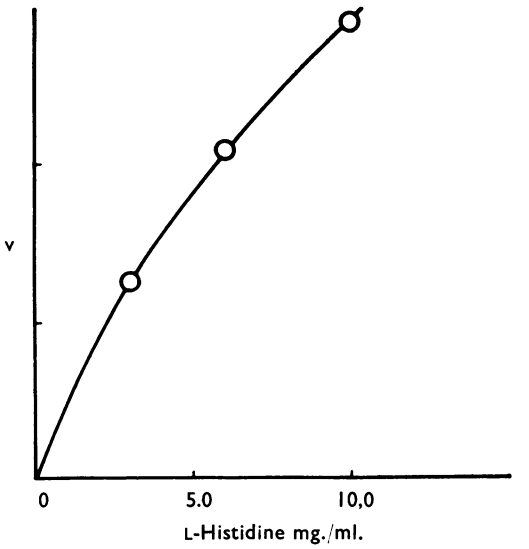


FIG. 1.—Influence of substrate concentration on histamine production by L-histidine decarboxylase in a guinea-pig kidney extract. Ordinate: v=initial rate of production of histamine (in arbitrary units). Abscissa: concentration of L-histidine in mg./ml.

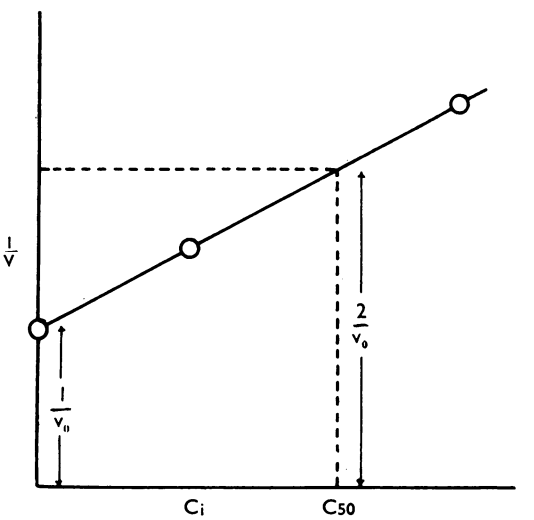


FIG. 2.—Inhibition of L-histidine decarboxylase in guinea-pig kidney extracts. Method of determining C_{50}. Ordinate: $\frac{1}{v}$ where v=initial rate of production of histamine (in arbitrary units). Abscissa: C_i=concentration of inhibitor in mg./ml. v_0= initial rate of the uninhibited reaction.

TABLE II

CONCENTRATIONS OF VARIOUS COMPOUNDS REQUIRED TO PRODUCE 50% INHIBITION (C_{50}) OF HISTIDINE DECARBOXYLASE

The concentration of L-histidine was 322×10^{-4} M, buffer *p*H 8·0, temperature 36°.

No.	Compound	$C_{50} \times 10^{-4}$ M
1	DL-α-Methyldopa	0·007
2	L-Dopa	0·18
3	L-2,4-Di-iodohistidine	40
4	D-2,4-Di-iodohistidine	40
5	L-Monoiodohistidine	50
6	DL-2-Methylhistidine	60
7	D-Histidine	86
8	DL-4-Methylhistidine	380
9	DL-β-(1-Methylimidazol-4-yl)alanine	420
10	L-β-(1-Methylimidazol-5-yl)alanine	910
11	Catechol	1·8
12	Salicylic acid	36
13	Phthalic acid	54
14	Imidazole	800
15	4-Methylimidazole	440
16	4-Methyl-5-nitroimidazole	34
17	Barbitone	330
18	L-Arginine	480
19	Creatine	2,000
20	Glycocyamine	2,600
21	Citrulline	3,200

DISCUSSION

The results given in Table II are grouped according to the chemical structures of the inhibitors. Compounds 18 to 21, though open-chain substances, were tested because they have some structural relationship to histidine. Although arginine was a fairly effective inhibitor, the other compounds were weak.

Substances 11 to 17 are all simple ring compounds with at least two electronegative atoms, either oxygen or nitrogen. Catechol was shown by Werle and Koch (1949) to be a potent inhibitor of histidine decarboxylase; this was confirmed. Of the imidazole analogues the 4-methyl-5-nitro-derivative was outstanding. Since the effect of the nitro-group is to withdraw electrons from the imidazole ring, thereby increasing the acidity of the nitrogen atoms in the ring, it is probable that this compound acts in a similar way to catechol. The acidic nitrogen atoms may be analogous to the acidic phenolic groups of catechol.

This idea may be tested quantitatively by calculating the concentrations of the anionic species present when the enzyme is 50% inhibited. Using the accepted *p*Ka values for catechol of 8.5 and 10.2, the concentrations of the mono- and di-valent catechol anions, present at *p*H 8.0 in a solution of catechol of concentration C_{50}, are approximately 45×10^{-6} M and 0.7×10^{-6} M respectively. For 4-methyl-5-nitroimidazole (*p*Ka = 9.9) the anion concentration at C_{50} is about 25×10^{-6} M. The charge of the 4-methyl-5-nitroimidazole anion can be partly distributed to the second nitrogen atom of the ring to form a species, obviously in lower concentration, analogous to the divalent catechol anion. However, since it was shown by Werle and Koch (1949) that phenol is a much less potent inhibitor than catechol, it is obvious that a simple mono-anion hypothesis is not acceptable, and that some part of the binding is due to the second electronegative atom.

Even with a pure compound several molecular species, dissociated or otherwise, are present in solution. Each species may contribute to the observed enzyme inhibition, and it cannot be assumed that they are all bound by the same mechanism, since both electrostatic interaction and hydrogen bonding are quite feasible. This may explain the behaviour of 4-methylimidazole in which the nitrogen atoms in the ring are more basic than in imidazole itself, and which, assuming electrostatic interaction, would be a weaker inhibitor than imidazole. 4-Methylimidazole was in fact somewhat more powerful than imidazole, and this could be due to the increased electron density in the nitrogen atoms resulting in greater ability of the undissociated base to form hydrogen bonds with the enzyme. As would be expected on the basis of either of the above hypotheses, barbitone was also an inhibitor of the enzyme.

Salicylic and phthalic acids, which may be regarded as derived from catechol by stepwise replacement of the phenolic groups by carboxyl groups, were progressively weaker inhibitors. This progressive decrease in potency may be due to steric factors, since the separation of the negatively-charged oxygen atoms of the ionized compounds increases in passing from catechol to phthalic acid. On the other hand, if hydrogen bonding is involved in the attachment of the ring to the enzyme, then interaction between the –C=O and –OH groups of the carboxyl-substituted compounds might lead to less efficient binding.

In the third group of inhibitors (Nos. 3 to 10, Table II) the iodohistidines were the most active, the acidity of the imidazole ring in these compounds being greater than in histidine itself. DL-2-Methylhistidine was surprisingly potent,

especially when compared with DL-4-methylhistidine, although in both compounds the methyl group would be expected to increase the basicity of the imidazole nitrogen atoms. D-Histidine, previously studied by Werle (1942), was also remarkably active. The D- and L-2,4-di-iodohistidines were prepared, and their potencies were found to be equal within experimental error. Further studies are required to elucidate the extent to which the D and L components contribute to the observed inhibitions by DL compounds. For this reason the relative activities of the two *N*-methylated histidines (Table II, Nos. 9 and 10) which were available only in the DL and L forms, respectively, were not strictly comparable. As expected, however, these were weak inhibitors, this result being analogous to the finding of Werle and Koch (1949) that phenol ethers are very weak inhibitors compared with the free phenols.

Werle (1942) found L-dopa to be a potent inhibitor of histidine decarboxylase. α-Methyldopa [DL-β-(3,4-dihydroxyphenyl)-α-methylalanine] was shown by Sourkes (1954) to be the most potent of a series of dopa analogues tested as inhibitors of dopa decarboxylase, and it also inhibits 5-hydroxytryptophan decarboxylase (Smith, 1960) and histidine decarboxylase (Udenfriend *et al.*, 1960). Quantitatively this compound (No. 1, Table II) was a considerably more potent inhibitor of histidine decarboxylase than is L-dopa itself (No. 2, Table II). This is not surprising since the α-methyl group increases the basicity of the α-amino-nitrogen atom, thus enhancing the ability of the latter to form a Schiff base with an aldehydic group, for example, that of pyridoxal 5′-phosphate.

It is likely that the views of Werle and Koch (1949) and of Metzler, Ikawa, and Snell (1954) regarding the mechanism of decarboxylation are basically correct. Histidine is probably attached to the holo-enzyme by the imidazole ring, the α-amino group, and the α-carboxyl group, the three-point attachment accounting for the optical specificity of the enzyme. However, since this specificity applies to the decarboxylation but not necessarily to the action of inhibitors, the actual interaction of carboxyl group and enzyme may not take place during sorption of substrate by the enzyme, but rather in an intermediate step preceding the subsequent breakdown to products. Although careful consideration of the results given here shows several discrepancies, it appears that increasing the acidity of the imidazole ring increases the binding of this portion of the molecule to the enzyme active centre.

The structural requirements of inhibitors of histidine decarboxylase and of dopa decarboxylase are clearly very similar. This similarity could mean that the enzymes are identical as implied by Udenfriend *et al.* (1960), but it might also arise from interaction of the inhibitors with the coenzyme, pyridoxal 5′-phosphate, which appears to be common to all decarboxylases.

2-Thiohistidine, 2-thioimidazole, ergothionine and cysteine were also tested as histidine decarboxylase inhibitors. The results are not included in Table II, because these compounds appear to act by a peculiar mechanism. Histamine production in the presence of these inhibitors was at first rapid, but decreased until, after about 2 hr., the enzyme was completely inactivated. Further studies on this type of compound are in progress.

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SOME PHARMACOLOGICAL ACTIONS OF SYNTHETIC ANALOGUES OF ANGIOTENSINAMIDE

BY

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In order to evaluate the importance of some structural features of asparagyl[1]-valyl[5]-angiotensin II (angiotensinamide) for its pharmacological actions, the relative potencies of angiotensinamide and five peptide analogues were studied on the blood pressure of the rat, the isolated rat uterus and the isolated guinea-pig ileum. All the modifications of the angiotensinamide structure that were studied led to a decrease of potency which, however, was not the same on all three preparations. The importance of the guanido group, the phenolic group and the length of the peptide chain for the pharmacological activities of these peptides is discussed.

The name *angiotensin* has been proposed by Braun-Menéndez and Page (1958) to replace the two trivial names, hypertensin and angiotonin, previously given to the product of renin action on the plasma α_2-globulins.

The knowledge of the structure of angiotensin peptides (Skeggs, Lentz, Kahn, Shumway and Woods, 1956; Elliott and Peart, 1957) and the development of methods for their synthesis (Rittel, Iselin, Kappeler, Riniker, and Schwyzer, 1957), as well as that of structural analogues, permitted the study of the relation of peptide structure to pharmacological activity. In a previous paper (Paiva and Paiva, 1960a) we have investigated the effect of modifications of the *N*-terminal grouping of valyl[5]-angiotensin II on its pressor and oxytocic activities, and concluded that a free β-carboxyl group in the *N*-terminal aspartyl residue, though not essential, is important for these two properties. A further study of the relation of angiotensin structure to activity was made possible by the availability of synthetic analogues prepared in the Ciba Laboratories. In the present paper we report a study of 6 such analogues, having in common an asparagyl instead of the aspartyl residue at the *N*-terminal end. Table I shows the amino acid sequences of valyl[5]-angiotensin II and of the 6 synthetic peptides studied. In order to simplify the reference to the different analogues, in this paper asparagyl[1]-valyl[5]-angiotensin II will be

TABLE I

THE SEQUENCE OF AMINO ACIDS IN VALYL[5]-ANGIOTENSIN II AND IN SIX SYNTHETIC ANALOGUES

The nomenclature used was derived from the name angiotensinamide (asparagyl[1], valyl[5]-angiotensin II) according to the rules proposed by Konzett and Berde (1959).

Peptide	Amino Acid Sequence
	1 2 3 4 5 6 7 8 9
Valyl[5]-angiotensin II	Asp.Arg.Val.Tyr.Val.His.Pro.Phe
Angiotensinamide	Asp(NH_2).Arg.Val.Tyr.Val.His.Pro.Phe
Isoleucyl[5]-angiotensinamide	Asp(NH_2).Arg.Val.Tyr.iLeu.His.Pro.Phe
Nitroarginyl[2]-angiotensinamide	Asp(NH_2).Arg.(NO_2).Val.Tyr.Val.His.Pro.Phe
Ornithyl[2]-angiotensinamide	Asp(NH_2).Orn.Val.Tyr.Val.His.Pro.Phe
Phenylalanyl[4]-angiotensinamide	Asp(NH_2).Arg.Val.Phe.Val.His.Pro.Phe
Homotyrosyl[4:5]-angiotensinamide	Asp(NH_2).Arg.Val.Tyr.Tyr.Val.His.Pro.Phe

referred to as *angiotensinamide*, and the name of the other peptides will be derived from this in the manner proposed by Konzett and Berde (1959) for the nomenclature of oxytocin analogues.

The pharmacological preparations used in the present study were the blood pressure of the rat, the isolated rat uterus and the isolated guinea-pig ileum. The first preparation was chosen because the pressor action is the most characteristic pharmacological action of angiotensin *in vivo*, and the two other preparations were chosen for being the most sensitive to angiotensin yet described (Ludueña, 1940; Picarelli, Kupper, Prado, Prado, and Valle, 1954; Schwartz, Masson, and Page, 1955).

Methods

The angiotensin analogues were all synthetic products prepared at Ciba Laboratories, Basle, and purified as free peptides in the final stage of the synthesis by counter-current distribution. The purity of the products was checked by paper chromatography and they were found to be either chromatographically pure or to contain small amounts (not more than 1%) of the corresponding aspartyl-peptides (formed by hydrolysis of the *N*-terminal asparagine amide group). All preparations contained 1 to 3 molecules of water and 1 to 2 molecules of acetic acid per molecule of peptide (Schwyzer, 1960).

Pharmacological Assays

Rat Blood Pressure.—Albino rats, weighing between 200 and 250 g., were anaesthetized with urethane 150 mg./100 g. body weight, and were kept on artificial respiration. The blood pressure was recorded with a mercury manometer connected to the carotid artery. Injections were made through a plastic cannula in a femoral vein. The rats were heparinized, and received an intravenous injection of hexamethonium bromide 5 mg./100 g. body weight. For the assay of *pressor activity* 0.1 or 0.2 ml. of the peptides, in isotonic solution, was injected.

Rat Uterus.—Virgin rats weighing 150 to 200 g. received subcutaneous injections of 10 μg. of stilboestrol per 100 g. body weight and were killed 16 to 20 hr. later by a blow on the head. The uterine horns were suspended in aerated de Jalon solution in a 2 ml. bath at 30°. The contractions were recorded with an isotonic lever with a six-fold magnification and a load of 1.0 to 1.5 g. In this paper, the activity of the peptides on this preparation is called *oxytocic activity*.

Guinea-pig Ileum.—Terminal segments of the ileum of guinea-pigs weighing 150 to 200 g. were suspended in a 2 ml. bath of aerated Tyrode solution at 35°. The contractions were recorded as described above for the rat uterus. The effect on the isolated guinea-pig ileum is called *gut-stimulating activity*.

The pressor, oxytocic and gut-stimulating activities of the 5 analogues of angiotensinamide were estimated by assays against standard solutions of angiotensinamide. For each analogue, a rough preliminary estimate of activity was made, and then approximately equipotent solutions of the peptide and of angiotensinamide were compared using a four-point design. The evaluation of potency was made by measuring the height of the recorded increases in blood pressure or contractions of the isolated smooth muscles, and submitting the data to the statistical treatment described by Schild (1942) for obtaining a potency ratio and fiducial limits. The relative potency of each peptide was defined as the ratio of the activity of one mole of peptide to the activity of one mole of angiotensinamide, which was arbitrarily taken as 100.

Results

All the peptides had detectable activity on the three pharmacological preparations. Qualitatively, no difference was detected between the pressor response to angiotensinamide on the rat and that of the other analogues. Although the oxytocic and gut-stimulating activities of isoleucyl^{5}- and homotyrosyl$^{4:5}$-angiotensinamides were qualitatively indistinguishable from angiotensinamide, the other 3 analogues produced a different type of response. On the isolated rat uterus, addition of nitroarginyl^{2}-, ornithyl^{2}- or phenylalanyl^{4}-angiotensinamide to the bath caused only a single contraction, instead of the rhythmic response characteristic of angiotensinamide (Paiva and Paiva, 1960b). On the isolated guinea-pig gut, the three last-mentioned analogues produced a tonus of shorter duration than that produced by

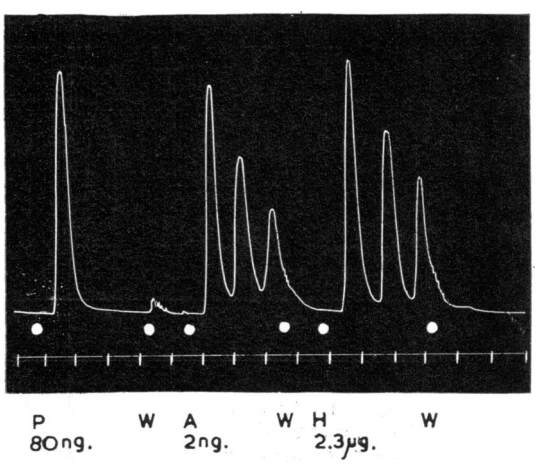

FIG. 1.—Isolated rat uterus suspended in a 2 ml. bath containing de Jalon solution at 30°. Responses to additions of phenylalanyl^{4}-angiotensinamide (P), angiotensinamide (A) and homotyrosyl$^{4:5}$-angiotensinamide (H). At W the preparation was washed three times. Time, min.

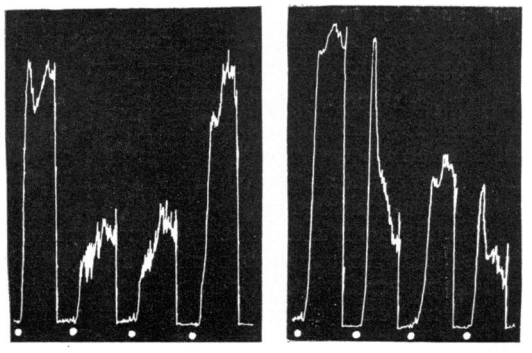

FIG. 2.—Isolated guinea-pig ileum suspended in a 2 ml. bath containing Tyrode solution at 35°. Contractions produced by angiotensinamide (A), homotyrosyl[4:5]-angiotensinamide (H), and phenylalanyl[4]-angiotensinamide (P).

angiotensinamide. To illustrate these qualitative differences, Figs. 1 and 2 show the response of the rat uterus and of the guinea-pig gut to angiotensinamide and to homotyrosyl[4:5]- and phenylalanyl[4]-angiotensinamide.

In spite of these qualitative differences, we decided to make a quantitative comparison of the oxytocic and gut-stimulating potencies of angiotensinamide with those of all the other peptides. The oxytocic activity was assessed from the height of the first contraction of the rat uterus, and the gut-stimulating activity from the maximum height of contraction of the ileum during the 90 sec. of contact with the drug. The results of the assays are summarized in Table II. No significant deviation of parallelism was found in any of the assays ($P>0.2$).

The possibility of competitive inhibition of angiotensinamide by homotyrosyl[4:5]-angiotensinamide was investigated in the three pharmacological preparations. The administration of mixtures of angiotensinamide with 100-fold concentrations of the homotyrosyl analogue resulted in simple summation of effects, without any sign of inhibition. Similar summation of effects was observed with mixtures of equipotent amounts of angiotensinamide with each of the other analogues; in these mixtures, the ratio between the molar concentration of the analogues and that of angiotensinamide ranged from 2, in the case of the pressor action of the mixture with isoleucyl[5]-angiotensinamide, to 350 in the case of the gut-stimulating action of the mixture with nitroarginyl[2]-angiotensinamide.

DISCUSSION

All the analogues studied were less active than angiotensinamide on the three pharmacological preparations. It is remarkable that relatively small alterations in the structure of angiotensinamide resulted in marked differences in the three pharmacological activities studied. One example of this is seen in Table II, where the ratios between the potencies of angiotensinamide and nitroarginyl[2]-angiotensinamide were found to be 4 for the pressor, 32 for the oxytocic, and 350 for the gut-stimulating activity. Another example illustrating that small changes in the structure of angiotensinamide resulted in marked alterations of pharmacological activity is where the presence of an extra methyl group, in isoleucyl[5]-angiotensinamide, resulted in a product that was about one-half to one-third as active as angiotensinamide. Furthermore, small activity was found for phenylalanyl[4]-angiotensinamide, which differs

TABLE II

RELATIVE POTENCIES OF SOME ANGIOTENSIN ANALOGUES ON THE RAT BLOOD PRESSURE, THE ISOLATED RAT UTERUS AND THE ISOLATED GUINEA-PIG ILEUM

Relative potencies were calculated on a molar basis, the value 100 being arbitrarily assigned to angiotensinamide. Figures in parentheses indicate the 95% fiducial limits.

Peptide	Rat Blood Pressure	Isolated Rat Uterus	Isolated Guinea-pig Ileum
Angiotensinamide	100	100	100
Isoleucyl[5]-angiotensinamide	52 (±8)	33 (±3)	35 (±5)
Nitroarginyl[2]-angiotensinamide	25 (±3)	3·1 (±0·2)	0·29 (±0·05)
Ornithyl[2]-angiotensinamide	8·9 (±0·9)	1·6 (±0·2)	0·73 (±0·08)
Phenylalanyl[4]-angiotensinamide	4·9 (±0·8)	2·5 (±0·4)	3·2 (±0·2)
Homotyrosyl[4:5]-angiotensinamide	0·33 (±0·05)	0·10 (±0·02)	0·10 (±0·02)

from angiotensinamide only by the absence of one phenolic hydroxyl group. These differences, however, are not so surprising, in view of the reported discrepancies (Berde, Doepfner, and Konzett, 1957; van Dyke, 1959) in the potencies of oxytocin and vasopressin analogues towards different biological tests.

Some conclusions regarding the importance of the guanido group of angiotensinamide for its pharmacological activities may be drawn from the results obtained with nitroarginyl^2- and ornithyl^2-angiotensinamide. The nitroarginyl analogue, in which the ionization of the guanido group is blocked, had a pressor activity that was one-quarter that of angiotensinamide, but still almost three times that of the ornithyl analogue, in which the guanido group was replaced by an amino group. This suggests that the importance of the guanido group for the pressor activity of angiotensinamide may not be attributed entirely to its basic character. The same is not so evident with regard to the oxytocic activity, since the two analogues have very low oxytocic potencies, nitroarginyl^2-angiotensinamide being still twice as active as ornithyl^2-angiotensinamide. The gut-stimulating activity, on the other hand, seems to be more dependent on the basicity of the guanido group, since this activity is negligible in nitroarginyl^2-angiotensinamide, whereas the ornithyl analogue is 2.5 times as potent. The inability of ornithine to replace arginine, especially in the case of the gut-stimulating activity, may be explained by the shortening of the side-chain interfering with the interaction of the basic group with the receptor. This explanation seems better than the one offered by Katsoyannis and du Vigneaud (1958) for the similar case of arginine and lysine vasopressins, namely, that the differences in the pK'_3 of the two amino acid residues might be responsible for the difference in the pharmacological activity of the two analogues. Since the two pK'_3 values are well above the physiological *p*H, the arginine guanido group and the ornithine amino group should both be completely ionized at that *p*H and, therefore, equally charged.

Homotyrosyl$^{4:5}$-angiotensinamide, although inducing the same type of response as angiotensinamide, was the least active of the analogues. The absence of competitive inhibition of angiotensinamide by this analogue suggests that its low activity may be due to a disproportionate distance, introduced by the extra tyrosyl residue, between two points of attachment to the receptor.

After this paper had been submitted for publication we had knowledge of a paper by Gross and Turrian (1960), in which the pressor activity of the first four analogues of Table II were studied on the nephrectomized rat.

These authors found that the isoleucyl analogue was as active as angiotensinamide, and that the arginyl and ornithyl analogues were, respectively, one-half and one-quarter as active as angiotensinamide. However, no measure of the error involved in their assays is given, and it is impossible to determine the significance of the difference between our results.

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EVALUATION OF ANTAGONISTS OF HISTAMINE, 5-HYDROXYTRYPTAMINE AND ACETYLCHOLINE IN THE GUINEA-PIG

BY

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Changes in the resistance to entry of air into the lungs of anaesthetized guinea-pigs have been used to study the effects of histamine, 5-hydroxytryptamine, and acetylcholine. Some known antagonists of these bronchoconstrictor agents have been investigated. The degree of antagonism has been expressed in terms of the dose ratio, previously used only with *in vitro* preparations.

In 1940 Konzett and Rössler described a simple method of recording the degree to which the lungs of anaesthetized animals resist inflation. Since then the method has been used on many occasions to study physiological changes in resistance, the effects of drugs upon resistance and the action of drugs upon responses produced by agents known to change resistance.

Many workers have assumed that any change in resistance was produced by changes in bronchiolar tone, and this point has been discussed by Konzett (1956). In the present paper a more detailed study is made of the increased resistance to inflation produced in anaesthetized guinea-pigs by histamine, 5-hydroxytryptamine and acetylcholine and of the effects upon these responses of some of the antagonists of these drugs. A method for estimating the potency of these antagonists using the dose ratio (Gaddum, Hameed, Hathway and Stephens, 1955) has been employed.

METHODS

Although the method used was originally evolved for this study it has since been described in detail by Collier, Holgate, Schachter, and Shorley (1960). Resistance to the entry of air into the lungs of guinea-pigs anaesthetized with urethane (1.25 g./kg.) injected intraperitoneally was recorded by the Konzett and Rössler (1940) apparatus connected to the tracheal cannula, the air being supplied by a miniature Starling pump. After the resistance to inflation had been increased by a drug, it was returned to the original level by clipping for 10 sec. the tube leading to the recording apparatus, thus forcing the full stroke volume of the pump into the lungs. This reopened collapsed lung segments. Constancy of the base line resistance was obtained by small alterations in pump stroke volume. Anaesthesia was maintained by further intraperitoneal doses of 0.1 g. urethane. Drugs dissolved in normal saline were given either into the jugular vein in volumes of 0.05 to 0.4 ml. washed in with 0.5 ml. saline containing 10 i.u. heparin/ml. or by stomach tube in volumes up to 10 ml. washed in with 2 ml. saline.

Histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate and acetylcholine chloride were used as agonists. As antagonists, chlorpheniramine maleate, diphenhydramine hydrochloride, mepyramine maleate, 2-bromolysergic acid diethylamide bitartrate, lysergic acid diethylamide tartrate and atropine sulphate were employed. All doses are given in terms of the active acid or base.

RESULTS

Agonists

As a preliminary to experiments using antagonists, control experiments were carried out in which only the agonists were given. With all 3 agonists the response increased over the first 3 to 6 doses, and these were excluded from subsequent analyses and from times given for the duration of experiments. In these control experiments 4 doses in geometric progression were chosen to cover the range of recordable response. These were given at intervals of 5 to 10 (usually 8) min. in random order over a period of $5\frac{1}{2}$ to $6\frac{1}{2}$ hr. with intervals of 30 to 60 min. interpolated between some of the sets of 4 doses.

The response measured in all experiments was the maximum excursion of the piston recorder of the Konzett and Rössler apparatus. This was taken to indicate the maximum resistance to air entry. Preliminary investigations had shown this

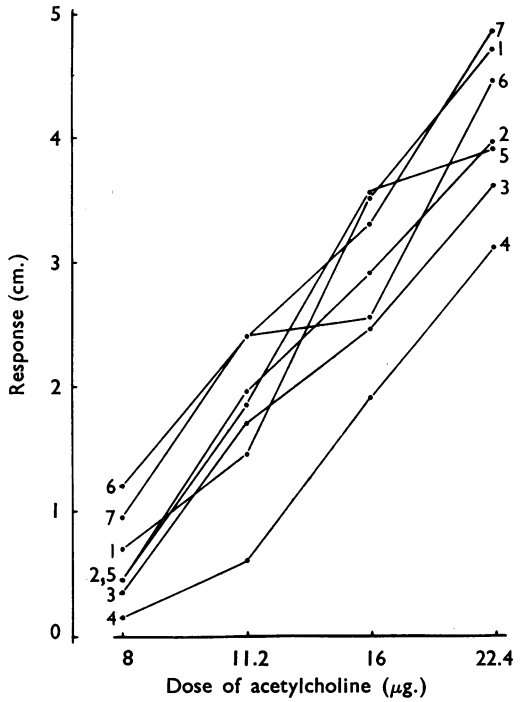

FIG. 1.—Log. dose-response curves for a control experiment with acetylcholine. Each line is drawn through points corresponding to 4 consecutive doses, and numbers indicate order of administration of each set of doses. Intervals of 30–60 min. were interpolated between sets 4, 5, 6, and 7. Responses were measured as the maximum excursion of the piston recorder in cm.

measure to be as informative as the area under the curve relating increase in excursion and time. The response was plotted against log dose of agonist as shown for an experiment on acetylcholine in Fig. 1.

Five such control experiments were carried out, 2 using histamine, 1 using 5-hydroxytryptamine and 2 using acetylcholine. From analysis of the results of these experiments it was found that, with 5-hydroxytryptamine and acetylcholine, the average dose-response curve was linear within the limits of experimental error. The deviations from linearity were significant in the experiments with histamine, but the biasing effect of this on the dose ratio (*vide infra*) was estimated as less than 4%.

In one experiment with histamine the slopes of the dose-response curves varied significantly as the experiment progressed, but in the remaining experiments with histamine and other agonists, differences in slope from one set of 4 responses to another were not significant.

In all control experiments the average level of response varied significantly between sets of responses. The degree of variation was expressed by determining the dose ratio for each set of 4 responses relative to the first set. Ideally this value should be unity since no antagonist had been given. The values obtained for the dose ratio are plotted against time in Fig. 2. In experiments involving acetylcholine the dose ratio showed progressive increase over a series of 4 sets of 4 doses with 8 min. intervals between doses and sets. In some preparations interpolation of

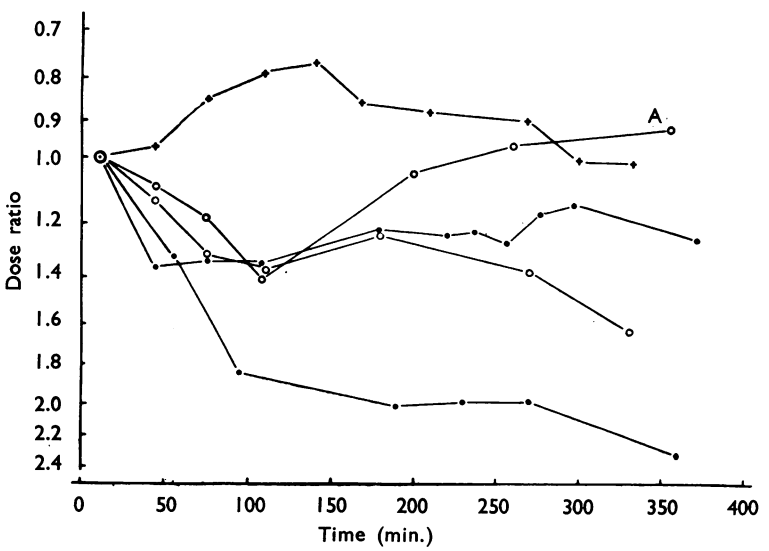

FIG. 2.—Control experiments. Dose ratios of sets of 4 responses relative to first set of 4 responses, plotted against time, taken as the mean for the period covered by a set of 4 observations. ●—●=Histamine; +—+=5-hydroxytryptamine; O—O=acetylcholine. Line A represents the results from the experiment illustrated in Fig. 1.

a 30 or 60 min. interval between 2 consecutive sets led to a decrease in dose ratio. Such a rest period did not, however, lead to a decrease in dose ratio in experiments with histamine.

Antagonists

Following Gaddum *et al.* (1955) the potency of an antagonist at a given concentration and a given time after administration is defined as the ratio A/A_0, where A_0 is the dose of the agonist producing a response (y) before the antagonist, and A is the dose of the agonist producing the same response (y) at the given time after the antagonist is administered. The dose ratio can be defined unambiguously only when the log. dose-response curves before and after giving the antagonist are parallel.

To obtain suitable data for the calculation of the dose ratio thus defined the following experimental procedure was adopted. Two doses of agonist were given repeatedly until the responses were stable. The antagonist was then given and the rise and fall of effect was followed by giving increasing and decreasing doses of agonist. Pairs of doses were chosen which, it was hoped, would produce responses comparable in magnitude to those obtained in the absence of antagonist. During the decline in the degree of antagonism intervals of up to 1 hr. were often allowed to elapse without doses of agonist being given. In the case of acetylcholine this was necessary in order to obtain maximal degree of recovery of the preparation (*vide supra*). When the dose ratio had fallen to 2 or less and stable responses were obtained to 2 doses of agonist, a further dose of antagonist was sometimes given.

From the data thus obtained the dose ratio could be determined graphically or by computation. The graphical method, suitable for routine investigation of antagonism, consists of plotting the responses against log. dose, estimating by eye the average slope within pairs of responses and drawing parallel lines through the centroid of each pair. The log. of the dose ratio is given by the horizontal distance between the parallel lines.

The computational approach, which is less subjective, has been used in all experiments reported here. The average slope within sets (usually a pair) over the duration of the experiment was calculated by standard regression procedures and the dose ratio determined from parallel lines with this slope fitted to sets of comparable responses.

In both methods where the degree of antagonism changes rapidly with time, individual responses may have to be used.

TABLE I

ANTAGONISM OF 5-HYDROXYTRYPTAMINE BY LYSERGIC ACID DIETHYLAMIDE

At 28 min. 50 μg./kg. lysergic acid diethylamide was given intravenously.

Dose No.	Time (min.)	Dose of 5-Hydroxy-tryptamine (μg.)	Response (cm.)
1	0	1	2·1
2	8	2	3·9
3	16	2	3·7
4	24	1	1·25
5	32	10	1·4
6	40	20	1·6
7	48	40	3·1
8	56	40	2·0
9	64	20	1·0
10	124	20	4·4
11	132	10	2·35
12	192	10	4·05
13	200	5	2·65
14	290	5	4·6
15	298	2·5	3·7
16	306	4	4·3
17	314	1	1·05
18	322	2	2·1

Table I gives the results of an experiment with 5-hydroxytryptamine and lysergic acid diethylamide; and Fig. 3 illustrates the method of determining from these data the dose ratio at different times.

Table II shows for 6 chosen antagonists dose ratios which are the maximal calculated during each experiment. When the antagonist was given intravenously the degree of antagonism often changed rapidly in comparison with the interval of 8 min. between doses of agonist. This may have resulted in underestimation of the maximal dose ratio.

Specificity of Antagonism

Specificity of antagonism was examined using diphenhydramine, mepyramine, lysergic acid diethylamide and atropine. Pairs of doses of each of the 3 agonists—histamine, 5-hydroxytryptamine and acetylcholine—were given at 5 min. intervals, usually in serial order with high and low doses alternating. When stable responses to each of these doses were obtained the antagonist was given intravenously. The sequence of doses of agonist was continued, increasing them where necessary to give responses

TABLE II

DOSE RATIOS CALCULATED FOR SIX ANTAGONISTS AT VARYING DOSES

Agonist	Antagonist	Dose (μg./kg.)	Route	Dose Ratio	No. of Experiments	Time at which Dose Ratio Measured (min.)
Histamine	Chlorpheniramine	1·6	Intravenous	1·5	1	4
		4		1·6	1	16
		16		3·1	1	4
		32		2·8	1	8
		40		5·4	1	12
		77		16	1	32
		400		27	1	32
		400	Oral	3·2	1	24
		1,700		52–330	3	44–80
	Diphenhydramine	3–6	Intravenous	0·8–1·4	4	8–32
		33–40		1·3–1·6	3	8–16
		80		1·8	1	4
		165		3·7	1	16
		320–400		3·0–6·8	4	8–28
		800		6·6	2	4–12
		2,000		32	1	24
		1,700–1,800	Oral	1·3–2·7	2	32–48
		17,000–18,000		40–62	3	64–84
	Mepyramine	4	Intravenous	2·4–4·2	2	12–24
		40		12–28	3	24–40
		400		3,500	1	32
		1,750–1,800	Oral	2.0–9·0	4	56–80
		8,750–9,000		12–110	3	40–104
		17,500		730	1	88
5-Hydroxytryptamine	Bromolysergic acid diethylamide	1	Intravenous	0·9–1·0	3	8–16
		10		1·9–6·2	3	16–24
		100		10–23	3	24–40
		100	Oral	1·0	1	16
		400–500		1·9–2·8	2	56–102
		2,000		3·9–4·9	2	72–160
	Lysergic acid diethylamide	1	Intravenous	1·0–2·5	3	16–32
		10		4·8–7·9	3	24–32
		50		32–34	2	32
		100		25–55	3	32–40
		50	Oral	6·1–14	2	32–88
		100		15	1	100
Acetylcholine	Atropine	1	Intravenous	1·1–1·3	2	8
		10		3·7-4·2	2	8–12
		100		10–20	2	4–8
		1,000	Oral	3·0–6·3	2	20–50
		5,000		22	1	188
		10,000		49	1	186

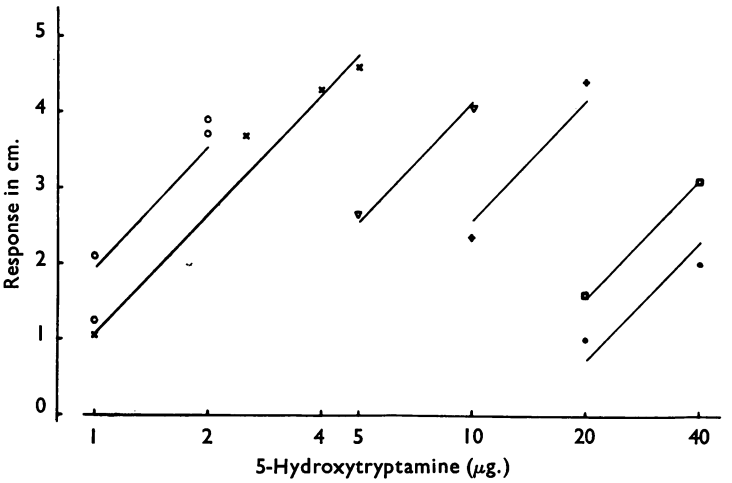

FIG. 3.—Log. dose-response curves illustrating data of Table I. O—O=Responses 1 to 4; □—□=responses 6 and 7; ●—●=responses 8 and 9; +—+=responses 10 and 11; ▽—▽=responses 12 and 13; and X—X=responses 14 to 18. For the 5 sets of responses after lysergic acid diethylamide the dose ratios were 24, 34, 7.6, 3.8, and 1.5 at 16, 32, 100, 168, and 278 min. respectively after giving the antagonist.

TABLE III

DOSE RATIOS OBTAINED IN SPECIFICITY EXPERIMENTS

Dose ratios given are the maxima or minima observed.

Antagonist	Intravenous Dose (μg./kg.)	Histamine		5-Hydroxytryptamine		Acetylcholine	
		Dose Ratio	Time (min.)	Dose Ratio	Time (min.)	Dose Ratio	Time (min.)
Diphenhydramine	400	7·8	37·5	1·9	12·5	2·0	102·5
Mepyramine ..	40	15	12·5	0·98	25	1·6	37·5
Lysergic acid diethylamide ..	50	0·83	15	53	32·5	0·76	10
Atropine ..	100	2·8	22·5	1·4	12·5	18	27·5
,, ..	100	2·4	10	1·7	15	27	20

similar to those obtained in the absence of the antagonist. The dose ratios could then be calculated for each agonist independently. The results are shown in Table III, in which dose ratios of less than 1 indicate potentiation. The 2 experiments with atropine showed good agreement.

DISCUSSION

This work shows that methods of evaluating the potency of an antagonist previously used *in vitro* can be applied to an *in vivo* preparation provided that the response to the agonist remains sufficiently stable over an adequate period of time. We believe that this has been achieved by certain refinements of the technique first reported by Konzett and Rössler (1940). These include keeping the preparation at a fairly constant degree of anaesthesia and maintaining aeration near the original level by reinflation of collapsed lung segments and slight alterations in pump stroke volume.

Sufficient stability of response was indicated in the control experiments previously described and illustrated in Fig. 1. The greatest loss of response resulted in a dose ratio of 2.3 over a period of 6 hr. Over shorter periods of time corresponding to times of maximal antagonism in Table II the change of response was always considerably less. The evidence of these control experiments is supported by many experiments in which antagonists have been given and full recovery of response observed after 5 to 6 hr. when the effects of the antagonists have disappeared.

When an antagonist has been given, the slopes of the log. dose-response curves before and after its administration have usually been comparable, the exception being when high doses of lysergic acid diethylamide were used. In this case the slope was decreased and it was impossible to calculate dose ratios.

The results given in Table II provide a basis for studying the variation in dose ratio between

animals given the same dose of antagonist. This variation is best measured by the coefficient of variation which is estimated from these results as about 35% for drugs given intravenously and about 70% for those given by mouth. Presumably this larger figure reflects variation in absorption of the antagonist.

From these results in an *in vivo* preparation, we can study the relative potency of a drug given intravenously and by mouth. In this short series of drugs remarkable differences can be seen as shown in Table IV. Even among the 3 antihistamines and the 2 antagonists of 5-hydroxytryptamine great differences exist.

TABLE IV

RATIOS OF ORAL TO INTRAVENOUS DOSES GIVING EQUAL DEGREES OF MAXIMAL ANTAGONISM

Antagonist	Approximate Ratio
Chlorpheniramine	4
Diphenhydramine	6
Mepyramine	200
Bromolysergic acid diethylamide..	100
Lysergic acid diethylamide ..	4
Atropine	60

In the specificity experiments given in Table III, dose ratios obtained against the primary agonist are reasonably comparable with those given in Table II, indicating that the use of other agonists in these experiments does not modify the primary antagonism. Both antihistamines showed specificity although this was greater for mepyramine than for diphenhydramine. Both antagonized acetylcholine to some extent, but the maximum dose ratio was obtained at a later time than that for histamine. The results for lysergic acid diethylamide were interesting in that the compound appeared to potentiate histamine and acetylcholine. This finding confirmed a previous experiment in which the responses before and after lysergic acid diethylamide were not sufficiently comparable to enable the dose ratio to be calculated.

In the 2 experiments using atropine, both histamine and 5-hydroxytryptamine were antagonized to some extent though the dose ratio is rather greater for histamine. This contrasts with the finding of Cambridge and Holgate (1955), using guinea-pig ileum, and suggests that 5-hydroxytryptamine is probably not acting by the liberation of acetylcholine. One would speculate that the 2 types of receptor suggested by Gaddum and Picarelli (1957) are not present in the bronchioles.

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Brit. J. Pharmacol. (1960), **15**, 567.

THE TRYPANOCIDAL ACTION OF HOMIDIUM, QUINAPYRAMINE AND SURAMIN

BY

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Homidium, quinapyramine, and suramin (Group II compounds) produce their trypanocidal effect *in vivo* only after a latent period of 24 hr. or more, during which time the trypanosomes may continue to multiply; this is in contrast to trivalent arsenical and diamidine compounds (Group I compounds), which begin to act immediately. Group II compounds also differ from Group I compounds in that (*a*) they have only a slight tendency to combine with trypanosomes, (*b*) they have little trypanocidal action *in vitro*, but (*c*) they make trypanosomes non-infective to fresh subinoculated mice. To explain these features it is postulated that homidium, quinapyramine, and suramin first combine in small amounts with some receptor on the trypanosome and then block some biochemical system which produces a hypothetical substance X which is needed for cell division of the trypanosome; the trypanosome is supposed to contain a preformed store of this substance X sufficient for several divisions to take place; and it is only when this store is exhausted that cell division is prevented and the trypanosome eventually dies.

The purpose of this paper is to describe certain characteristics of the trypanocidal action of homidium (Ethidium ; B.Vet.C.Supp. 1959, p. 27), quinapyramine (Antrycide ; B.Vet.C., 1955, p. 553), and suramin, and to offer a hypothesis to explain them. These compounds differ from other trypanocidal compounds such as trivalent arsenicals and diamidines in that their trypanocidal action *in vivo* is manifested only after a long latent period. Experiments were undertaken to study quantitatively the behaviour of the trypanosomes during this latent period.

METHODS

Rats or mice were infected with trypanosomes by intraperitoneal inoculation When the blood contained scanty trypanosomes, blood from the tail was diluted in a W.B.C. or R.B.C. pipette with a fluid which lysed the erythrocytes and stained the trypanosomes ; the trypanosomes were then counted in a haemocytometer slide under the microscope. A suitable fluid for this purpose was: 1% methylene blue, 2 ml. ; glacial acetic acid, 0.25 ml. ; water, 50 ml. In the case of *Trypanosoma evansi*, the number of trypanosomes in a drop of blood from the tail was probably the same as that in the main volume of circulating blood. *T. congolense*, on the other hand, tends to accumulate in the capillaries of the tail, and the count recorded may often have been much greater than that in the circulating blood ; presumably, however, the two were proportional, and this discrepancy would not affect the general argument. The animal was then treated intraperitoneally with a dose of the drug approximately double the dose which was sufficient to remove all visible trypanosomes from the blood within four days. Counts of the trypanosomes were made at suitable intervals (usually morning and evening) until no further trypanosomes were found.

The trypanosomes used were *T. evansi*, Mathura strain, described in a previous paper (Sen, Sharma, and Hawking, 1960) which was studied in rats ; and *T. congolense* (N.I.M.R. strain) which had been maintained in mice for some years and which was very sensitive to quinapyramine and homidium.

RESULTS

The results of typical experiments are shown in Fig. 1 in which the time is plotted horizontally and the logarithm of the number of trypanosomes in the blood is plotted vertically. In the case of *T. evansi*, the trypanosomes of the untreated control rat increase about 25 times (average) every 24 hr., that is, 4.7 divisions per 24 hr., or 1 division approximately every 5 hr. In the case of *T. congolense*, the trypanosomes of untreated control mice increased approximately 8 times every 24 hr, that is, approximately 1 division

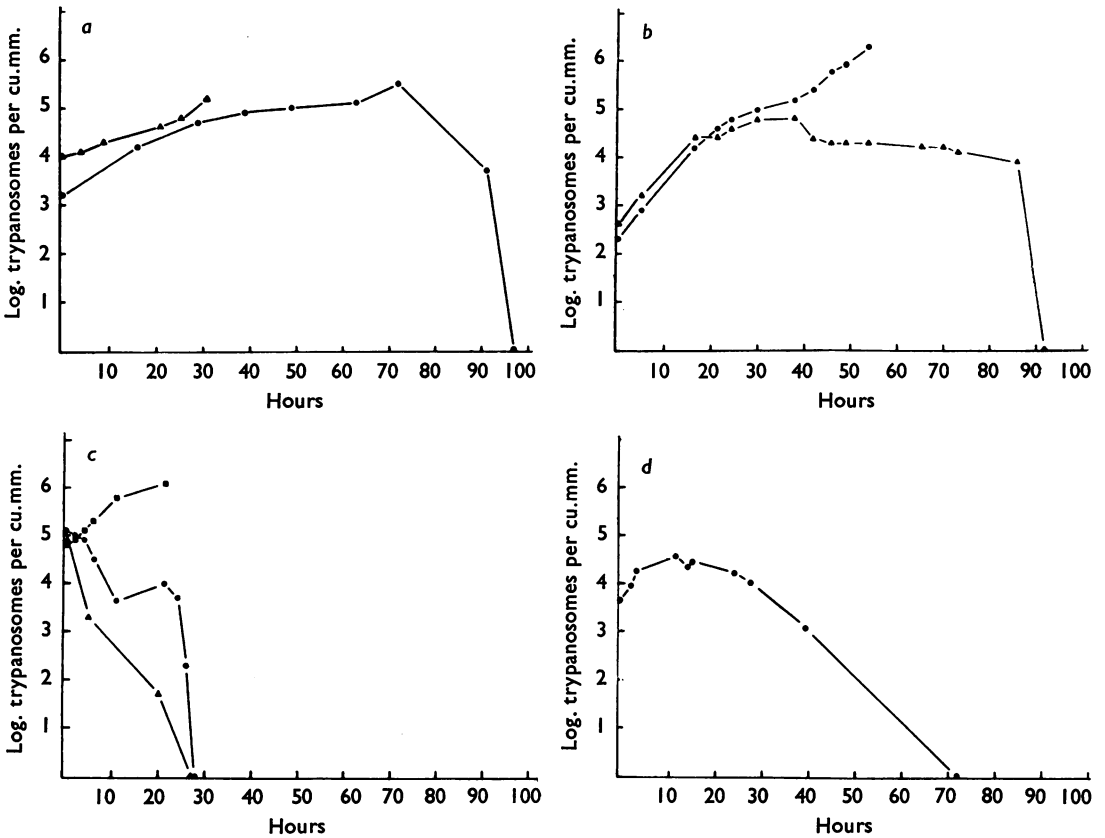

FIG. 1.—The vertical scales show the log. of the number of trypanosomes per cu.mm. tail blood; failure to see trypanosomes is arbitrarily indicated as log. 0. The horizontal scales show the time in hours after treatment. Drugs were given by intraperitoneal injection. Suramin 0.03 mg./100 g. ●—● (control ▲—▲) in (*a*). Quinapyramine 0.01 mg./100 g. ▲—▲ (control ●—●) in (*b*); melarsoprol 0.01 mg./100 g. ▲—▲, stilbamidine 0.2 mg./100 g. ●—● (control ■—■) in (*c*); were tested on *T. evansi* in rats. Homidium 0.004 mg./20 g., ●—●, in (*d*), was tested on *T. congolense* in mice.

every 8 hr. After treatment with two compounds (melarsoprol and stilbamidine), which are believed to exert a direct lethal action on the trypanosomes, the number of trypanosomes begins to fall immediately after the treatment, and reaches zero in about 27 hr. After treatment with homidium, quinapyramine, and suramin the trypanosomes continue to multiply for 24 to 72 hr.; then there is a period during which the trypanosomes remain approximately constant in number; finally they diminish rapidly and disappear. Depending on the dose, the drug, and the sensitivity of the trypanosome, these three phases vary in duration, but they can still be seen even when the dose is raised considerably. The essential point is that the trypanosomes continue to multiply for a considerable period after they have come into contact with the drug. In the case of suramin and quinapyramine the number may increase by 128 times, that is, 7 divisions; in the case of homidium it increased 8 times, that is, 3 divisions.

DISCUSSION

Two types of trypanocidal compounds can be recognized, according to their biological action as summarized in Table I.

It is considered that the first group—trivalent arsenicals (for example, trivalent tryparsamide, melarsoprol), acriflavine, diamidines (for example, stilbamidine, berenil)—are specifically absorbed by the trypanosomes, so that a high concentration is reached inside the cells, and that they then cause the death of the trypanosomes by direct action on some vital mechanism (Hawking,

TABLE I

TYPES OF TRYPANOCIDAL COMPOUNDS

	Group I Trivalent Arsenical Compounds	Group II Homidium and Other Phenanthridines, Quinapyramine, Suramin
Absorption by trypanosomes	Marked and immediate	Inconspicuous
Trypanocidal action *in vitro*	Marked	Slight or absent
Effect on infectivity ..	Slight	Marked
Trypanocidal action *in vivo*	Immediate	Manifest only after a period of multiplication

1938). The other compounds (homidium, quinapyramine, and suramin) are less simple in their mode of action.

Little absorption of the compound by the trypanosome, either *in vitro* or *in vivo*, can be shown by direct chemical methods, although a slight absorption probably does occur. Suramin could not be shown to be absorbed by trypanosomes, when a somewhat insensitive method was used (Hawking, 1939). Prothidium (a phenanthridine compound with pyrimidine side chain) is absorbed to a slight extent according to Taylor (1960). By contrast trivalent arsenicals and acriflavine are absorbed by trypanosomes in large amounts (Hawking, 1938) and the same is true of stilbamidine (Hawking, 1944; Fulton and Grant, 1955); this absorption can easily be demonstrated *in vitro* and *in vivo* and can be studied quantitatively.

The trypanocidal action *in vitro* of homidium and quinapyramine is moderate and that of suramin is slight. Suramin kills *T. rhodesiense* (old laboratory strain) (37°, 24 hr. exposure) only at 1/3,000 (Hawking, 1939). Under the same conditions quinapyramine kills *T. equiperdum* at 1 in 1 to 4 million (Hawking and Thurston, 1955) and dimidium kills *T. rhodesiense* (old laboratory strain) 1 in 1 million (Lock, 1950). These concentrations are all greater than are reached *in vivo* after therapeutic doses. By contrast the trivalent arsenicals and similar compounds under the same conditions kill trypanosomes at low concentrations like 1 in 50 million or 1 in 200 million.

When trypanosomes are exposed *in vivo* or *in vitro* for several hours to homidium, quinapyramine or suramin in suitable concentrations the trypanosomes appear unharmed and may continue to wriggle actively for 24 hr.; but if they are inoculated into fresh animals, no infection develops, showing that some profound change has been produced in them (Hawking, 1938; Lock, 1950; Ormerod, 1951; Hawking and Thurston, 1955). By contrast, when trypanosomes are exposed to arsenicals and similar compounds, their infectivity is not usually diminished, unless all the trypanosomes have been obviously killed or damaged before they are inoculated.

As demonstrated above, the *in vivo* trypanocidal action of homidium, quinapyramine, and suramin is exerted only after a latent period during which 3 to 7 cell divisions may occur, while the *in vivo* trypanocidal action of trivalent arsenicals and diamidine is manifested almost immediately.

It is suggested that these special features of the action of Group II compounds might be explained by a hypothesis on the following lines.

The drug first combines in small amounts with some receptor on the trypanosome. It then blocks some biochemical system which produces a hypothetical substance X which is needed for the cell division (multiplication) of the trypanosome. It is postulated that the trypanosome contains a preformed store of this substance X sufficient for several divisions to take place; when this store is exhausted, no further divisions can occur (perhaps the cell cannot even continue to function), and after a further period all the trypanosomes die.

The chief evidence for combination between group II drugs and trypanosomes (fixation) is that if trypanosomes are exposed to these drugs *in vitro* or *in vivo* they become non-infective to fresh animals, although they continue to wriggle for long periods. In its early stages part of this combination is probably reversible, but, judging by the period of exposure needed to make most of the trypanosomes non-infective, an irreversible combination seems to occur with dimidium in 0.5 hr. (Lock, 1950), with quinapyramine in 2.5 hr. (Ormerod, 1951), and with suramin in 1 to 2 hr. (Hawking, 1939). Newton (1957) found that *Strigomonas oncopelti* exposed to homidium (10 μg. per ml.) absorbed 3 μg. per 10^8 organisms in the first 3 hr., and that this combination was irreversible.

The phenomena of drug resistance show that homidium, quinapyramine, and suramin do not all act on the same site in the trypanosome (although there may be some overlapping between homidium and quinapyramine). Accordingly it may be supposed that either the compounds are

first fixed on different receptors (drug resistance depending on a diminished avidity of such receptors) after which all three act on the same biochemical system; or homidium blocks the production of a substance X, quinapyramine blocks the production of a similar substance Y, and suramin the production of a substance Z.

Substance X (or Y or Z) might be imagined to be a compound concerned with the synthesis of ribonucleic acid. In this connexion it may be noted that homidium rapidly inhibits the synthesis of deoxyribonucleic acid in *Strigomonas oncopelti* while the synthesis of ribonucleic acid and protein continues for several hours after addition of the drug (Newton, 1957). McIlwain (1946) has pointed out that in bacteria the molecules of many important substances, such as vitamins and co-enzymes, are limited in number per cell, for example, a cell may contain only 200 to 1,200 molecules of folic acid, and possibly only a few molecules of certain fundamental enzymes. Something the same may be true of trypanosomes, and substance X may be one of the molecules which are present only in limited numbers.

According to fluorescent appearances quinapyramine (Ormerod, 1951) and prothidium (Taylor, 1960) usually collect in the kinetoplast and in granules, in the cytoplasm of trypanosomes, and not in the nucleus to any large extent. (A similar distribution is shown by stilbamidine and acriflavine, which kill trypanosomes in a more direct manner). Either the hypothetical biochemical processes blocked by quinapyramine and prothidium take place mainly in the kinetoplast rather than in the nucleus, or only a small proportion of the drug absorbed by the trypanosome is responsible for the death of the organisms, the greater proportion being diverted into inactive combinations.

The morphological effects produced on trypanosomes by the administration of homidium, quinapyramine, or suramin to infected rats consist of: (i) Diminution in the percentage of dividing forms. (ii) Sometimes the occurrence of giant multinuclear trypanosomes in which the nucleus has divided once, or twice, but the cytoplasm has not; this is evidence of disordered cell division, but the disorder is greater in the cytoplasm than in the nucleus: this occurs with *T. rhodesiense* but it is rare with *T. equiperdum* or *T. congolense*. (iii) The appearance in the trypanosome cytoplasm of basophilic inclusion bodies which are best developed after 24 hr. Apparently these contain ribonucleic acid protein and drug (Ormerod, 1951). (iv) Diminished mobility in the trypanosomes, which can be seen under the microscope to wriggle less actively than usual (Mr. P. Walker, unpublished observation).

In discussing the mode of action of homidium upon *S. oncopelti* with particular reference to the metabolism of ribonucleic acid and deoxyribonucleic acid, Newton (1957) postulates that there is first a rapid combination of the drug with "primary binding sites" which does not interfere with growth and cell division, and then a slower combination with "secondary binding sites" which become available to the drug during growth; it is this secondary uptake which progressively inhibits growth. The conception of a rapid combination with primary binding sites (fixation), followed by a slower combination with secondary binding sites, is supported by many phenomena in trypanocidal action, and it is generally accepted. The suggestion that these secondary binding sites for homidium, etc., only become available during growth is an alternative hypothesis to the one submitted above in the attempt to explain the biochemical mechanism by which a drug can produce its inhibitory effect only after a latent period which may be 24 hr. or more. Whatever hypothesis is eventually proved to be correct, it is to be expected that it should apply to quinapyramine and suramin as well as to the phenanthridine compounds.

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THE EXCRETION OF SODIUM 6-(2,6-DIMETHOXYBENZAMIDOPENICILLANATE) MONOHYDRATE IN RATS

BY

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The excretion of sodium 6-(2 : 6 dimethoxybenzamido) penicillanate monohydrate has been studied. When injected intramuscularly it was rapidly excreted in the bile and urine of rats, but only in trace amounts in the faeces. The excretion products in the bile (2 to 3% of the dose) and urine (26–84% of the dose) are microbiologically active. The product in the bile may be in conjugated form.

Sodium 6-(2:6 dimethoxybenzamido) penicillanate monohydrate (BRL 1241) is a synthetic derivative of 6-aminopenicillanic acid which is almost immune to the action of penicillinase and has marked bactericidal activity against strains of *Staphylococcus aureus* resistant to natural forms of penicillin (Rolinson, Stevens, Batchelor, Cameron-Wood, and Chain, 1960).

Pharmacological studies in patients (Knudsen and Rolinson, 1960; Stewart, 1960) have shown that BRL 1241 is rapidly excreted in the urine in amounts up to about 75% of each injected dose. In laboratory animals (Brown, personal communication) about 30% is excreted in 6 hr. Of the remainder, a proportion can be detected in bile (Stewart, 1960), but there is no exact data on the amounts excreted by different routes.

The present study describes the mode and rate of excretion of BRL 1241 in bile, compared with urine and faeces.

METHODS

BRL 1241 was injected intramuscularly as a sterile neutral solution in water in doses of 100 and 300 mg./kg. Standard solutions for assay were made up from the pure amorphous powder.

Rats.—Male and female albino rats, 250 to 400 g. weight, bred in these laboratories, were used. The bile duct was cannulated at laparotomy, under ether and/or nembutal anaesthesia, with polythene tubing (0.50 mm. bore × 0.20 mm. wall) through which bile was collected into small graduated tubes for measured intervals of time. After recovery from the anaesthetic, the rats were restrained in close-fitting, flexible wire-mesh cages.

Assays.—BRL 1241 was assayed in test and standard solution on square agar plates 33 × 33 cm. against *Sarcina lutea* and the Oxford strain of *Staphylococcus aureus*. Standards were normally prepared in water, but, in some experiments, normal rat bile, plasma and urine were used as solvents. Concentrations of the drug in test fluids were estimated by interpolation on graphs relating inhibitory zone-diameter to the logarithms of concentrations of standards. Chromatographic bio-assay was performed by the ascending technique on 25 cm. square Whatman No. 1 paper. The solvent was butanol:ethanol:0.05 M phosphate 25:10:20, at *p*H 7.0. After a 17-hr. run, chromatograms were dried with a current of air at room temperature, cut into 7 mm. strips, and placed on seeded agar plates for overnight incubation. By this technique, BRL 1241 gave a mean R_F value of 0.91 ± 0.07.

Solutions for assay were kept at 4° or lower, and standards were made up freshly from a pure, stable preparation of BRL 1241 in powder form; when made up, these standard solutions were kept at −20°.

Urine and faeces from rats were collected over 24-hr. periods in metabolism cages. Urine was measured and assayed directly after dilution in water to 1:100 or higher. Faeces were weighed, homogenized in water with minimal dilution, and filtered. Before assay, urine and faeces were heated to 75 to 80° and kept at this temperature for 10 min. to minimize contamination. There was a fractional (15 to 20%) loss in the activity of BRL 1241, but this varied according to *p*H and other factors and no allowance has been made for it in the assay figures in the tables.

For the assays liver was washed clear of blood, blotted, weighed and homogenized in water. The homogenate was centrifuged and the supernatant inserted directly into cups in agar, without dilution, as with serum or plasma.

RESULTS

BRL 1241 was regularly present in the bile of healthy adult rats of both sexes in relatively high concentrations within 1 hr. of an intramuscular injection. After 100 mg./kg., 0.14 to 0.4 mg. was excreted in the first hour and further comparable

quantities in the second hour. Thereafter, the amount excreted dropped sharply and was hardly detectable after 4 hr. (Fig. 1). Excretion began within 10 min. of an injection, but the rate of excretion thereafter varied according to the dose injected. At 100 mg./kg. the peak values occurred between 30 and 90 min., whereas 300 mg./kg. gave sharper and disproportionately higher peak values at about 30 min. (Fig. 2). The actual concentrations of these peaks (0.60 mg./ml. and 2.6 mg./ml. respectively) were much higher than corresponding levels in the serum and in the liver (Table I).

At this rate of excretion in bile 0.32 to 1.08 mg. BRL 1241 was transferred to the duodenum during the 3 hr. after an injection of 100 mg./kg., equivalent to about 1 to 3.5% of the total dose or a mean excretion of 2.6 mg./kg. (Table II). After 300 mg./kg. the excretion in one rat was

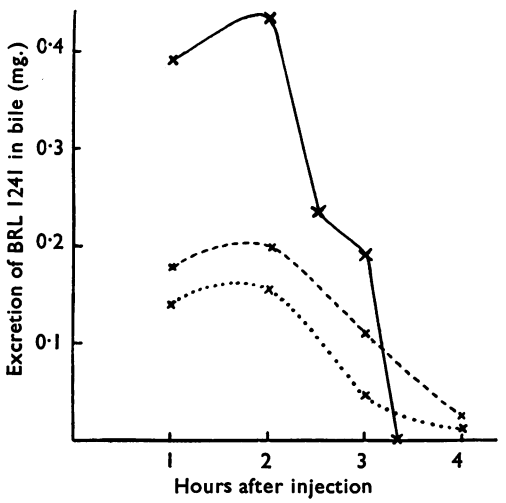

FIG. 1.—Rate of excretion of BRL 1241 in the bile of rats. —— Rat 1, 300 mg./kg. - - - - Rat 2, 100 mg./kg. . . . . Rat 3, 100 mg./kg.

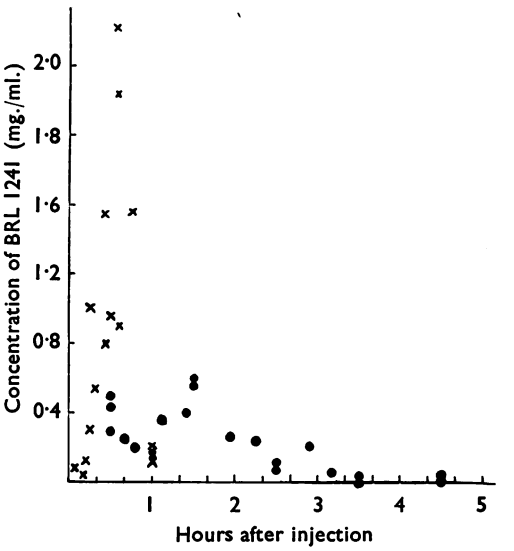

FIG. 2.—Concentration of BRL 1241 in the bile of rats injected intramuscularly. X 300 mg./kg. ● 100 mg./kg.

TABLE I

CONCENTRATIONS OF BRL 1241 IN LIVER, SERUM, BILE AND URINE OF RATS INJECTED WITH 100 MG./KG.

Hours after Dose	BRL 1241			
	Serum mg./ml.	Liver mg./g.	Bile mg./ml.	Urine mg./ml.
1·5	0·029	0·013	0·420	9·320
3·5	0·005	0·002	0·110	10·300

TABLE II

EXCRETION OF BRL 1241 IN BILE, URINE, AND FAECES OF RATS

Dose (mg./kg.)	No. of Rats	24-hour Excretion of BRL 1241				Total (% of Dose)
			Bile	Urine	Faeces	
300	3	Mean (mg./kg.)	10·6	120	Trace	40–46
		% of dose	3·6	40		
100	14	Mean (mg./kg.)	2·6±0·98	53±23	0–0·2	27–86
		% of dose	1·1–2·7	26–84	0–0·2	

5.1 mg., nearly 6% of the dose, the mean excretion being 10.6 mg./kg.

Chromatographic bio-assays show that BRL 1241 had a lower R_F in bile than in water, irrespective of whether it was excreted in the bile *in vivo* or added *in vitro* (Table III). When incorporated *in vivo*, the drug appeared to be relatively more active against *Staph. aureus* than against *Sarcina lutea* (Table IV).

TABLE III
CHROMATOGRAPHIC BIO-ASSAY OF BRL 1241. R_F VALUES IN WATER AND BILE

BRL 1241	No. of Tests	R_F Values		
		Range	Mean	S.D.
Aqueous	8	0·89–0·93	0·91	0·07
Excreted in bile ..	10	0·66–0·85	0·78	0·08
Added to bile ..	8	0·66–0·88		

TABLE IV
ASSAYS OF BRL 1241 IN BILE AGAINST *SARCINA LUTEA* AND THE OXFORD *STAPHYLOCOCCUS*

Sample of BRL 1241	Expt. No.	Concentration (μg./ml.) as Assayed Against	
		Sarcina lutea	Oxford *Staph.*
Excreted in bile	1	450	2,000
	2	530	1,600
	3	210/290	1,550/1,000
	4	260/270	800/780
	5	260/375	975/990
	6	320/255	360/400
Added to bile ..	1	18·5	22·5
	2	18·0	18·5
Added to bile and incubated	1	25·5	28·5
	2	20·0	20·0
	3	10·0	10·0

In urine, the mean excretion after a dose of 100 mg./kg. was 53 mg./kg., the range being 26 to 84% of the injected dose (Table II). Samples obtained during the 4 hr. after injection gave assay values of 25 mg./kg. or more, indicating that excretion occurred rapidly, as in bile. Individual rats showed considerable variation in the quantities of drug detectable in urine. This appeared to be due to differences in urinary flow and to partial inactivation of BRL 1241 in acid solution, especially in 24 hr. samples. A further 15 to 20% loss occurred in heating urine (and faeces) at 75°, which was necessary to minimize contamination of the assay-plates. If allowance was made for these variables, it was clear that the bulk of the injected dose was rapidly excreted in the urine.

In the faeces, BRL 1241 was absent or barely detectable (Table II), accounting at maximum for only 0.2% of the injected dose.

DISCUSSION

It is known that BRL 1241 is rapidly excreted in the urine, in man as in laboratory animals (Knudsen and Rolinson, 1960; Douthwaite and Trafford, 1960; Stewart, 1960; Brown, personal communication). The present results show that excretion also occurs in bile, in rats, with comparable rapidity. The amount detectable in bile (usually 2 to 3% of the injected dose) is small compared with that in the urine, but is sufficient to produce concentrations (0.32 to 5.1 mg./ml.) which are in excess of those (0.001 to 0.005 mg./ml.) at which the drug is bactericidal to pyogenic cocci. Quantities of this order are above the levels present in serum and in the liver, so that the drug is concentrated 10 to 100 fold in the biliary tract and is presumably excreted directly after passage through the hepatic cells, without storage. The moiety of drug thus transferred to the intestine is presumably re-absorbed or inactivated, as little or none is detectable in the faeces.

Chromatographic studies have shown (Stewart, 1960) that BRL 1241 is excreted in human urine essentially unchanged. This is also true when the drug is excreted by rats in the urine but not in bile. The biliary excretion product shows changed chromatographic and microbiological properties. The rapid rate of excretion, and the absence of a metabolite in the urine, make it unlikely that the change is due to the formation of a hepatic metabolite, though it may be a physical conjugation with a substance present in the bile. Whatever the explanation, the resulting compound apparently retains full antibacterial activity, and may even show an apparent enhancement of activity against *Staph. aureus*.

BRL 1241 has also been found, in active form and in high concentration, in human bile (Stewart, 1960). In this respect it is similar to natural forms of benzylpenicillin.

BRL 1241 was kindly supplied by Beecham Research Laboratories.

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THE MECHANISM OF THE INHIBITORY EFFECT OF GLUTEN FRACTIONS ON THE PERISTALTIC REFLEX

BY

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The mechanism of the inhibitory effect of gluten fractions on the peristaltic reflex was studied using, first, a filtrate of the autoclaved peptic-tryptic digest of gluten (fraction III A.F.) and, second, the ultrafiltrate of an aqueous extract of gluten (fraction G.U.F.). The fractions did not act as antagonists to naturally-occurring agents such as acetylcholine or substance P, but they did depress the twitch response of the isolated guinea-pig jejunum preparation stimulated coaxially. The output of acetylcholine from cholinergic nerve endings in gut was decreased during rest as well as during electrical stimulation. In large amounts, the gluten fractions depressed acetylcholine synthesis *in vitro*. It was concluded that the mechanism of inhibition of the peristaltic reflex by gluten fractions was chiefly by decreasing acetylcholine release.

Intolerance to dietary wheat gluten has been established as a major factor in the pathogenesis of coeliac disease as well as of idiopathic steatorrhoea. Certain fractions of gluten depress the peristaltic reflex of isolated loops of rat jejunum (Schneider, Bishop and Shaw, 1960). The present work is an attempt to analyse the mechanism of this depression of the peristaltic reflex.

Two gluten fractions were used. The first was a filtrate of the peptic-tryptic digest of gluten after protein had been denatured by autoclaving (fraction III A.F.). The second was an ultrafiltrate of an aqueous extract of gluten (fraction G.U.F.).

METHODS

Antagonism to Drug-induced Contractures in the Guinea-pig Gut.—This was tested by conventional methods using the isolated guinea-pig ileum preparation.

Twitch Response of the Guinea-pig Jejunum Stimulated Electrically.—The method of Paton (1957) was used.

Assay of Acetylcholine Output.—This was carried out on isolated terminal ileum of the guinea-pig set up in Tyrode solution containing neostigmine and morphine as described by Paton (1957).

Assay of Acetylcholine Synthesis by Guinea-pig Small Intestine.—The incubation system used was that of Feldberg and Lin (1949) and acetylcholine was assayed on the terminal ileum preparation of Paton (1957).

Preparation of Gluten Fractions.—The fractions were prepared by the methods described by Schneider, Bishop, and Shaw (1960).

RESULTS

The Effect of the Two Gluten Fractions on Contractions of the Longitudinal Muscle of Isolated Guinea-pig Intestine Induced by Acetylcholine and Substance P.—Both gluten fractions were used in the amounts which had been found to abolish the peristalic reflex of the isolated

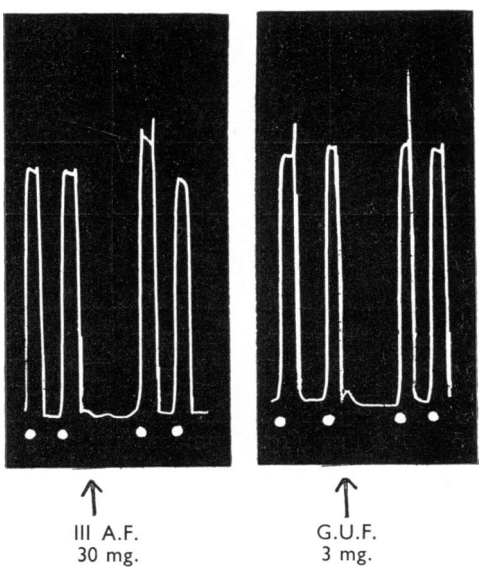

FIG. 1.—Effect of the two gluten fractions on the acetylcholine induced spasm of guinea-pig ileum. Bath volume, 15 ml.

rat jejunum in the earlier studies of Schneider, Bishop and Shaw (1960), namely, for III A.F., 2.0 mg./ml., and G.U.F., 0.2 mg./ml. A 2 min. cycle was used, and the intestine was in contact with the gluten fraction for 30 sec. There was no antagonism of the spasm induced by acetylcholine (Fig. 1), nor to that induced by substance P (Fig. 2). In both instances, the spasms in the presence of the gluten fractions were often slightly larger than those in control. The failure of the two gluten fractions to depress the responses either to acetylcholine or to substance P excluded both the possibility of a specific antagonism to either substance or of a general paralysing effect on intestinal muscle.

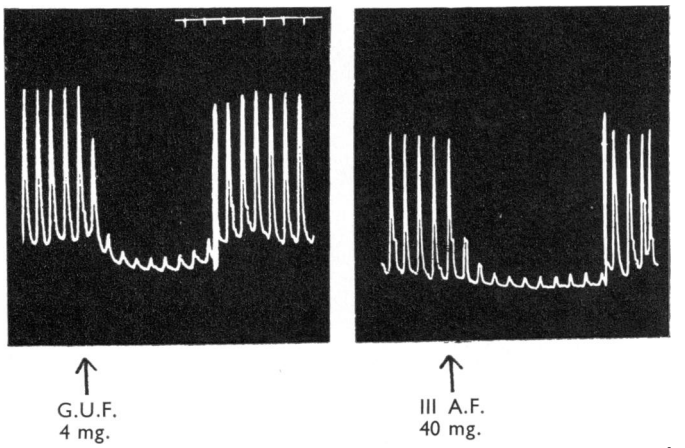

FIG. 3.—Effect of the two gluten fractions on the twitch response of coaxially stimulated guinea-pig ileum. Bath volume, 50 ml. Time, 10 sec.

The Effect of the Two Gluten Fractions on the Contraction of the Isolated Guinea-pig Jejunum Stimulated Coaxially.—Paton (1957) has shown that this preparation was sensitive to the blocking action of atropine, but was resistant to ganglionic blocking agents. Thus any depression of the response to electrical stimulation could be attributed to effects at the peripheral cholinergic nerve endings. Both gluten fractions caused a marked depression of the twitch response (Fig. 3). Usually 0.8 mg./ml. of III A.F. and 80 μg./ml. of G.U.F. decreased the response compared with 2.0 mg./ml. and 0.2 mg./ml. respectively for the peristaltic reflex. This preparation proved to be about two or three times more sensitive to the action of the gluten fractions than did the peristaltic reflex of the isolated rat jejunum.

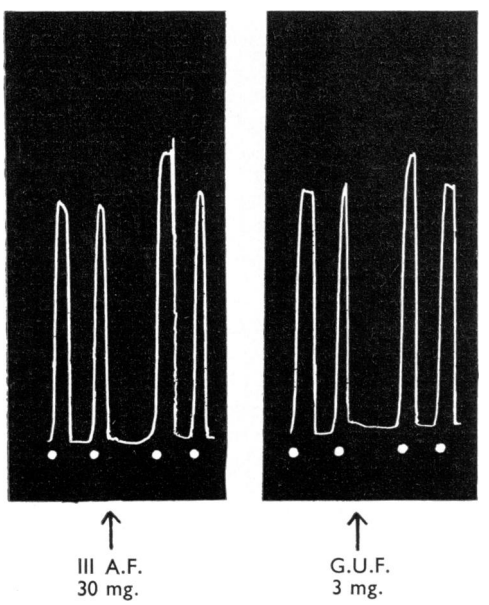

FIG. 2.—Effect of the two gluten fractions on the substance P induced spasm of guinea-pig ileum. Bath volume, 15 ml.

The Effect of the Two Gluten Fractions on the Output of Acetylcholine from the Isolated Guinea-pig Jejunum.—Results are summarized in Table I. Both gluten fractions decreased the output of acetylcholine significantly. This occurred

TABLE I

ACETYLCHOLINE OUTPUT FROM GUINEA-PIG INTESTINE

Gluten Fraction	No. of Expts.	Mean Acetylcholine Output (ng./min.)		Difference of Means (ng.)	*P*
		Control	Test		
III A.F. 40 mg./ml.					
Rest. ..	13	16·8	10·0	6·8	0·001
Stim. ..	13	30·3	17·8	12·5	0·01–0·001
G.U.F. 4 mg./ml.					
Rest. ..	11	18·6	10·9	7·7	0·01–0·001
Stim. ..	13	26·0	15·5	10·5	0·001

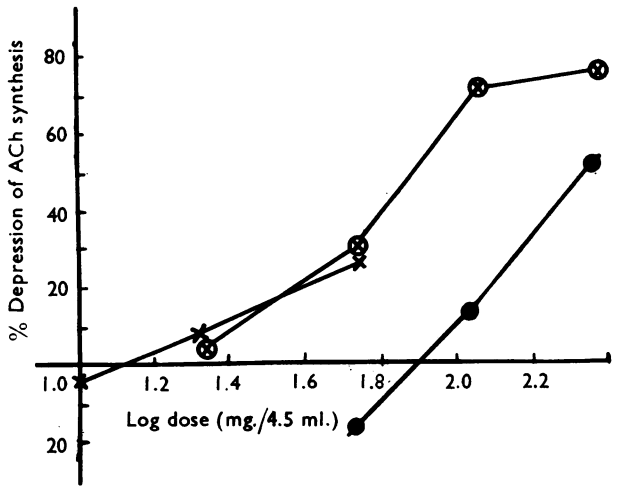

FIG. 4.—Effect of the two gluten fractions. G.U.F. X—X, III A.F. ⊗—⊗, and the peptic-tryptic digest of gelatin, Gel. III A.F. ●—●, on acetylcholine synthesis in guinea-pig small intestine.

in the resting state as well as during stimulation at 50 pulses/min. for 5 min. The ultrafiltrate G.U.F. was again about 10 times more potent than the peptic-tryptic digest III A.F.

The Effect of the Two Gluten Fractions on the Synthesis of Acetylcholine.—Addition of either of the gluten fractions to the incubation system caused a decrease in acetylcholine synthesis which became more marked with higher doses (Fig. 4). When the amount of III A.F. was raised from 4.4 to 11 mg./ml., the decrease in synthesis rose from 4 to 27% and reached 70% when 22 mg./ml. was used. Amounts of G.U.F. of 22 mg./ml. and over could not be tested, for they were spasmogenic. With the lower doses the curves for the two gluten fractions relating dose to decrease in acetylcholine synthesis were superimposable. In order to determine whether this depression of synthesis was a characteristic feature of gluten fractions, or whether it was a non-specific effect, experiments were carried out with a peptic-tryptic digest of gelatin (Gel. III A.F.) which had no action in depressing the output of acetylcholine from the isolated guinea-pig jejunum. Depression of synthesis was again found, but the gelatin digest was less potent than the corresponding gluten fraction (Fig. 4).

DISCUSSION

There are several ways in which the two gluten fractions might depress the peristaltic reflex. (1) Complete paralysis of both longitudinal and circular muscle could abolish the reflex. This mechanism may be excluded, for, in the presence of the gluten fractions, the longitudinal muscle of the intestine remained sensitive to acetylcholine and substance P. (2) Ganglionic blockade could depress the peristaltic reflex, but this mechanism was unlikely as the two gluten fractions depressed the twitch response of the guinea-pig jejunum to coaxial stimulation and this preparation is insensitive to the action of ganglionic blocking agents (Paton, 1957). (3) Antagonism to a substance occurring physiologically and which stimulates smooth muscle directly such as acetylcholine or possibly substance P might have caused depression. This possibility was excluded, for the gluten fractions did not antagonize the effects of either. Using the rat fundus preparation of Vane (1957), antagonism to 5-hydroxytryptamine could not be demonstrated as both gluten fractions caused a spasm which would have masked any possible depressant effect (Schneider and Bishop, unpublished observation). Antagonism to 5-hydroxytryptamine, however, is unlikely, as severe 5-hydroxytryptamine depletion does not abolish the peristaltic reflex (Bülbring and Crema, 1959). (4) The remaining possible mode of action was by depression of acetylcholine production at cholinergic nerve endings as has been claimed for morphine (Schaumann, 1957, and Paton, 1957). In fact the acetylcholine output from the resting as well as by the electrically stimulated isolated guinea-pig jejunum was decreased by both gluten fractions. The clinical observations of Ingelfinger and Moss (1942) are in accord with our findings. Using an intraluminar balloon technique, these workers found depression of small intestinal motility and tone in two cases of "sprue." Function of the small intestine was restored by the administration of methacholine but not by the anticholinesterase, neostigmine. The authors concluded that, in the syndrome, there was an inability of the intestinal autonomic nervous system to liberate acetylcholine.

Such depression of acetylcholine production could be due either to depressed synthesis or depressed liberation from its inactive precursor. Experiments on the acetylcholine synthesizing system in the guinea-pig small intestine showed that the addition of either gluten fraction could depress synthesis and suggested that there was a relationship between dose and effect. However, with the doses which decreased acetylcholine

output (4.0 mg./ml. for III A.F. and 0.4 mg./ml. for G.U.F.) there was no depression of acetylcholine synthesis. With larger doses (11 mg./ml.) both gluten fractions were equally potent in depressing synthesis, even though G.U.F. was at least 10 times more potent than III A.F. in decreasing acetylcholine output. As this depression of acetylcholine synthesis might be a non-specific effect of peptides due to the large amount of material used, the peptic-tryptic digest of gelatin (Gel III A.F.) was tested and found in large amounts (22 mg./ml.), to depress acetylcholine synthesis. It was, however, less potent than fraction III A.F. From these experiments it was concluded that both mechanisms might contribute to the depression of acetylcholine output by gluten fractions, but that depression of liberation of acetylcholine was more important than depression of synthesis.

These studies are part of a collaborative programme of work on the pathogenesis of gluten-induced enteropathy which is being undertaken in this department under the general direction of Professor A. C. Frazer. We wish to thank Mrs. B. Matthews for technical assistance and the Energen Foods Co. for supplies of gluten.

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TREMOR IN CATS: THE EFFECT OF ADMINISTRATION OF DRUGS INTO THE CEREBRAL VENTRICLES

BY

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In cats, tremor produced by intraperitoneal pentobarbitone sodium or by intramuscular chlorpromazine was abolished by injection into the cerebral ventricles of a few gamma of adrenaline or noradrenaline, but not of other sympathomimetic amines or of anti-Parkinsonian drugs. Chloralose, urethane, calcium chloride, and magnesium chloride produced anti-tremor activity when administered in this way. When adrenaline or noradrenaline was perfused from a lateral ventricle to the aqueduct during pentobarbitone sodium tremor, tremor was inhibited when less than 1 μg. had been perfused. Adrenaline was at least four times as active as noradrenaline. Isoprenaline inhibited tremor when perfused in higher concentrations, but perfusion of ephedrine, amphetamine or of the anti-Parkinsonian drugs in high concentrations was ineffective. Perfusion of 2 μg. of chloralose or of 20 μg. of calcium chloride was sufficient to inhibit tremor. Intraventricular injections of 250 to 500 μg. of 5-hydroxytryptamine initiated tremor. Possible physiological implications of these findings are discussed.

The present experiments deal with the action of drugs in initiating and abolishing tremor when administered by the intraventricular route, that is, by injection into the cerebral ventricles or by perfusion from a lateral ventricle to the aqueduct. Feldberg and Malcolm (1959) observed that tubocurarine perfused in this way produced tremor in cats anaesthetized with chloralose, and that the tremor was abolished when adrenaline or noradrenaline was added to the perfusing fluid. The concentration of the amines employed in these experiments was strong, as was the concentration of tubocurarine required to produce tremor. The question therefore arose as to whether adrenaline or noradrenaline exerted anti-tremor activity, when used in more physiological concentrations, on tremor produced by other means.

Intraperitoneal pentobarbitone sodium (Hall and Goldstone, 1940; Lippold, Redfearn and Vuco, 1959) and intramuscular chlorpromazine (Kaelber and Joynt, 1956) produce tremor in cats, and Hall and Goldstone have shown that the pentobarbitone sodium tremor is abolished by an intravenous infusion of adrenaline, 4 μg./kg. per min.; an intravenous injection of 80–150 μg./kg. first accentuated and then abolished tremor. It has now been found that both the pentobarbitone sodium and the chlorpromazine tremors are abolished by small amounts of adrenaline or noradrenaline administered by the intraventricular route. Of other sympathomimetic amines examined in this way, isoprenaline was found to have anti-tremor activity but only when administered in much larger amounts than adrenaline or noradrenaline.

Another well-known action of intraventricular adrenaline and noradrenaline is sedation, or an anaesthesia-like condition (for references see Feldberg, 1958). A similar effect occurs with intraventricular chloralose, urethane, calcium and magnesium (Feldberg and Sherwood, 1957; Feldberg, 1958). These substances were therefore also examined by the intraventricular route, and found to inhibit the pentobarbitone sodium and chlorpromazine tremor. On the other hand, anti-Parkinsonian drugs administered in this way did not affect these tremors. Finally, while investigating the effect of intraventricular 5-hydroxytryptamine, the substance itself was found to initiate tremor.

METHODS

The experiments were carried out on cats. For the injection of drugs into the cerebral ventricles a Collison cannula was implanted aseptically into the left lateral ventricle during pentobarbitone sodium anaesthesia as described by Feldberg and Sherwood (1953). After recovery from the operation the cats

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were used for experiments with intraventricular injections once or twice a week. The drugs were injected intraventricularly in a volume of 0.25 ml.

Perfusion from Lateral Cerebral Ventricle to Aqueduct.—The cats were anaesthetized with intraperitoneal pentobarbitone sodium (30 mg./kg.). The trachea was cannulated and, with the cat lying on its back, the head was fixed in a raised and flexed position so that the surface of the skull was horizontal to the ear bars and mouth piece of a head holder, similar to the Horsley Clark stereotaxic instrument. The method of perfusion was that described by Bhattacharya and Feldberg (1958). The inflow was through a Collison cannula implanted into the left lateral ventricle, the outflow through a polythene tube inserted through the opened cisterna magna, so that the tip of the cannula lay in the middle of the aqueduct. This fact was ascertained at the end of several experiments by perfusing the dye bromophenol blue and then making a sagittal section through the brain. The perfusion fluid was the artificial cerebrospinal fluid described by Merlis (1940) but without the bicarbonate. The bicarbonate was replaced by an equivalent amount of NaCl in order to diminish the destruction of adrenaline and noradrenaline in the alkaline fluid. The composition of the perfusion fluid was as follows (g./l.): NaCl 8.98; KCl 0.25; $CaCl_2$ 0.14; $MgCl_2$ 0.11; NaH_2PO_4 0.07; urea 0.13; and glucose 0.61.

Perfusion was maintained by a continuous slow injector (C. F. Palmer) at a rate of 0.1 ml./min. The fluid in the syringe of the injector passed by means of polythene tubing first through a small air chamber where the inflow could be checked, then through a water jacket in which water at 38° was circulating by means of an air lift, and finally through the shaft of a fine syringe needle which was inserted through the rubber diaphragm closing the top of the Collison cannula. The substances to be perfused were dissolved in the artificial cerebrospinal fluid and a second continuous slow injector was filled with the solution. The switch-over of perfusion from one injector to the other was done by withdrawing the needle of the one and inserting that of the other in its place; this took less than a second.

Recording of Tremor.—In cats anaesthetized with pentobarbitone sodium records of tremor were obtained from the hind legs by attaching the paw or the tendon of the tibialis anterior muscle by means of a thread to a light tension lever, the movements of which were traced on a smoked drum.

Substances Applied by the Intraventricular Route.—The following substances were injected into or perfused through the cerebral ventricles: adrenaline bitartrate; noradrenaline bitartrate; 5-hydroxytryptamine creatinine sulphate; creatinine sulphate; amphetamine sulphate; ephedrine sulphate; phenylephrine hydrochloride (Neosynephrine); isoprenaline sulphate; atropine sulphate; hyoscine hydrochloride; benzhexol (Artane); diethazine hydrochloride (Diparcol); chloralose; urethane; calcium chloride and magnesium chloride. When the substances used were salts, the doses given in the text refer to the salts.

RESULTS

Chlorpromazine Tremor

Intramuscular injection of 25 mg./kg. chlorpromazine hydrochloride produced tremor, but whereas relaxation of the pupils, ataxia and sedation occurred within a few minutes, tremor did not begin until 1 to 4.5 hr. after the injection. It then continued sometimes for several hours and was most clearly observed when the animal was lying on its side. Chlorpromazine tremor is known to be related to hypothermia, which in its turn depends on the environmental temperature. This, in the present experiments, was between 19 and 23°.

Sympathomimetic Amines. — Intraventricular injection of 5 μg. of either adrenaline or noradrenaline stopped tremor for 10 to 25 min. In some cats the intraventricular injection of 2.5 μg. of either amine decreased or stopped tremor for a few minutes, but in others this dose was ineffective. Intraventricular injections of amphetamine or phenylephrine tested in doses of up to 500 μg. did not affect tremor.

Chloralose.—Intraventricular injection of 0.5 mg. chloralose stopped tremor for 1 to 2 hr.

Calcium Chloride.—Intraventricular injection of 0.5 mg. calcium chloride stopped tremor for 10 to 20 min.; an injection of 0.25 mg. or less was ineffective.

Anti-Parkinsonian Drugs.—Atropine, hyoscine and benzhexol injected intraventricularly in amounts of up to 0.5 mg. did not affect tremor.

Pentobarbitone Sodium Tremor

Intraperitoneal injection of 30 mg./kg. pentobarbitone sodium initiated a fine fast tremor or shivering 30 to 150 min. after the injection. Tremor continued for at least 1 hr. and sometimes for as long as 5 hr. When the cat was lying on its side, tremor usually began in the flank, gradually spread over the hind limbs and later involved the whole body, including the neck and the head. Often the intensity of tremor increased during this period. In different cats the intensity of tremor showed pronounced variations, but in the same cat tremor did not vary, or varied only a little, when the pentobarbitone sodium was injected on different days. In several experiments the rate of tremor recorded on a fast-moving smoked drum was found to vary between 10 and 18/sec.

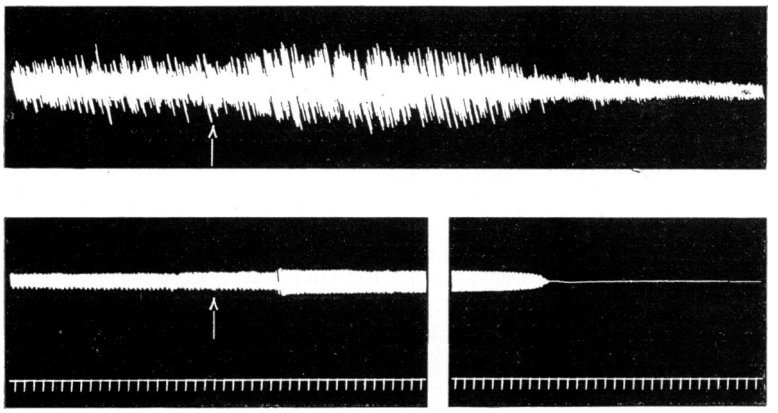

FIG. 1.—Tremor recorded from left hind leg of a cat anaesthetized with pentobarbitone sodium. At the arrows intraperitoneal injections of pentobarbitone sodium (9 mg./kg.). Lower record taken approximately 0.5 hr. after upper record. The interval between the two sections of the lower record was 5 min. Time signals in 10 sec.

When during the recovery phase from the anaesthesia the cat sneezed, or spontaneous movements occurred, tremor ceased but returned once the movement was completed.

In most cats the onset of tremor occurred when the rectal temperature had fallen to between 35.5 and 36.5°. During tremor the temperature rose, but tremor continued for some time after the temperature had returned to the normal level, or had reached a level above normal, that is, 41°. In a few cats tremor occurred without a fall in rectal temperature, or with a slight fall only, so that in these there was no correlation between tremor and fall in rectal temperature.

When additional pentobarbitone was injected intraperitoneally during the period of tremor it caused diminution or cessation of tremor. This was often preceded by a short period of increased strength of tremor. An example of this is shown in Fig. 1. Intraperitoneal injection of 9 mg./kg. pentobarbitone sodium caused first an increase and then a decrease in the amplitude of tremor, but no cessation (upper record). A subsequent injection caused only slight initial accentuation of tremor and then cessation (lower record).

Effects of Intraventricular Injections of Drugs on Pentobarbitone Sodium Tremor

Sympathomimetic Amines. — Intraventricular injection of adrenaline or noradrenaline abolished tremor. In some cats a dose of 2.5 μg. abolished tremor, in others it caused attenuation and in still others it had no effect. Most cats responded to 5 μg. either with abolition or with diminution of tremor and 10 μg. always abolished it. Following the injection, tremor either ceased abruptly within 1 or 2 sec. or disappeared gradually within 30 to 90 sec. After 5 μg. tremor returned within 10 to 20 min., and after 10 μg. within 20 to 35 min.

Intraventricular injections of ephedrine, amphetamine, isoprenaline or phenylephrine, in doses ranging from 5 to 500 μg., did not affect the tremor.

Chloralose, Urethane.—Intraventricular injection of 0.25 mg. of chloralose decreased or abolished tremor for about 20 min.; 0.5 mg. abolished tremor for as long as 2 hr. Urethane had transient anti-tremor effect when injected intraventricularly in large doses. Less than 10 mg. was ineffective whereas 20 to 30 mg. decreased or stopped tremor for 3 to 6 min.

Calcium Chloride.—Intraventricular injection of 0.35 to 0.5 mg. decreased or stopped tremor for 20 to 50 min. Such an experiment is illustrated in Fig. 2.

Magnesium Chloride.—Intraventricular injection of 0.5 mg. decreased tremor for about 10 min. The effect began after a latency of about 10 min. After an injection of 1 or 2 mg. the latency was shorter and tremor decreased or ceased for 30 to 50 min. In the experiment, shown in Fig. 3, the injection of first 1 mg. and then 2 mg. decreased the amplitude of the temor but did not abolish it. In another experiment these doses of magnesium chloride caused cessation of tremor.

Anti-Parkinsonian Drugs. — Intraventricular injections of atropine, hyoscine, diethazine and trihexiphenidyl in doses ranging from 5 to 500 μg. did not affect the tremor. With atropine and hyoscine it was further found that they did not affect the responses to subsequent intraventricular injections of adrenaline or noradrenaline.

Effects of Drugs Perfused from Lateral Ventricle to Aqueduct on Pentobarbitone Sodium Tremor

Sympathomimetic Amines.—On perfusion the anti-tremor effect of adrenaline or noradrenaline

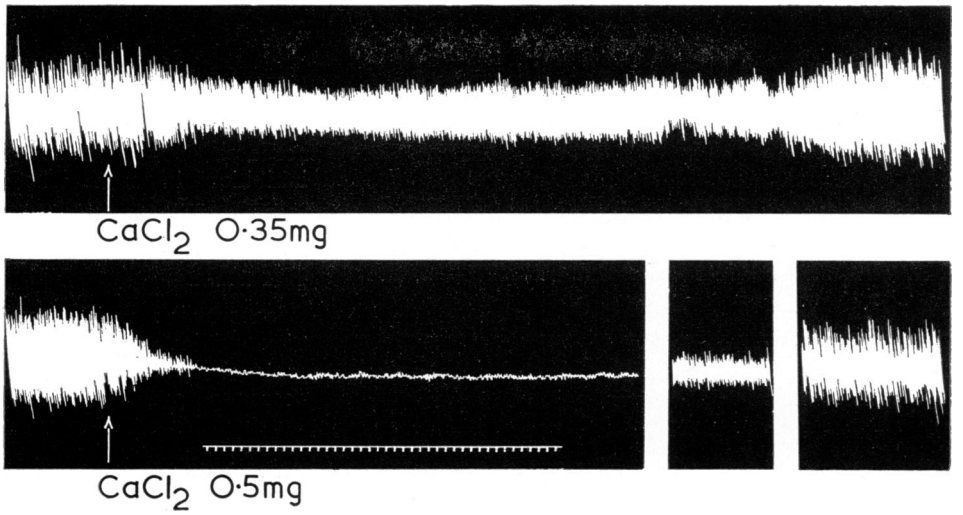

FIG. 2.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. At the arrows intraventricular injection of 0.35 mg. (upper record) and of 0.5 mg. (lower record) of calcium chloride. In the lower record there was an interval of approximately 0.5 hr. between each section of the record. Time signals in 10 sec.

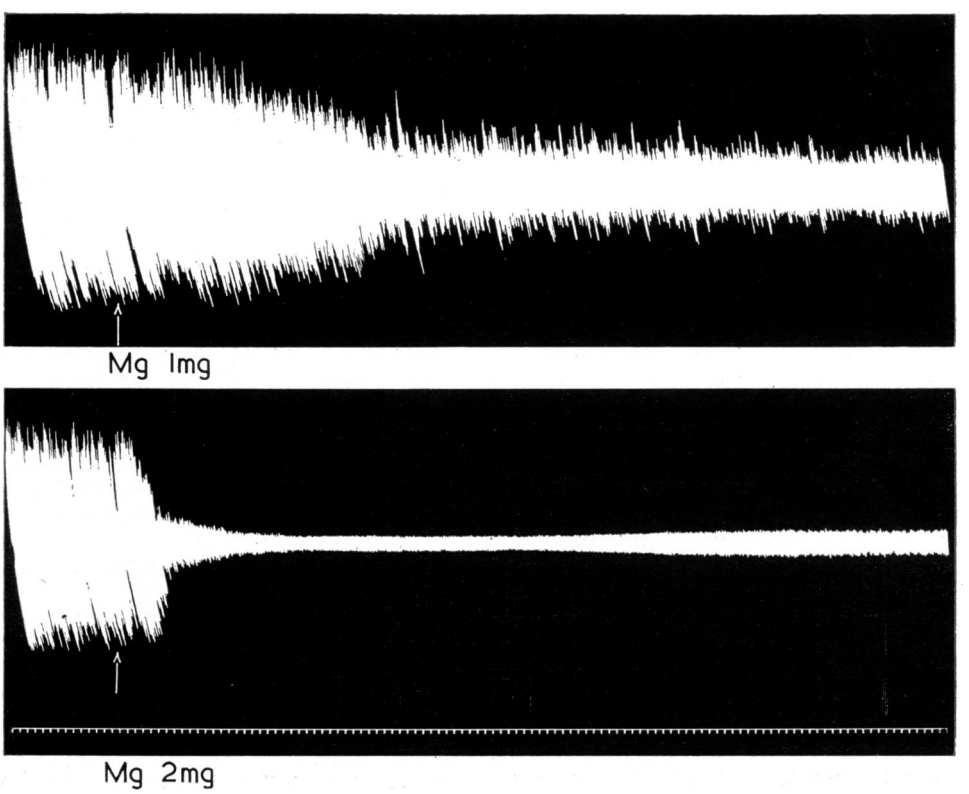

FIG. 3.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. At arrows intraventricular injections of 1 mg. (upper record) and 2 mg. (lower record) of magnesium chloride. The 2 mg. was injected 40 min. after the 1 mg. Time signals in 10 sec.

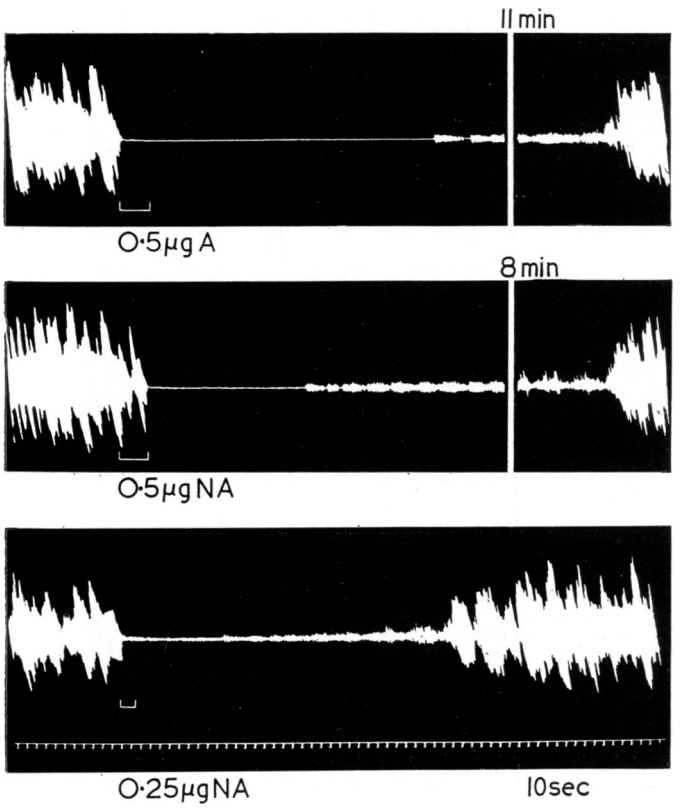

FIG. 4.—Tremor recorded from left tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. At the signals perfusion was with adrenaline (A) or noradrenaline (NA) 1/100,000 from lateral ventricle to aqueduct at a rate of 0.1 ml./min. for 30 sec. (0.5 µg.) or for 15 sec. (0.25 µg.). In the upper two records the intervals between the two sections were 11 and 8 min. respectively. Time signals in 10 sec.

was obtained with even smaller doses than those effective on intraventricular injection. In the experiment of Fig. 4, the amines were perfused in a concentration of 1/100,000. The upper two records show cessation of tremor on perfusion for 30 sec. and the lower record on perfusion for 15 sec., that is, when 0.5 and 0.25 µg. respectively entered the cerebral cavities. As the effects were readily reproducible during succeeding periods of perfusion the relative potencies of the two amines could easily be ascertained. Fig. 5 illustrates the finding that adrenaline is at least four times more potent than noradrenaline in inhibiting tremor. Perfusion with adrenaline 1/250,000 (upper record) was much more effective than a similar concentration of noradrenaline (middle record), and perfusion with adrenaline 1/1,000,000 (lower record) was slightly more effective than the perfusion with noradrenaline 1/250,000. Onset of inhibition in each case occurred within 1 min. of perfusion, that is, after 0.4 µg. of the stronger and 0.1 µg. of the weaker solution had been perfused.

Isoprenaline which on intraventricular injection did not inhibit the pentobarbitone sodium tremor in doses up to 500 µg. did so when perfused from the lateral ventricle to the aqueduct although it was less potent than adrenaline or noradrenaline. The upper record of Fig. 6 illustrates the result obtained on perfusion with isoprenaline 1/10,000. The strength of tremor was affected within 0.5 min. of perfusion, that is, after less than 5 µg. had entered the cerebral ventricles.

Amphetamine and ephedrine did not affect the tremor when perfused through the cerebral ventricles in a concentration as strong as 1/2,000. This is shown in the middle two records of Fig. 6. The lowest record serves as a control and shows that adrenaline was effective in a low concentration.

Chloralose.—As shown in Fig. 7, perfusion with chloralose 1/50,000 for 10 min. abolished tremor. The effect began after 1 min. perfusion, that is, after 2 µg. chloralose had entered the cerebral ventricles, and persisted for nearly 10 min. after the perfusion solution was changed back to artificial cerebrospinal fluid. On perfusion with stronger solutions of chloralose the effect lasted longer.

Calcium Chloride.—Perfusion in a concentration of 1/100,000 calcium chloride decreased, and of 1/50,000 abolished, tremor. The effect began after about 2 min. perfusion, that is, after 20 and 40 µg. respectively had entered the cerebral ventricles.

Anti-Parkinsonian Drugs.—Atropine, hyoscine, trihexiphenidyl and diethazine did not affect tremor when perfused from the lateral ventricle to the aqueduct in a concentration as strong as 1/2,000.

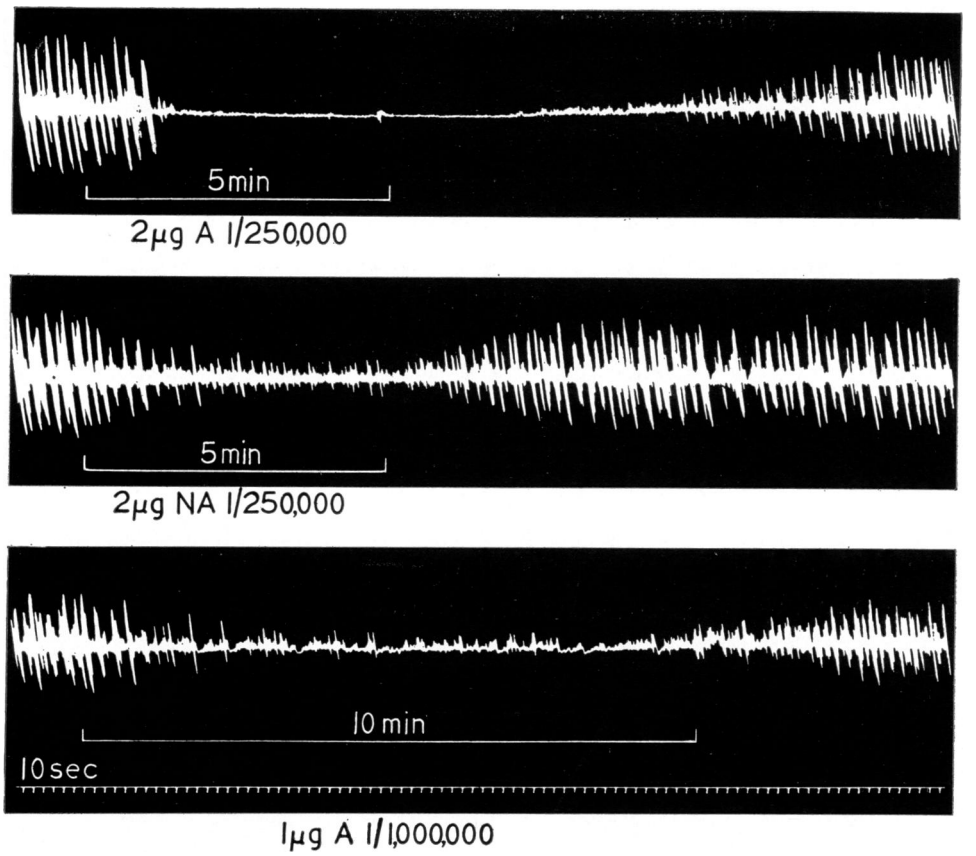

FIG. 5.—Tremor recorded from left tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. At the signals perfusion was with adrenaline (A) or noradrenaline (NA) from lateral ventricle to aqueduct. Upper two records: perfusion of the amines in a concentration of 1/250,000 for 5 min. at a rate of 0.1 ml./min. (2 μg.). Lower record: perfusion of adrenaline in a concentration of 1/1,000,000 for 10 min. at a rate of 0.1 ml./min. (1 μg.). Time signals in 10 sec.

5-Hydroxytryptamine Tremor

In non-anaesthetized conscious cats intraventricular injections of 500 μg. 5-hydroxytryptamine produced tremor which started within 1 min. or 2 min. after the injection. Usually it appeared first in the ears, then the hind legs became involved and finally there was tremor over the whole body. Tremor was continuous, but sometimes bursts of greater amplitude occurred. Tremor lasted from 20 min. to over 2 hr. At the height of activity, tremor could be seen in the legs of the standing cat and the entire head shook due to the strength of the tremor. Intraventricular injection of 250 μg. was also effective ; tremor lasted for 10 to 20 min. During, and following, the period of tremor the cat was usually less alert than before the injection and would be sitting or lying in the cage with its head erect. There was a tendency for the eyelids to close. The ears were hot due to dilatation of their vessels.

During the initial stage of a pentobarbitone sodium anaesthesia before the pentobarbitone sodium induced tremor occurred, tremor could be initiated by intraventricular 5-hydroxytryptamine and this tremor was recorded. Such an experiment is illustrated in Fig. 8. It shows the sudden onset of tremor and its rate when recording was on a fast-moving drum. The rate was approximately 17/sec.

Creatinine sulphate itself injected intraventricularly in equivalent amounts did not initiate tremor.

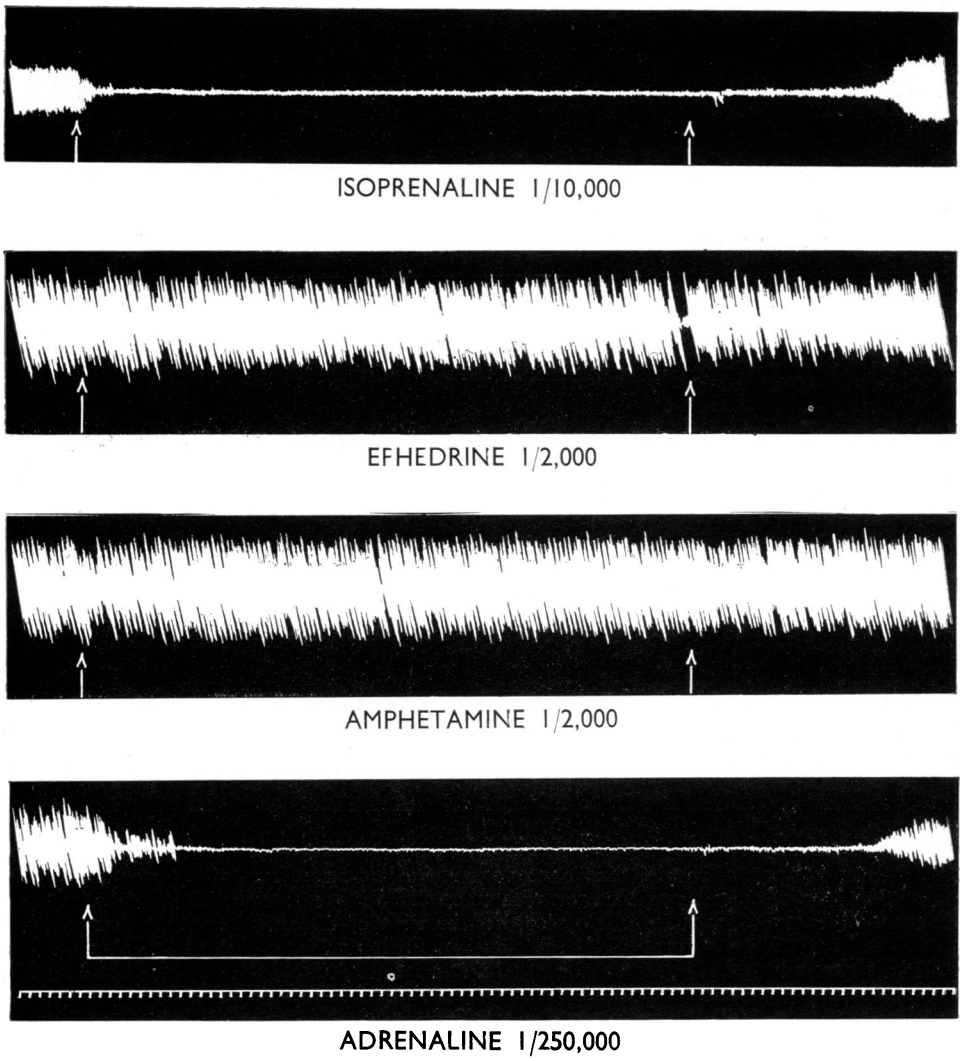

FIG. 6.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. Between the arrows perfusion was with isoprenaline 1/10,000 (upper record), ephedrine 1/2,000 (second record from top), amphetamine 1/2,000 (third record from top) and adrenaline 1/250,000 (lowest record), from lateral ventricle to aqueduct for 10 min. at a rate of 0.1 ml./min. Time signals in 10 sec.

DISCUSSION

No distinction has been made when describing the results between tremor and shivering. Lippold, Redfearn and Vučo (1959) looked upon the rhythmical muscular activity which occurred in cats anaesthetized with pentobarbitone sodium as shivering, and likened it to the shivering which occurs in man exposed to cold. Hall and Goldstone (1940) combined pentobarbitone sodium anaesthesia with submersion of the cat in cold water in order to produce shivering. In most of our experiments the onset of tremor occurred when the rectal temperature of the cat had fallen during the pentobarbitone sodium anaesthesia to between 35.5 and 36.5°, but tremor continued after the temperature had returned to normal or

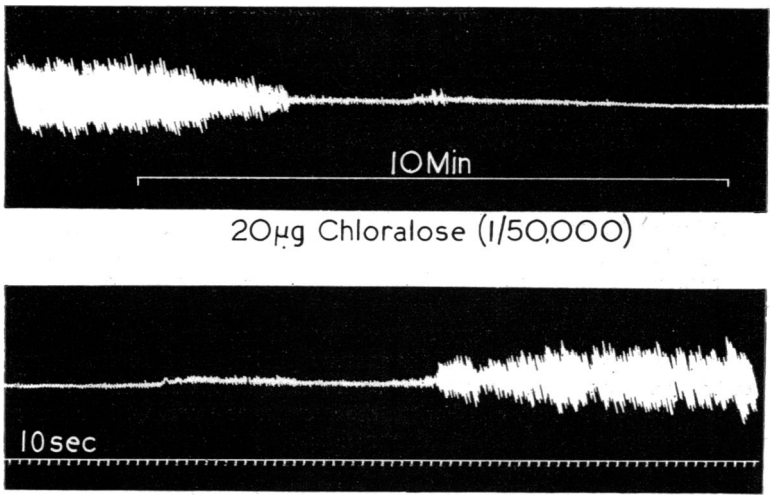

FIG. 7.—Tremor recorded from right tibialis anterior muscle of a cat anaesthetized with pentobarbitone sodium. At the signal perfusion was with chloralose 1/50,000 from lateral ventricle to aqueduct for 10 min. at a rate of 0.1 ml./min. (20 µg.). Lower record continuation of upper record. Time signals in 10 sec.

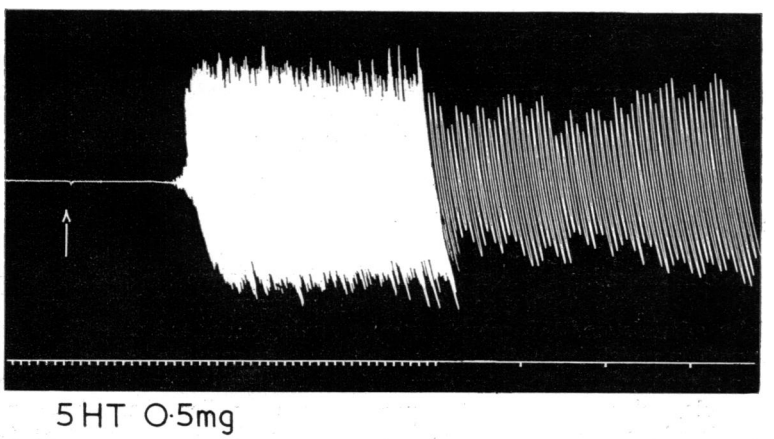

FIG. 8.—Tremor recorded from right hind leg of a cat anaesthetized with pentobarbitone sodium. At the arrow intraventricular injection of 0.5 mg. 5-hydroxytryptamine. Left half of record taken on a slow-moving drum: time signals in 10 sec. Right half of record taken on a fast-moving drum: time signals in 1 sec.

above normal level. In some cats tremor began without any change in rectal temperature. We know that rectal temperature does not necessarily reflect the temperature of the blood passing through the brain. The absence of a correlation between rectal temperature and onset of tremor therefore does not exclude the lowering of blood temperature as the cause of the pentobarbitone sodium tremor. Yet the transient increase in the strength of tremor observed on deepening the anaesthesia by additional pentobarbitone sodium, injected intraperitoneally, would be difficult to explain on the assumption that the pentobarbitone sodium tremor is solely dependent on a fall in temperature. The tremors initiated by cold or by anaesthesia, though not dependent on each other, may nevertheless be essentially the same phenomenon. In this connexion, it is interesting to note that adrenaline, which has such a strong action on the pentobarbitone sodium tremor, also affects shivering produced by cold. Tournade, Raynaud and Chardon (1939) found that intravenous adrenaline decreases shivering in dogs exposed to cold.

Since chlorpromazine causes depression of body temperature (Eriksson and Wasz-Höckert, 1959), its mode of action in producing tremor is probably similar to that of pentobarbitone sodium and cold. Weight is added to this view in that the same agents which depressed pentobarbitone sodium tremor also depressed tremor produced by chlorpromazine. The fact that both types of tremor were refractory to the action of drugs employed in the treatment of Parkinsonism may mean that a different mechanism is responsible for Parkinson's tremor.

The finding that a number of substances influence tremor or shivering when given by the intraventricular route poses the question of where they act when producing this effect. It is known that tremor-like movements are produced by electrical stimulation of the medial portions of the brain stem which in monkeys reach from the mesencephalon to the medulla (Jenkner and Ward, 1953) but which in cats extend further rostrally to the hypothalamus (Birzis and Hemingway, 1957). It is reasonable to assume that the substances given intraventricularly influence tremor activity by acting on some of these structures. Those in the medulla and caudal portion of the mesencephalon can be excluded as the main site of action because the effects were also obtained when the substances were perfused from the lateral ventricle to the middle of the aqueduct. The assumption that the drugs act on the midline structures in the diencephalon and rostral part of the mesencephalon, electrical stimulation of which results in tremor-like movements, implies that they are able to penetrate into the brain substance lining the third ventricle and the aqueduct. Previous experiments in which histamine or the dye bromophenol blue was perfused from the lateral ventricle to the aqueduct showed that these substances penetrate into the grey matter surrounding these cavities (Draskoci, Feldberg, Fleischhauer and Haranath, 1960; Feldberg and Fleischhauer, 1960) and it is likely that other substances penetrate in a similar way.

Whenever a substance artificially administered acts in minute amounts on structures in which it is usually found, one is led to consider the possibility that the observed pharmacological effect mimics a physiological function. Fractions of a microgram of either adrenaline or noradrenaline when perfused from the lateral ventricle to the aqueduct were sufficient to suppress tremor. Vogt (1954) has shown that the highest concentrations of noradrenaline occur in the midline structures of diencephalon and mesencephalon. The concentration of noradrenaline in the perfusion fluid necessary to produce anti-tremor activity approximates that found in these mid-brain structures, but the concentration attained must be smaller than in the perfusion fluid itself due to dilution during its passage to the effector sites. Could it be that the physiological release of the brain stem noradrenaline is involved in the control of tremor and shivering? At present this question cannot be answered because the relevant physiological experiments have not been performed. However, the results obtained with minute doses of noradrenaline perfused from the lateral ventricle to the aqueduct at least point in this direction. The same question is applicable to the brain stem adrenaline. Although it contributes only a small proportion of the brain stem sympathin (Vogt, 1954) our finding that it is at least four times more potent than noradrenaline in anti-tremor activity is compatible with the view that adrenaline shares in this central nervous function.

It appears that the structure of the sympathomimetic amines essential for anti-tremor activity is the catechol ring, since it was found that only those having this moiety, that is, adrenaline, noradrenaline and isoprenaline, possessed this action.

The anti-tremor activity of adrenaline and noradrenaline was shared by a number of substances which, like these amines, produce sedation or an anaesthesia-like condition on intraventricular injection, suggesting a relationship between the central depression which leads to anaesthesia and anti-tremor activity. This suggestion is supported by the finding that deepening of the pentobarbitone sodium anaesthesia by additional pentobarbitone sodium into a cat during the period of tremor often increased its amplitude for a short time before tremor decreased and finally ceased. This would imply that before the injection the cat was above the level of anaesthesia at which maximal strength of tremor is exhibited and that it passed through this level as the anaesthesia deepened.

Noradrenaline and adrenaline are not the sole amines which occur normally in the central nervous system. Amin, Crawford and Gaddum (1954) showed that 5-hydroxytryptamine has a similar distribution in the brain stem although the absolute quantities are smaller than those of noradrenaline. It has now been found that 5-hydroxytryptamine initiates tremor on intraventricular injection. This amine may, therefore, also be involved in regulation of tremor activity. The amounts necessary to produce the effect, however, were large. They were much greater than those of noradrenaline or adrenaline required to suppress tremor. This may be due to the remoteness of the site of action of 5-hydroxytryptamine from the ventricular cavity and aqueduct which would entail more dilution due to the greater distance traversed. If the brain stem 5-hydroxytryptamine were to exert a tremor inducing function, the higher concentration of the anti-tremor amine, noradrenaline, in this region may serve to prevent a continuous state of tremor.

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TRANSMISSION FAILURE IN SYMPATHETIC NERVES PRODUCED BY HEMICHOLINIUM

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It has been shown by others that hemicholinium (α,α′-dimethylethanolamino-4,4′-biacetophenone) inhibits the synthesis of acetylcholine, an effect which is reversed by choline. Hemicholinium produces a failure of response to nerve stimulation in the following sympathetically innervated preparations: guinea-pig isolated vas deferens, rabbit isolated uterus, rabbit isolated colon, perfused rabbit ear, cat isolated atria and the piloerector muscles in the cat's tail. The blocking action of hemicholinium on the responses to postganglionic sympathetic stimulation resembles its blocking action against cholinergic nerve stimulation observed on rabbit isolated atria with vagus nerves, rabbit isolated vagina with pelvic nerves, and guinea-pig isolated diaphragm with phrenic nerve. The failure of transmission produced by hemicholinium in sympathetic nerves and in cholinergic nerves can be reversed by choline. It is suggested that if there were a cholinergic junction at sympathetic nerve endings the mechanism of the blocking action of hemicholinium at these endings could be explained by inhibition of acetylcholine synthesis.

Hemicholinium (α,α′-dimethylethanolamino-4,4′-biacetophenone) produces a failure of response to cholinergic nerves (MacIntosh, Birks, and Sastry, 1956; Reitzel and Long, 1959a, b; Wilson and Long, 1959). MacIntosh *et al.* (1956) and Gardiner (1957) found that hemicholinium inhibits the synthesis of acetylcholine by nervous tissues. This observation led them to suggest that the transmission failure is caused by the loss from the nerve endings of acetylcholine which cannot be replaced by resynthesis in the presence of hemicholinium.

Rand and Chang (1960) observed that hemicholinium blocks the responses of the guinea-pig isolated vas deferens to stimulation of the sympathetic hypogastric nerve. This blocking action of hemicholinium has a number of features in common with the block of cholinergic nerves, and they suggested that the site of the block was similar and that there was a cholinergic mechanism involved in sympathetic nerves.

We have now carried out further investigations on the effects of hemicholinium on the responses of a number of organs to nerve stimulation.

METHODS

Sympathetically Innervated Preparations. — The guinea-pig vas deferens with hypogastric nerve attached was prepared by the method developed by Huković (described by Huković, 1960b, and Boyd, Chang, and Rand, 1960); the rabbit uterus with hypogastric nerve by the method of Varagić (1956); the rabbit colon with lumbar sympathetic nerve as described by Finkleman (1930). The nerves were passed through a tube containing bipolar platinum electrodes of the type described by Burn and Rand (1960a). These organs were suspended in McEwen (1956) solution or in Krebs' bicarbonate solution bubbled with 95% oxygen and 5% carbon dioxide in a 50 ml. bath at 30°.

Experiments on the sympathetically innervated perfused rabbit ear were carried out by the method of Gaddum and Kwiatkowski (1938). The superior cervical ganglion was stimulated with bipolar platinum electrodes; one was placed distal to the ganglion, the other on the ganglion. The outflow of drops of perfusion fluid from the ear was measured with a Gaddum or a Thorp recorder (C. F. Palmer, London).

Observations were made with isolated sympathetically innervated atria of the cat heart. Huković (1959) has described the preparation of isolated sympathetically innervated rabbit atria in which he stimulated the cervical sympathetic trunk close to the stellate ganglion. In order to ensure that the sympathetic stimulation was postganglionic we used a different procedure. Anaesthesia was induced with ether in small cats (1 to 2 kg.) and continued with intravenous urethane. The trachea was cannulated and artificial respiration given. The chest was opened in the midline and retracted back on the right side. The right stellate ganglion was exposed and ligatures tied around the cervical sympathetic nerve near to its

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intersection with the subclavian artery, around the thoracic sympathetic chain in the second intercostal space and around the branches of the stellate ganglion which entered the brachial plexus. The tissue between these ligatures, which included the stellate ganglion, was separated from the right posterior surface of the pleural cavity. The cat was bled out through a carotid cannula. The heart was lifted at the apex and cut away together with the lungs, trachea, oesophagus, and the muscles as close as possible to the underlying vertebral column and ribs. These tissues were transferred to oxygenated McEwen solution at 20°. In further dissection the lungs, pericardium, oesophagus, and ventricles were cut away. The atria were suspended in a 100 ml. bath of McEwen solution at 27°. The stellate ganglion was stimulated with bipolar platinum electrodes.

Observations on the pilomotor response to sympathetic stimulation were carried out as described by Burn and Rand (1960b).

Parasympathetically Innervated Preparations.—Rabbit atria with intact vagus nerves were prepared by the method of Burn and Rand (1957) and suspended in a 100 ml. bath of McEwen solution at 27°.

Some observations were made on the parasympathetically innervated rabbit vagina. The vagina was separated from the rectum and at its lower end, on the posterior surface, two blood vesels with adjacent nerves were identified. The vagina was suspended in McEwen solution in a 50 ml. organ bath to record longitudinal contractions. One of the two nerves entering the posterior wall was passed through a bipolar electrode tube.

Motor Nerve.—Observations on guinea-pig hemidiaphragm with its phrenic nerve were made by the method described by Bülbring (1946) for the rat.

Details of the electrical stimulation applied to nerves are given in the Results.

Hemicholinium (α, α' dimethylethanolamino 4, 4′-biacetophenone dibromide) was a generous gift to one of us (M. J. R.) from Dr. J. P. Long, of the Department of Pharmacology, State University of Iowa, Iowa City. The amounts of hemicholinum referred to in the text are as this salt.

Results

Guinea-pig Vas Deferens with Hypogastric Nerve.—Preliminary experiments showed that after hemicholinium in concentrations of 20 to 50 μg./ml. there was a gradual decrease of the contractions of the vas deferens in response to hypogastric nerve stimulation (Rand and Chang, 1960). The record from one experiment is shown in Fig. 1. The responses to stimulation of the nerve with 2 msec. pulses at 10/sec. for 10 sec. periods in every 2 min. were completely abolished 100 min. after hemicholinium was added to the bath. Observations in a number of similar experiments indicated that the time taken for hemicholinium to produce a complete block of response was related to the frequency of stimulation of the nerve. In the experiment shown in Fig. 2 hemicholinium (30 μg./ml.) was allowed to act for 100 min. during which time the nerve was stimulated at 5/sec. for 10 sec. periods in every 2 min., which produced only a

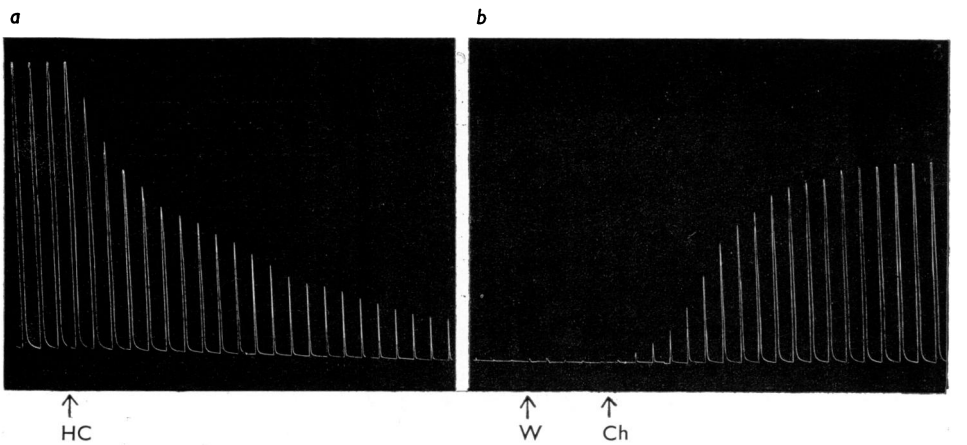


FIG. 1.—Guinea-pig vas deferens. Contraction in response to hypogastric nerve stimulation with 2 msec. pulses at 10/sec. for 10 sec./2 min. At (HC), 20 μg./ml. of hemicholinium was added to the bath, which produced a gradual decrease in response and complete failure 100 min. later in (*b*). The hemicholinium was washed out at (W), and after choline chloride (0.5 mg./ml.) was added at (Ch) the contractions were restored.

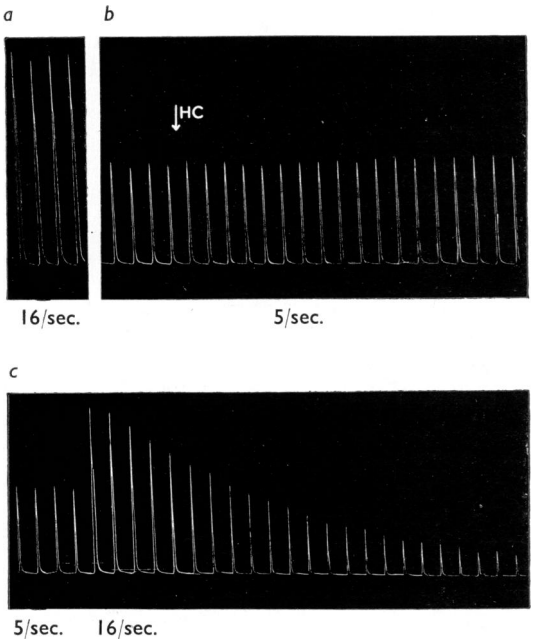


FIG. 2.—Guinea-pig vas deferens. Hypogastric nerve stimulated with 2 msec. pulses for 10 sec. periods every 2 min. In (*a*) the frequency of stimulation was 16/sec.; in (*b*) 5/sec. During stimulation at 5/sec., hemicholinium (30 μg./ml.) was added to the bath at (HC), and 100 min. later, in (*c*), the frequency was again altered to 16/sec. There was only a slight reduction of responses to stimulation at 5/sec., but at 16/sec. failure developed rapidly.

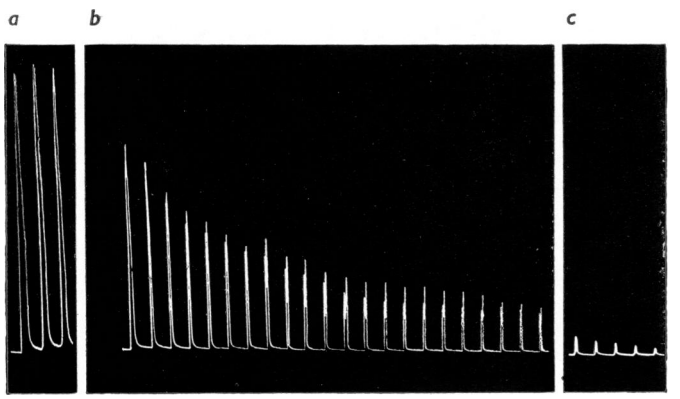


FIG. 3.—Guinea-pig vas deferens. In (*a*), hypogastric nerve stimulated at 16/sec. Between (*a*) and (*b*), hemicholinium (40 μg./ml.) was added to the bath and no stimuli were applied until 100 min. later. In (*b*), periods of stimulation at 16/sec. were recommenced. Failure was almost complete after 100 min. of stimulation (in *c*), that is, 200 min. after hemicholinium.

slight decrease in response. However, when the frequency of stimulation was increased to 16/sec. the responses rapidly decreased. Fig. 3 illustrates an experiment in which the nerve was not stimulated for 100 min. after adding hemicholinium (40 μg./ml.) to the bath. During this period hemicholinium produced a 30% decrease in the contraction in contrast to Fig. 1 in which the nerve was regularly stimulated and the response was completely blocked by hemicholinium after 100 min.

When hemicholinium had reduced, but not yet abolished contractions, we observed that the speed of the contraction was decreased, the contraction was not sustained, and the latent period of the response was increased. These effects are shown in Fig. 4 in which the responses were recorded at a faster kymograph speed.

After hemicholinium had produced failure of the response to nerve stimulation, choline chloride in concentrations of 0.1 to 1.0 mg./ml. in the bath gradually restored contractions (Fig. 1b). The observations made during the restoration of responses by choline were the reverse of those made during the development of failure by hemicholinium. The contractions gradually increased in size, were more sustained and the latent period decreased (Fig. 4).

The response of the vas deferens to noradrenaline was enhanced when the response to nerve stimulation had been abolished by hemicholinium (Fig. 5). The response of the vas deferens to nicotine was abolished as shown in Fig. 6b. However, the interpretation of this observation was not clear because choline restored the contractions in response to nerve stimulation to 61% of their original height, but only restored the response to nicotine to 15% of its original height (Fig. 6c). In other experiments it was found that hemicholinium immediately blocked the response to nicotine on the vas deferens, when the response to nerve stimulation was still unaffected. The response to acetylcholine was not affected by hemicholinium.

Atropine (3 μg./ml.) had no effect on the contractions of the vas deferens to hypogastric nerve stimulation before the addition of hemicholinium to the bath or after the restoration of responses by choline, although contractions produced by acetylcholine were blocked.

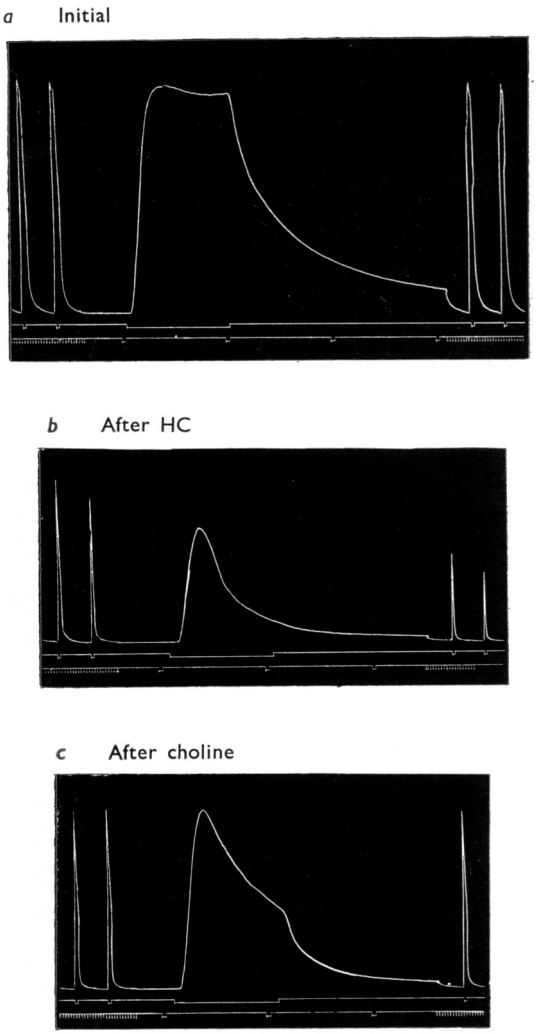


FIG. 4.—Guinea-pig vas deferens. In each panel, the upper trace is the record of contraction of vas deferens, the middle trace is a signal indicating the application of stimulus to the hypogastric nerve and the lower trace is a time signal showing 10 sec. intervals. The rate of kymograph movement was increased just before the 3rd response in each panel. Stimulation was with 2 msec. pulses at 16/sec., applied for 10 sec. in every 2 min. Control records are shown in (*a*). Between (*a*) and (*b*) 20 μg./ml. of hemicholinium was added to the bath, and (*b*) shows the record when the response had declined to about half of the control height. Between (*b*) and (*c*), 200 μg./ml. of choline chloride was added to the bath, and (*c*) shows the record when contractions were restored to about three-quarters of the control height.

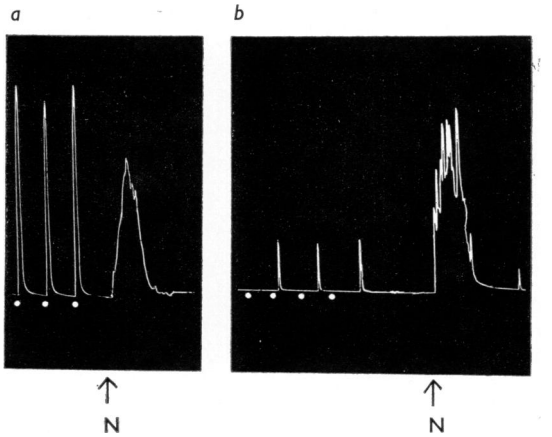


FIG. 5.—Guinea-pig vas deferens. The hypogastric nerve was stimulated at the white dots for 10 sec. at 10/sec. At (N) noradrenaline (10 μg./ml.) was added to the bath. Between (*a*) and (*b*), 20 μg./ml. of hemicholinium was added and stimulation was continued until failure was complete; the three small contractions in panel (*b*) were spontaneous.

Rabbit Uterus with Hypogastric Nerve.—The isolated rabbit uterus contracted regularly in response to hypogastric nerve stimulation with 2 msec. pulses at 25/sec. for 10 sec. in every 2 min. We confirmed Varagić's (1956) observation that the relaxation from the first contraction was slow, but after a few successive periods of stimulation the relaxation was more rapid.

The effect of hemicholinium (40 μg./ml.) is shown in Fig. 7. There was a gradually increasing irregularity of response to nerve stimulation; occasional responses reached the same height and were of even longer duration than observed initially, but other responses were small. The latent period of response after the start of nerve stimulation increased from 5 sec. to 15 sec. A second addition of hemicholinium to the bath to give a total concentration of 80 μg./ml. produced an increase of tone which appeared to be initiated by nerve stimulation and which then gradually declined. The responses to nerve stimulation were finally abolished, although some spontaneous rhythm of the uterus was present. The addition of choline chloride (800 μg./ml.) produced a contraction. After the choline was replaced by fresh McEwen solution the contractions of the uterus in response to nerve stimulation gradually recovered. At first the latent period was 15 sec. (i.e., contraction began 5 sec. after the end of the 10 sec. period of

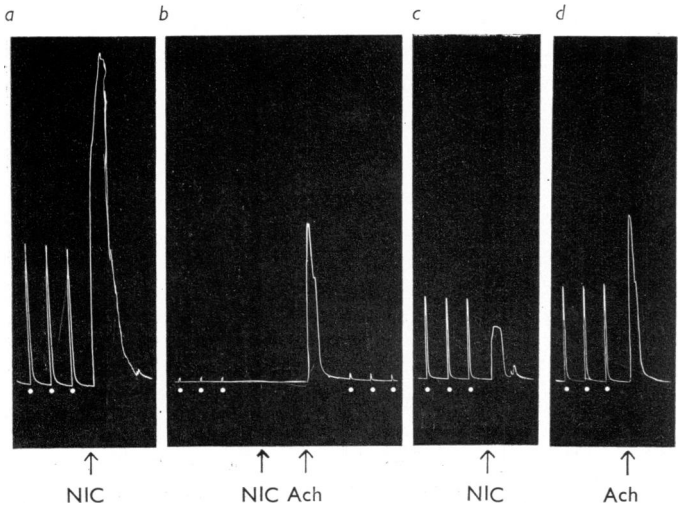


FIG. 6.—Guinea-pig vas deferens. Hypogastric nerve stimulation at white dots (12/sec. for 10 sec. periods). At (NIC), nicotine acid tartrate (20 μg./ml.), and at (Ach) acetylcholine chloride (20 μg./ml.) was added to the bath. Between (*a*) and (*b*) hemicholinium (20 μg./ml.) was added to the bath and stimulation was continued until failure developed. Between (*b*) and (*c*), choline chloride (0.4 mg./ml.) was added to the bath fluid and remained in (*c*) and (*d*).

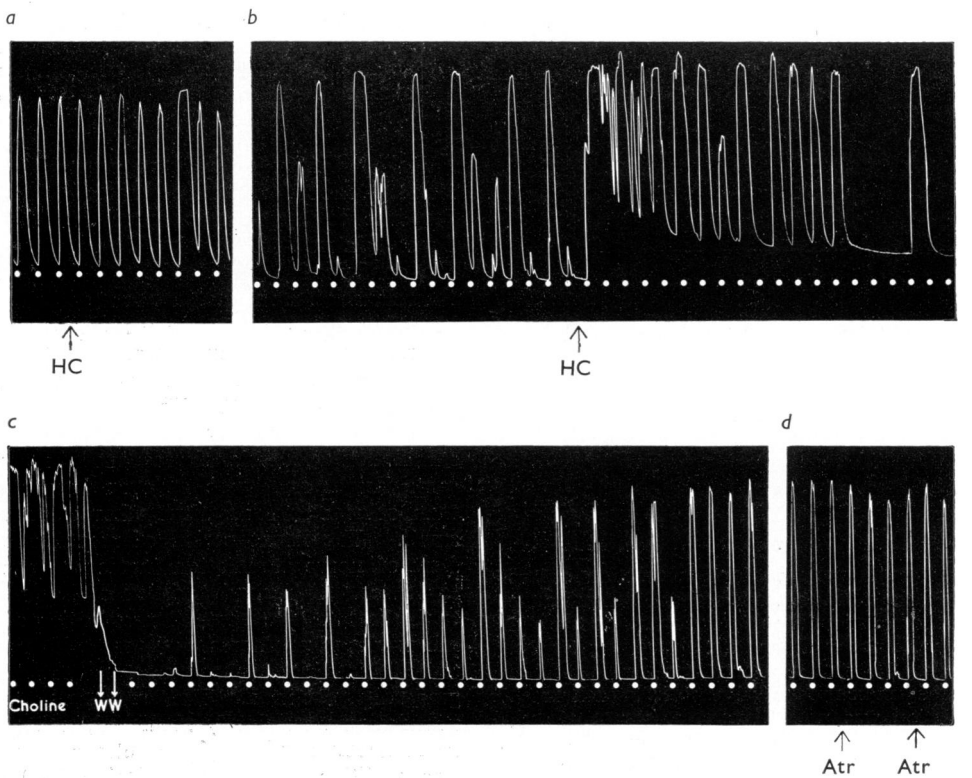


FIG. 7.—Isolated rabbit uterus. The hypogastric nerve was stimulated with 2 msec. pulses at 25/sec. for 10 sec. in every 2 min. as indicated by the white dots. In (*a*), hemicholinium (40 μg./ml.) was added to the bath at (HC). Between (*a*) and (*b*) 46 min. elapsed. In (*b*), a second addition of hemicholinium was made. Between (*b*) and (*c*), choline chloride (0.8 mg./ml.) was present in the bath, and it was washed out at W. Responses to nerve stimulation were gradually restored. In (*d*), atropine sulphate (Atr.) (0.5 and 1.0 μg./ml.) had no effect on the responses.

stimulation); when the contractions had been fully restored the latent period was 7 sec. The restored contractions to nerve stimulation were not affected by atropine (1 μg./ml.), a result which indicated that they were not produced by the direct action of acetylcholine from cholinergic nerve fibres on the uterine smooth muscle.

Rabbit Colon with Lumbar Sympathetic Nerves.—Constant relaxations of the colon were obtained in response to nerve stimulation at 25–50/sec. applied for 30 sec. in every 4.5 min.

The effect of hemicholinium (50 μg./ml.) in one experiment is illustrated in Fig. 8. The inhibition produced by sympathetic nerve stimulation was gradually reduced and almost abolished by the 20th period of stimulation after hemicholinium. In this experiment a slight restoration of the response was seen after washing out the hemi-

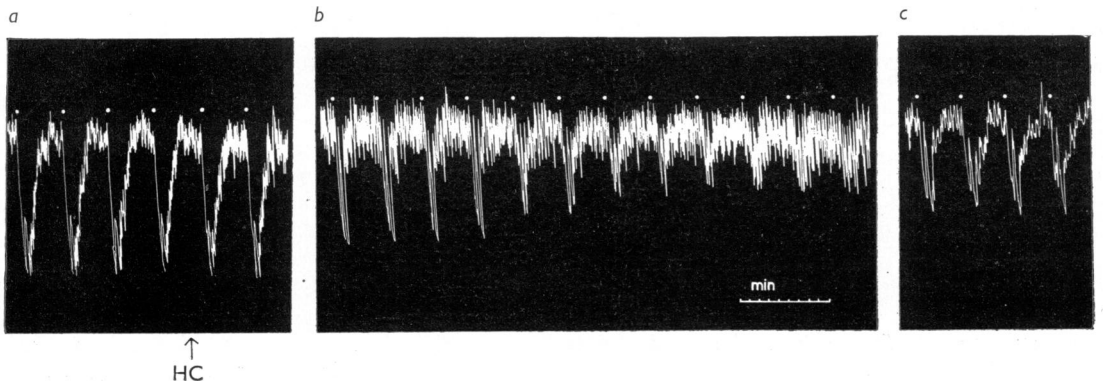


FIG. 8.—Rabbit distal colon in Krebs' bicarbonate solution. The sympathetic nerve supply to the colon was stimulated with 2 msec. pulses at 50/sec. for 30 sec. in every 4.5 min. at the white dots. In (*a*), hemicholinium (50 μg./ml.) was added at (HC). The interval between (*a*) and (*b*) was 45 min. Between (*b*) and (*c*), the bath was washed out and stimulation was stopped. In (*c*), 1 hr. later, relaxations were partly restored.

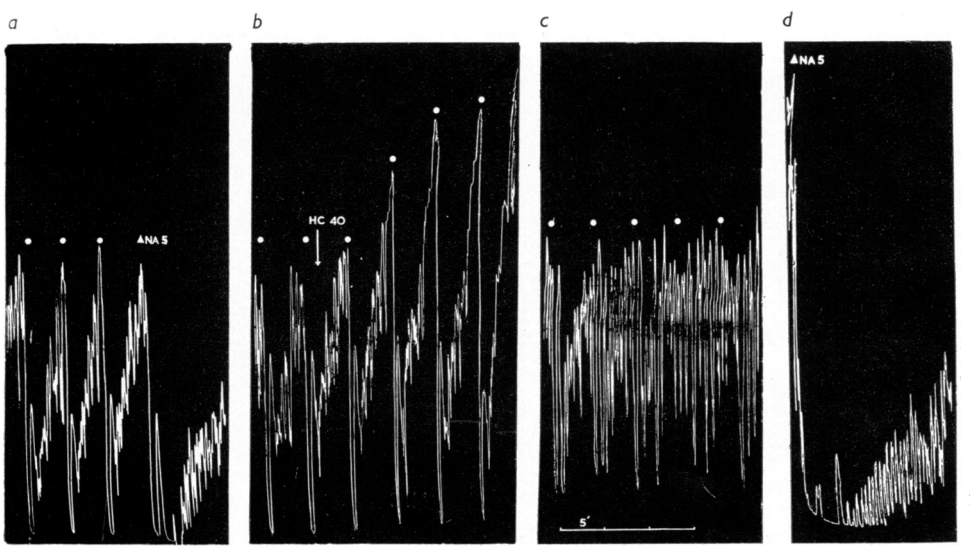


FIG. 9.—Rabbit colon in McEwen solution. The sympathetic nerves were stimulated with 2 msec. pulses at 25/sec. for 30 sec. in every 4.5 min. at the white dots. In (*a*) noradrenaline (5 μg./ml.) was injected at (NA) (white triangles). In (*b*), hemicholinium (40 μg./ml.) was added to the bath. In (*c*), 160 min. after hemicholinium, the relaxations in response to nerve stimulation were abolished, but in (*d*) noradrenaline still produced relaxation.

cholinium and resting from stimulation for one hour.

In another experiment hemicholinium produced an increase in tone of the colon (Fig. 9). At first the relaxations produced by nerve stimulation were greater than before hemicholinium, but subsequently the responses were abolished, although the tone remained enhanced and there was an increased amplitude of the spontaneous rhythm. The relaxation produced by noradrenaline was greater during a hemicholinium-induced failure of nerve stimulation than it had been initially.

Sympathetically Stimulated Perfused Rabbit Ear.—The vasoconstriction produced by postganglionic sympathetic stimulation was blocked by hemicholinium in each of 5 experiments. In 2 experiments a concentration of 20 μg./ml. of hemicholinium in the Locke solution perfusing the ear produced failure of the response to sympathetic stimulation in 1 hr. and in 6 hr., and in 3 experiments using hemicholinium in a concentration of 50 μg./ml. the response to sympathetic stimulation failed after 3 hr., 4 hr., and 6 hr. respectively.

After perfusing with hemicholinium the latency of response was increased. In one experiment in which stimulation was applied for 1 min. in every 5 min. the vasoconstriction reached its maximum after 30 sec. of stimulation, but 1 hr. after perfusing with hemicholinium (20 μg./ml.) the maximum response was reached after 45 sec. of stimulation.

In 2 experiments the response to sympathetic stimulation increased at first after perfusion with hemicholinium was begun (Fig. 10, panel *c*) and was subsequently abolished (panel *d*).

The response to sympathetic nerve stimulation could be partially restored by changing to fresh Locke solution and injecting choline into the arterial cannula at intervals (Fig. 11).

When the response to sympathetic stimulation had been abolished by hemicholinium the responses to noradrenaline (Fig. 11) and to nicotine (Fig. 10) were still present; in fact, the response to noradrenaline was somewhat larger than it had been and that to nicotine showed a considerable potentiation.

Isolated Cat Atria with Sympathetic Innervation.—Observations were made on the amplitude of contraction of the atria recorded with a light spring lever and on the rate of contractions. In Fig. 12 the stellate ganglion was stimulated with 2 msec. pulses at 20/sec. for 30 sec. in every 5 min. The increases in rate and amplitude of atrial beating were constant. The

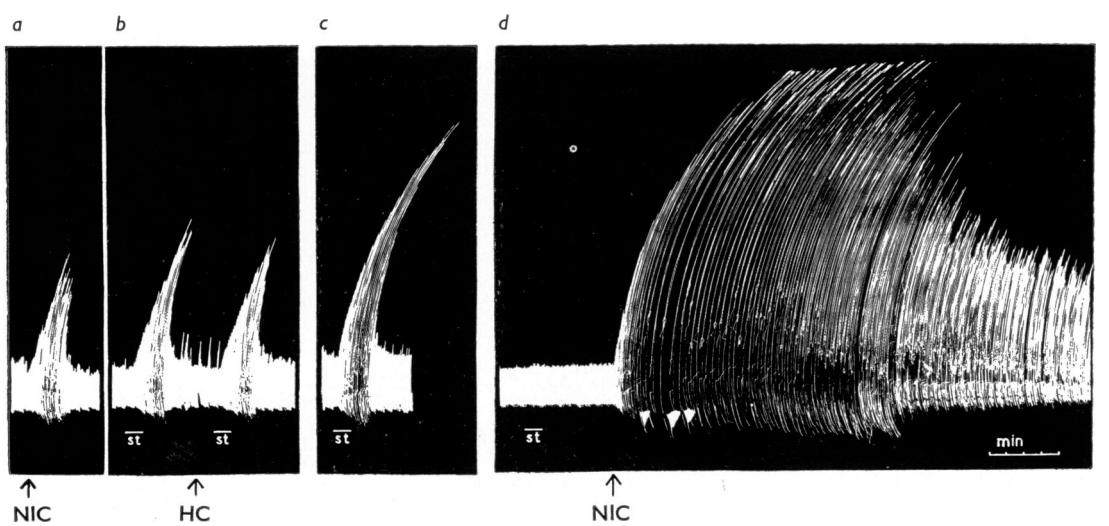


FIG. 10.—Outflow from perfused rabbit ear recorded by Gaddum's method. In (*a*) nicotine acid tartrate (1 mg.) was injected at (NIC). In (*b*) the superior cervical ganglion was stimulated at *st* with 2 msec. pulses at 50/sec. for 50 sec. Stimulation was repeated regularly at 5 min. intervals. In (*b*) hemicholinium (50 μg./ml.) was added to the perfusion fluid at (HC). Two hours later, in (*c*), the vasoconstriction produced by nerve stimulation was enhanced, but 6 hr. later, in (*d*), the response to nerve stimulation was abolished although the response to nicotine was greatly enhanced.

concentration of hemicholinium required to affect the responses to sympathetic stimulation was greater than in the preparations described above. After hemicholinium (500 μg./ml.) had been added to the bath the resting rate of atrial beating was increased and then gradually declined to its previous level. Initially, stimulation of the stellate ganglion produced an increase in rate of 50 to 58%. One hour after hemicholinium it was approximately 14%, and 3 hr. after hemicholinium there was no response. The latent period gradually increased during the development of failure of response. A slight recovery of response was seen when hemicholinium was washed from the bath and the preparation rested for 30 min.

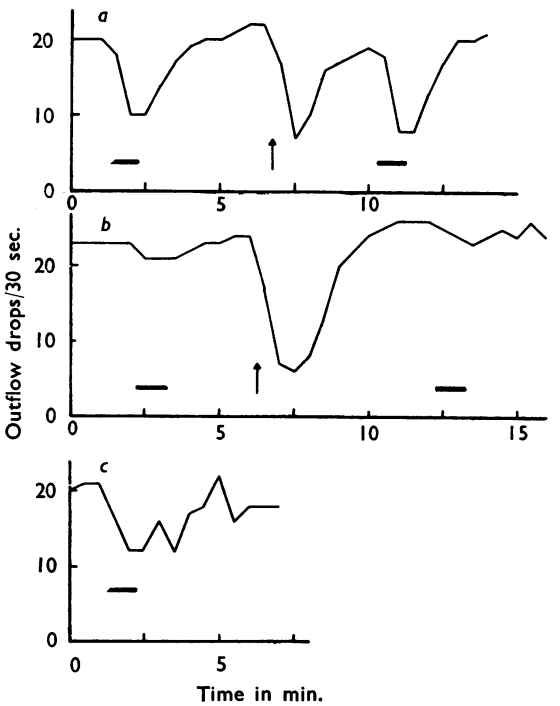


FIG. 11.—Outflow from perfused rabbit ear recorded in drops/30 sec. The horizontal lines indicate periods of stimulation of the superior cervical ganglion with 2 msec. pulses at 25/sec. for 50 sec. Periods of stimulation were repeated at 5 min. intervals throughout the experiment. At the arrows, noradrenaline (1 μg.) was injected into the arterial cannula. (*a*) shows the control observations; (*b*) is 3 hr. after perfusing with 50 μg./ml. of hemicholinium; the responses to nerve stimulation were abolished, but the response to noradrenaline was enhanced. Between (*b*) and (*c*) the ear was perfused with Locke solution without hemicholinium and four injections of 100 mg. of choline chloride were given. The stimulation applied in (*c*) was once more effective.

In another preparation (Fig. 13) the response to nerve stimulation was restored by adding choline to the bath. This slowed the rate of atrial contractions, but after the choline was washed out the responses to stellate ganglion stimulation were fully restored.

Piloerection in the Cat Tail.—Stimulation of the right lumbar sympathetic trunk with pulses at 20/sec. produced piloerection which reached its peak 2 sec. after the beginning of stimulation. The hairs were still maximally erected at the end of a 3 min. period of stimulation. Hemicholinium solutions were injected intradermally beneath alternate tufts of hair. In the experiment of Fig. 14, 0.05 ml. of each solution containing 500 μg./ml., 1 mg./ml., 2 mg./ml., and 5 mg./ml. of hemicholinium was injected beneath the tufts numbered 2, 4, 6, and 8 respectively. Tufts 1, 3, 5, 7, and 9 served as untreated controls.

Since the effect of hemicholinium in diminishing responses to nerve stimulation depended on the number of stimuli delivered, and since an increase in the latent period of the response has consistently been observed, the effect of hemicholinium was tested in the following manner. Photographs of the hair tufts were taken 3 sec. after the beginning of stimulation (Fig. 14, panel D); the tufts which had been injected with hemicholinium were slower to erect, although at the end of 10 sec. of stimulation the photograph shows that they were erected to the same extent as the untreated tufts (panel E). However, at the end of 3 min. of stimulation the tufts injected with hemicholinium had already partly relaxed, but the untreated tufts were still erected (panel F).

Rabbit Atria with Vagus Nerves.—The inhibition of atrial contractions in response to vagus nerve stimulation was reduced by hemicholinium (40 μg./ml.). In the experiment of Fig. 15 the effect of hemicholinium is shown on the response to vagal stimulation at 25/sec. for 1 min. periods in every 3 min. There was a clear reduction of responses at the 5th and 6th periods of stimulation and almost complete failure of response to the 10th and 11th periods of stimulation.

Responses to vagal stimulation were slightly restored after washing hemicholinium from the atria, and almost completely restored after treating the atria with choline chloride (0.4 mg./ml.).

Rabbit Isolated Vagina with Parasympathetic Innervation.—Regular contractions of the longi-

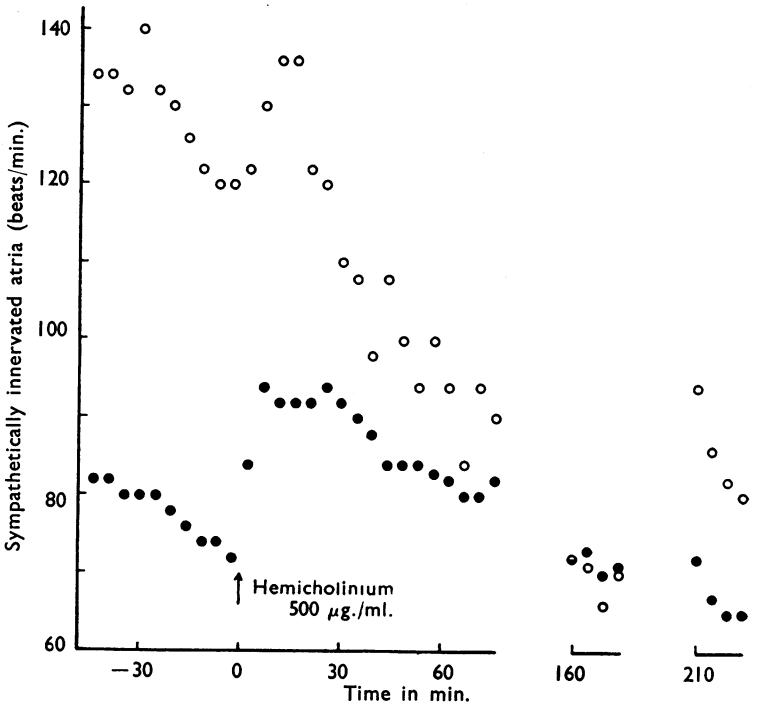
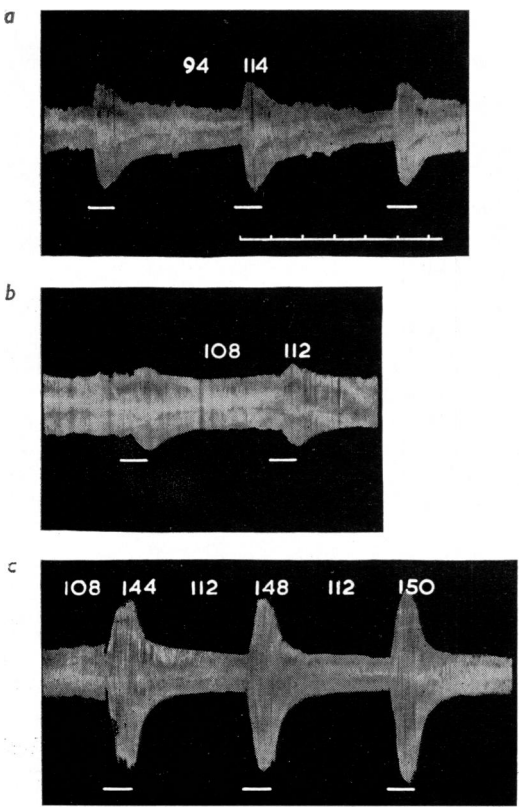


FIG. 12.—Rate of beating of isolated cat atria; ●, before sympathetic stimulation; O, during sympathetic stimulation. Sympathetic stimulation was by 2 msec. pulses at 20/sec. applied for 30 sec. at 5 min. intervals. After hemicholinium (500 μg./ml.) had been added to the bath the response to sympathetic stimulation decreased and was finally abolished. After 180 min. the bath was washed out and stimulation was stopped for 30 min., then recommenced.



tudinal muscle of the vagina were produced by stimulating the nerve for 10 sec. in every 2 min. Fig. 16 shows the effect of hemicholinium (24 μg./ml.) in inhibiting the contractions, and the restoration of response in the presence of choline chloride (400 μg./ml.). The restored responses were blocked by 0.4 μg./ml. of atropine, thus demonstrating that the contractions were due to the direct action of nervously released acetylcholine.

Guinea-pig Isolated Diaphragm with Phrenic Nerve.—Fig. 17 shows the effect of hemicholinium in diminishing the contractions of the diaphragm in response to phrenic nerve stimulation at the rate of 1/4.6 sec. After 40 min. in the presence of 200 μg./ml. of hemicholinium the diaphragm contracted to 20% of the height of the initial contraction. Choline chloride (1 mg./ml.) restored the contractions to their original height.

FIG. 13.—Isolated cat atria. The stellate ganglion was stimulated at 50/sec. for 50 sec. every 4.5 min. as indicated by the white lines. The numerals over the record refer to the rate in beats/min.; (*a*) shows initial observations. In (*b*), 4½ hr. after hemicholinium (500 μg./ml.), the response to stimulation was almost abolished. Between (*b*) and (*c*) the bath contained 1 mg./ml. of choline chloride, which was washed out before (*c*) was obtained. The time marker in (*a*) indicates 1 min. periods.

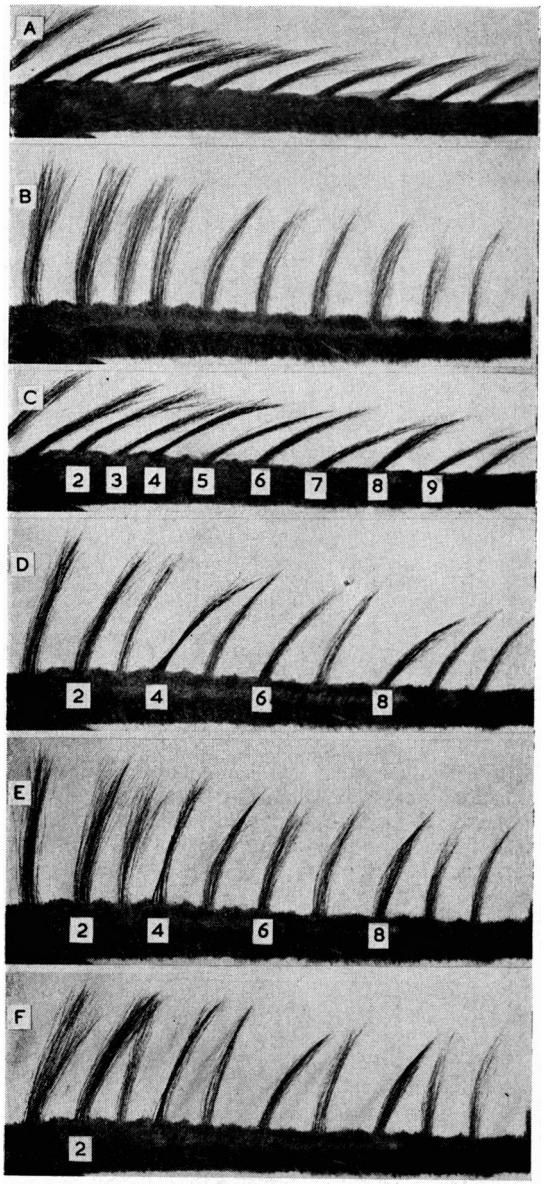


FIG. 14.—Photographs of tufts of hair on dorsal surface of cat's tail. (*A*) No stimulation. (*B*) Lumbar sympathetic trunk stimulated at 20/sec. for 10 sec. In (*C*) tufts 1, 3, 5, 7, and 9 were untreated; intradermal injections of hemicholinium were given beneath tufts 2 (0.5 mg./ml.), 4 (1 mg./ml.), 6 (2 mg./ml.), and 8 (5 mg./ml.). Between (*C*) and (*D*) several bursts of stimulation were applied. Then the lumbar sympathetic trunk was stimulated and (*D*) was taken after 3 sec. of stimulation, (*E*) after 10 sec. and (*F*) after 3 min. The tufts injected with hemicholinium were slower to erect and the erection did not persist during stimulation.

DISCUSSION

Hemicholinium was at first considered to act primarily on the central nervous system to produce depression of respiration (Long and Schueler, 1954; Schueler, 1955; Kasé and Borison, 1958). The findings of Longo (1958) that the phrenic nerve action potentials were not impaired when hemicholinium had abolished respiratory movements suggested that the inhibition of respiratory movement was peripheral, although the experiments of Kasé and Borison (1958) clearly indicate that there are also central effects. That hemicholinium blocks at the myoneural junction has been adequately shown by Long and his colleagues (Wilson and Long, 1959; Reitzel and Long, 1959b).

Reitzel and Long (1959b) reported that hemicholinium was more effective in blocking at the myoneural junction (sciatic nerve-gastrocnemius muscle) when the nerve was stimulated at 1/sec. than when it was stimulated at 1/10 sec. However, they pointed out that similar observations had been made for tubocurarine (Preston and Van Maanen, 1953; Gesler and Hoppe, 1956), although there were points of difference between the blockade produced by hemicholinium and that by tubocurarine, perhaps the principal one being that hemicholinium did not block the action of acetylcholine on the frog rectus muscle. Hemicholinium blocked transmission in the superior cervical ganglion and its blocking action depended on the frequency of preganglionic nerve stimulation (MacIntosh *et al.*, 1956). The ganglionic blocking drugs also have a greater blocking action against high frequencies of stimulation (Paton, 1951; Green, 1956). Thus there appears to be, in general, a relationship between the degree of block and the frequency of stimulation with a diverse range of blocking drugs, but the mechanisms involved may be very different.

Perry (1957) has distinguished seven phases in ganglionic transmission, and presumably all are susceptible to drug action leading to block. The difference in mechanism between a hemicholinium-induced failure of transmission and the blockade produced by the ganglionic blocking drug pentamethonium has been revealed by studies with the cat perfused superior cervical ganglion. MacIntosh *et al.* (1956) found that hemicholinium reduced the amount of acetylcholine released into the perfusion fluid on preganglionic nerve stimulation, which is in contrast to the finding of Paton and Zaimis (1951)

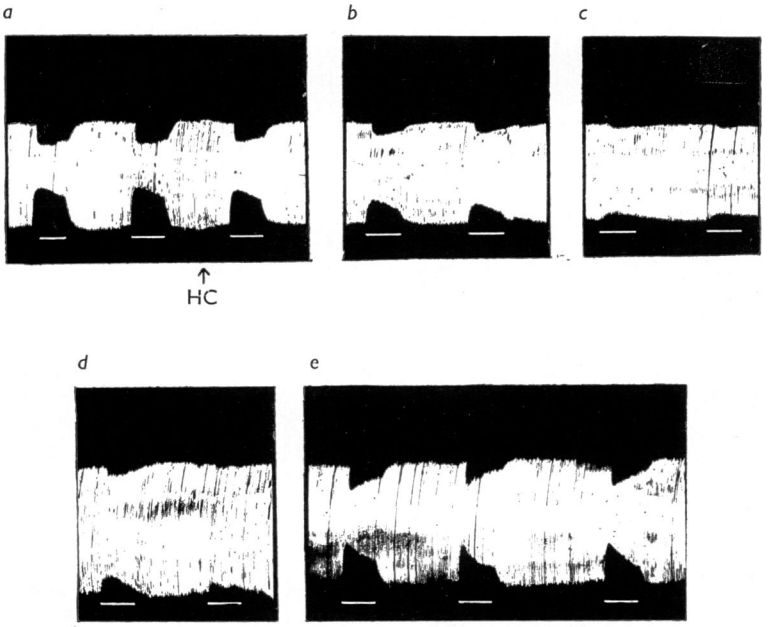


FIG. 15.—Isolated rabbit atria. The vagus nerves were stimulated at 20/sec. for 1 min. at 3 min. intervals as indicated by the horizontal lines. In (*a*) hemicholinium (40 μg./ml.) was added to the bath at (HC) ; (*b*) shows the 5th and 6th responses to vagal stimulation after hemicholinium and (*c*) shows the 10th and 11th. Between (*c*) and (*d*) the bath was washed out and stimulation was stopped for 2 hr. Between (*d*) and (*e*) choline chloride (0.4 mg./ml.) was added to the bath and then washed out.

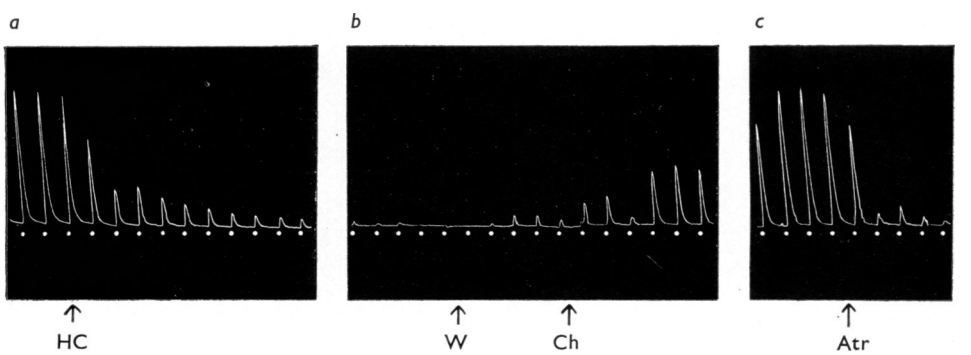


FIG. 16.—Isolated rabbit vagina. Parasympathetic nerve was stimulated at 60/sec. for 10 sec. in every 2 min. as indicated by white dots. Hemicholinium (24 μg./ml.) was added to the bath at (HC) in (*a*). In (*b*) the bath was washed out (W) 52 min. after hemicholinium, then choline chloride (0.4 mg./ml.) (Ch) was added, which restored responses. In (*c*) responses were blocked by 0.4 μg./ml. of atropine sulphate (Atr).

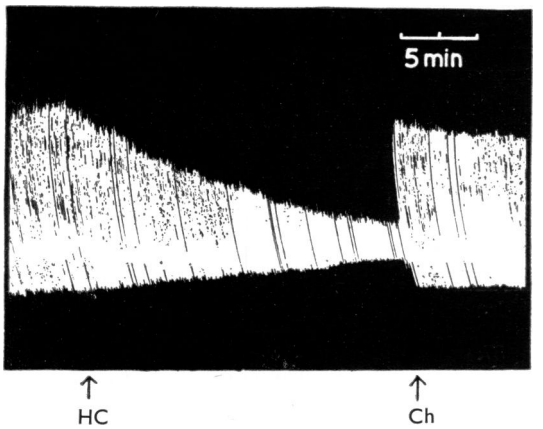


FIG. 17.—Contractions of guinea-pig diaphragm. The phrenic nerve was stimulated every 4.6 sec. At (HC) 200 μg./ml. of hemicholinium was added to the bath. At (Ch) the drum was stopped, the bath washed out and 1 mg./ml. of choline chloride was added. The drum was restarted 40 min. later.

that a blocking dose of pentamethonium did not interfere with the release of acetylcholine.

The blocking action of hemicholinium at the sympathetic nerve endings was only observed at high frequencies of stimulation. However, bretylium, whose blocking action is also exerted at sympathetic nerve endings, is also more effective at higher frequencies of stimulation (Boura and Green, 1959), although Nasmyth and Andrews (1959) found that the block produced by the related drug choline 2,6-xylyl ether did not depend on frequency. The effects of nicotine and of acetylcholine (in the presence of atropine) in constricting the vessels of the rabbit's ear are essentially similar (Kottegoda, 1953; Burn and Rand, 1958). One difference between the hemicholinium block at sympathetic nerve endings and the block produced by bretylium is that in the perfused rabbit ear nicotine produced vasoconstriction after hemicholinium had blocked sympathetic responses, whereas bretylium blocked both sympathetic responses and the vasoconstriction produced by acetylcholine (Huković, 1960a).

In the experiments of MacIntosh *et al.* (1956) with the cat perfused superior cervical ganglion, they found that hemicholinium decreased the rate of acetylcholine release into the perfusion fluid as the ganglion lost its ability to transmit impulses, and the store of acetylcholine in the ganglion was diminished. These effects of hemicholinium were reversed by the addition of choline to the perfusate. The synthesis of acetylcholine by mouse brain mince was inhibited by hemicholinium and the inhibition was reversed by choline (MacIntosh *et al.*, 1956, confirmed by Gardiner, 1957). However, the acetylcholine synthesizing ability of reconstituted acetone-dried powders of rat brain was less affected by hemicholinium. Gardiner (1957) concluded that the inhibiting action of hemicholinium was lessened when the degree of tissue disruption was increased. From these observations it was concluded that hemicholinium did not affect the enzyme choline acetylase, but that it acted on a system (carrier) which transported choline to the site of the acetylation (MacIntosh *et al.*, 1956; Gardiner, 1957; Birks and MacIntosh, 1957).

The reversal of hemicholinium block at the myoneural junction (sciatic nerve-gastrocnemius muscle) by choline has been extensively studied by Reitzel and Long (1959a). They found that choline itself was the only active substance in a series of choline analogues. Esters of choline which could be hydrolysed by cholinesterase were also effective, but their reversing action was abolished by the anticholinesterase TEPP, which suggested that the esters must first be split to yield choline. The reversing action of choline was enhanced when cholinesterase was blocked, which suggested that acetylcholine was formed from the choline. These observations support the conclusion of MacIntosh *et al.* (1956) that the transmission failure produced by hemicholinium was due to the impairment of acetylcholine synthesis, so that responses to nerve stimulation failed because acetylcholine became depleted, and that the reversal by choline was due to the restoration of acetylcholine synthesis.

Broadly speaking, one of two diametrically opposed conclusions arises from our findings that hemicholinium blocked responses to stimulation of sympathetic nerves. Firstly, it may be that hemicholinium exerts its nerve blocking activity through specific interference with acetylcholine synthesis. Alternatively, it may be that the sympathetic blocking action of hemicholinium and its reversal by choline are unrelated to acetylcholine, in which case arguments arising from the use of hemicholinium in the investigation of "cholinergic" nerves must be discounted. The similarity in the actions of hemicholinium in blocking sympathetic nerves and in blocking cholinergic nerves is striking: the effective concentrations of hemicholinium were approximately equal as far as isolated preparations are concerned, the block depended on the frequency of stimulation, and choline reversed the block.

We prefer a single theory to explain the action of hemicholinium on both "cholinergic" and "adrenergic" nerves rather than to suggest that it acts on "cholinergic" nerves by inhibiting the synthesis of acetylcholine and on "adrenergic" nerves in a different way.

The possibility that sympathetic effects may be mediated *via* acetylcholine has been discussed by Burn and Rand (1959, 1960b). Evidence has been obtained for cholinergic fibres in the sympathetic nerve supply to the vessels of the dog hind leg (Bülbring and Burn, 1935), to the cat nictitating membrane (Bacq and Fredericq, 1935; Burn and Rand, 1960b), to the rabbit ear (Burn and Rand, 1960b), to the rabbit ileum (Gillespie and Mackenna, 1959), to the rabbit atria (Huković, 1959), to the cat spleen (Burn and Rand, 1960b; Brandon and Rand, 1960), to the cat uterus (Burn and Rand, 1960b), to the rabbit uterus (Varagić, 1956), to the dog tongue and lips (von Euler and Gaddum, 1931), and to the guinea-pig vas deferens (Boyd, Chang, and Rand, 1960). In various organs acetylcholine (like nicotine) has sympathomimetic effects which are exerted at a site peripheral to the sympathetic ganglion cells, and it has been shown that these effects depend on the presence of noradrenaline stores in the organ (Burn, Leach, Rand, and Thompson, 1959). Therefore the possibility exists that the acetylcholine from sympathetic cholinergic fibres might be exerting a sympathomimetic effect by releasing noradrenaline.

The present findings that hemicholinium produces a block of responses to sympathetic nerve stimulation which is reversed by choline can be understood if acetylcholine were necessary at sympathetic nerve endings to release noradrenaline.

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ANALGESIC ANTIPYRETIC DRUGS AS ANTAGONISTS OF BRADYKININ

BY

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The antagonism between analgesic antipyretic drugs and bradykinin was examined quantitatively, using the bronchoconstrictor response of guinea-pigs *in vivo*. The dose of bradykinin required to overcome antagonism by calcium acetylsalicylate increased with the dose of acetylsalicylate given, the ratio being roughly constant. Fifty times the quantity of acetylsalicylate which just antagonized bradykinin did not modify bronchoconstriction due to small doses of histamine, 5-hydroxytryptamine, or acetylcholine. A method of measuring the potency of this anti-bradykinin action was developed. Acetylsalicylic acid, phenylbutazone, amidopyrine, and phenazone had a high potency; paracetamol, cinchophen, sodium salicylate, and acetanilide had a moderate potency; and phenacetin, salicylamide, and 4-hydroxyisophthalic acid had little or none. Cortisone, hydrocortisone, aldosterone, amodiaquine, and morphine were ineffective or their action was non-specific. In sensitized guinea-pigs, an injection of antigen caused bronchospasm. This response was greatly lessened by pretreatment with mepyramine, but was not affected by calcium acetylsalicylate, lysergic acid diethylamide, or atropine. Acetylsalicylic acid, phenylbutazone, and amidopyrine did not specifically antagonize the action of bradykinin on the capillaries of guinea-pig skin *in vivo*, on guinea-pig ileum *in vitro* or on rat duodenum *in vitro*.

Collier, Holgate, Schachter, and Shorley (1959, 1960) found that bradykinin causes bronchoconstriction in the guinea-pig, that small doses of acetylsalicylic acid, phenylbutazone and amidopyrine suppress this response without affecting those to histamine or 5-hydroxytryptamine, and that increasing the dose of bradykinin overcomes this suppression. Since the relationship between these analgesic and antipyretic drugs and bradykinin in its bronchoconstrictor action shows the main features of pharmacological antagonism, we thought it worth while to study this relationship quantitatively, to measure the potency of antagonism and to explore how effective were other antipyretic, analgesic, anti-inflammatory and anti-rheumatic drugs. Since bradykinin might take part in anaphylactic bronchospasm, we investigated whether pre-treatment with acetylsalicylate lessened the intensity of this spasm. We examined also whether the antagonists of bradykinin in its bronchoconstrictor action likewise antagonized some of its other actions.

METHODS

Materials.—Bradykinin was prepared by the action of crystalline trypsin on heated beef serum globulin and was purified chromatographically. The potencies of preparations, standardized against pure bradykinin obtained with trypsin (Elliott, Lewis, and Horton, 1960), ranged from 0.6 to 16 μg. pure bradykinin/mg. Substances tested in acute experiments as antagonists of bradykinin are given in Table III, which shows the salts used. Histamine acid phosphate, 5-hydroxytryptamine creatinine sulphate and acetylcholine chloride were also employed as bronchoconstrictor agents and mepyramine maleate, lysergic acid diethylamide tartrate and atropine sulphate as their respective antagonists. Weights of salts are expressed as acid or base. Where possible, drugs were administered dissolved in 0.9% sodium chloride solution. Acetylsalicylic acid, salicylamide, 4-hydroxyisophthalic acid, cinchophen, acetanilide, phenacetin and amodiaquine were suspended in 5 to 10% gum acacia solution. Cortisone and cortisone acetate were suspended in 0.9% sodium chloride solution. Acetylsalicylic acid was given intravenously after mixing 10 parts by weight with three of calcium carbonate and one of citric acid and dissolving in 0.9% sodium chloride solution immediately before use. We refer to acetylsalicylic acid in this soluble form as calcium acetylsalicylate or more briefly as acetylsalicylate.

Guinea-pig Lungs in vivo.—To measure bronchoconstriction, we used the method of Konzett and Rössler (1940), with details as described by Collier *et al.* (1960). We estimated antagonism to bradykinin bronchoconstriction by giving intravenous

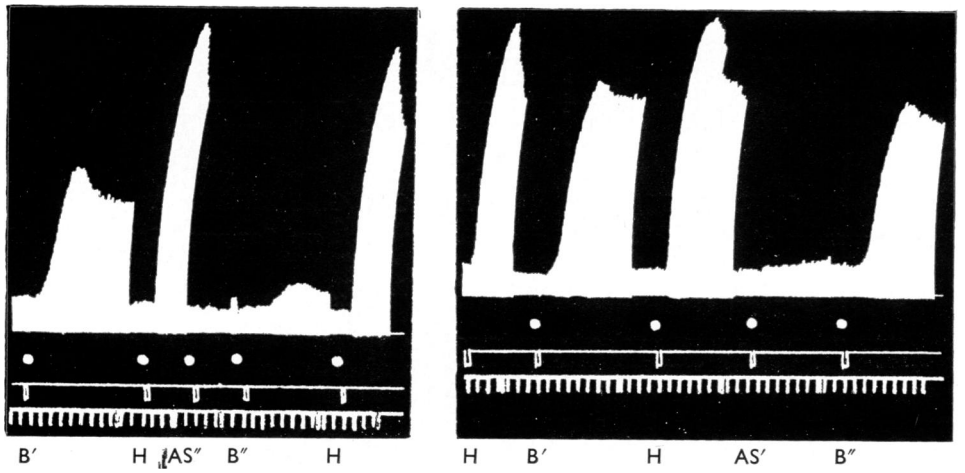

FIG. 1.—Resistance to inflation of guinea-pig lungs *in vivo*. Estimation of intravenous minimal effective dose of calcium acetylsalicylate. B′, 0.4 μg., and B″, 0.8 μg. of bradykinin; H, histamine, 2 μg.; AS′, 1 mg./kg., and AS″, 2 mg./kg. of calcium acetylsalicylate. All doses were given intravenously. Guinea-pig weights: left, 450 g.; right, 380 g. Time, 10 sec.

bradykinin and histamine before and after an intravenous, intraduodenal or oral dose of antagonist. Intravenous injections were spaced at 5 to 10 min. intervals. Bradykinin and histamine were injected 0.5 to 1 hr. after an intraduodenal or an oral dose of antagonist and subsequently. Because responses to repeated doses of bradykinin decline, the second dose of this substance was doubled. A dose of antagonist was considered effective if it reduced the second response to bradykinin to less than half the preceding one. An effective dose was regarded as specific if it did not depress the histamine response. Different doses of antagonist were given, each in a different guinea-pig, at a factor of 0.5 on the scale 512, 256, 128 . . . 4, 2, 1 and the minimal effective dose was taken as the lowest effective dose on this scale . This procedure is illustrated for acetylsalicylate in Fig. 1.

In some experiments, antagonists were administered daily for 3 to 4 days and then the bronchoconstrictor response to bradykinin compared with that of controls.

Three-week-old guinea-pigs were sensitized by injecting 10% egg albumen in saline, 1 ml. subcutaneously and 1 ml. intraperitoneally. About three weeks later, the effects of antagonists on anaphylactic bronchospasm were studied.

Guinea-pig Skin Capillaries in vivo.—We used the method of Miles and Miles (1952), applied to bradykinin by Holdstock, Mathias, and Schachter (1957). Guinea-pigs were depilated with Sleek (Elizabeth Arden) over a dorsal area lying between the pectoral and pelvic girdles and extending 4 cm. on either side of the mid-line Bradykinin and histamine were administered intradermally at 4 to 9 sites half to one hour after injecting intravenously 1.2 ml./kg. of 5% pontamine blue 6BX (Gurr) in saline. Half an hour after these control injections, antagonist was given intraperitoneally or subcutaneously and, half an hour later, bradykinin and histamine were again injected intradermally at other sites. All intradermal injections were randomized in relation to agent, site, dose, and time. Responses were measured as areas by tracing the zone of blueing 10 min. after intradermal injection on to mm. squared paper.

Guinea-pig Ileum in vitro.—Isotonic contractions were recorded from pieces of ileum, suspended in Tyrode solution in a 15 ml. bath at 32°.

Rat Duodenum in vitro.—Isotonic movements were recorded from pieces of isolated rat duodenum, suspended in de Jalon solution in a 15 ml. bath at 32°, according to the method of Gaddum and Horton (1959).

RESULTS

Resistance to Inflation of Guinea-pig Lungs

In 9 guinea-pigs, using three doses of acetylsalicylate, we determined approximately how much bradykinin was needed to restore the response 5 min. after acetylsalicylate to the same magnitude as that to 0.4 μg. of bradykinin before it. The results are expressed in Table I, which shows that the dose of bradykinin required to overcome antagonism by acetylsalicylate increased with the dose of acetylsalicylate given, the ratio at three doses being roughly constant. To save bradykinin, we did not give higher doses than 4 mg./kg. of acetylsalicylate.

Table II shows the percentage decrease in the response to bradykinin in 11 guinea-pigs, obtained by the procedure described for determining the minimal effective dose in the section on methods,

TABLE I

RESTORATION OF BRONCHOCONSTRICTOR RESPONSE OF GUINEA-PIGS TO BRADYKININ AFTER CALCIUM ACETYLSALICYLATE

A standard response was first obtained to 0.4 μg. of bradykinin. After administering acetylsalicylate at three doses, the doses of bradykinin were determined which evoked a response similar to the standard. All doses were given intravenously.

Dose of Acetylsalicylate (mg./kg.)	Dose of Bradykinin to Restore Response (μg.)	Dose-Ratio
1	1·6–3·2	4–8
2	6·4–12·8	16–32
4	12·8–25·6	32–64

using intravenous doses of 0.5, 1, or 2 mg./kg. of acetylsalicylate. From these results, if any of the observed responses to a particular dose were equally likely, the choice of the minimal effective dose would be 2 mg./kg. on two-thirds, 1 mg./kg. on one-quarter and 0.5 mg./kg. on one-twelfth of the occasions. From Tables I and II it will be seen that the minimal effective dose for intravenous acetylsalicylate (2 mg./kg.) corresponds to a dose-ratio (Gaddum, Hameed, Hathway, and Stephens, 1955) of 16 to 32. Cutting both vagi in the neck did not affect the potency of acetylsalicylate. This test was used to examine other potential antagonists of bradykinin. Table III gives each minimal effective dose which is based on determinations in at least three animals. Of the eight analgesic antipyretic drugs given intravenously, seven showed measurable anti-bradykinin activity, the descending order of potency being: calcium acetylsalicylate, phenylbutazone, amidopyrine and phenazone, paracetamol, cinchophen and sodium salicylate. Of the eleven drugs given orally, only five were active. We therefore gave drugs by the intraduodenal route which Corne and Edge (1958) had found better than the oral for pempidine in anaesthetized cats. Table III shows that sodium

TABLE II

REDUCTION OF BRONCHOCONSTRICTOR RESPONSE OF GUINEA-PIGS TO BRADYKININ BY SINGLE DOSES OF CALCIUM ACETYLSALICYLATE

In 11 guinea-pigs a reference response was first obtained to 0.4 μg. of bradykinin. After administering acetylsalicylate, a response to 0.8 μg. bradykinin was obtained. The % reduction was measured by the extent to which the second response to bradykinin was reduced in comparison with the first. All doses were given intravenously.

% Reduction of Bradykinin Response at Doses of Acetylsalicylate		
0·5 mg./kg.	1·0 mg./kg.	2·0 mg./kg.
−12	14	56
−9	37	61
26	50	82
68		100

TABLE III

POTENCIES OF VARIOUS AGENTS IN SUPPRESSING BRONCHOCONSTRICTION DUE TO BRADYKININ IN GUINEA-PIGS

The minimal effective dose (MED) is the least dose of an antagonist that reduces the response to an intravenous dose of bradykinin, which is twice the preceding dose, to less than half the preceding response, without reducing that to histamine. * Administered as calcium salt; § administered as sodium salt; —, not tested; I.P., intraperitoneal; I.M., intramuscular.

Agent	MED (mg. Acid or Base/kg.)			
	Intravenous	Oral	Intraduodenal	Other Routes
Acetylsalicylic acid	2*	32	64	—
Sodium salicylate	64	>512	256	—
Salicylamide	—	>256	>512	—
4-Hydroxyisophthalic acid	>64§	—	>512	I.P >512
Cinchophen	32§	>512	256	—
Sodium phenylbutazone	4	16	16	—
Amidopyrine	8	16	16	—
Phenazone	8	64	128	—
Acetanilide	—	>512	256	—
Phenacetin	—	>512	512	—
Paracetamol	16	512	128	—
Amodiaquine phosphate	—	>512	>512	—
Cortisone	—	—	—	I.M. >25
Hydrocortisone sodium succinate	Non-specific at 100	—	—	—
D,L-Aldosterone	>2	—	—	—
Morphine sulphate	>32	—	—	—
1-(1-Phenylcyclohexyl)piperidine hydrochloride	>16	—	—	—

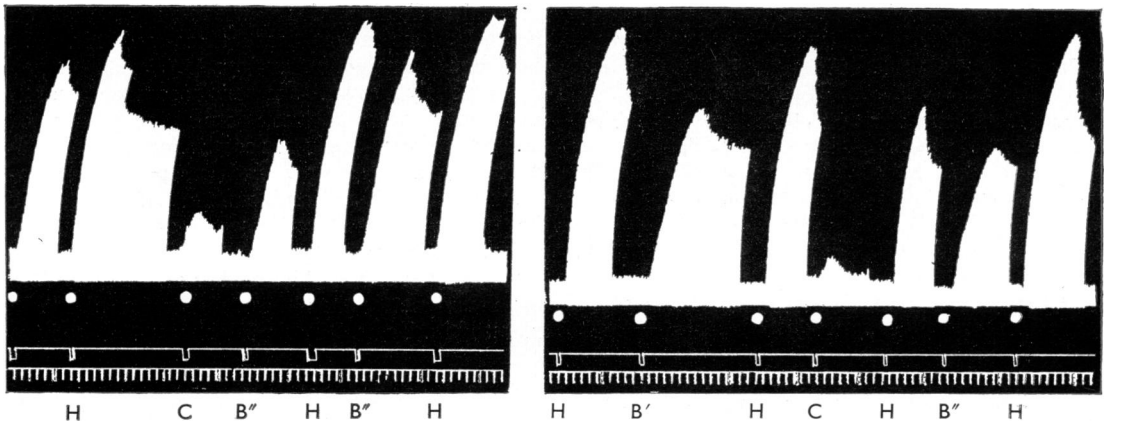

FIG. 2.—Resistance to inflation of guinea-pig lungs *in vivo*. Depression of responses to bradykinin and histamine by a large dose of hydrocortisone. B′, 0.4 μg., and B″, 0.8 μg. of bradykinin; H, histamine, 1.0 μg.; C, hydrocortisone sodium succinate, 100 mg./kg. All doses were given intravenously. Guinea-pig weights: left, 515 g.; right, 650 g. Time, 10 sec.

salicylate, cinchophen, acetanilide and phenacetin were active intraduodenally though not by mouth.

Amodiaquine in single large oral or intraduodenal doses was ineffective. Repeated administration of this drug did not lower the response of guinea-pig bronchioles to bradykinin. Two guinea-pigs each received by mouth 50 mg./kg. amodiaquine daily on 4 successive days. One hour after the last treatment, the bronchiolar responses of each animal to bradykinin were tested and found to be normal. In a similar subacute experiment with chloroquine phosphate in 3 guinea-pigs, bronchiolar responses also remained normal.

Since a single intramuscular dose of cortisone (25 mg./kg.) failed to reduce the bradykinin response (Table III), 3 guinea-pigs were given daily on 3 successive days 25 mg. of cortisone acetate subcutaneously. One hour after the third dose, the bronchiolar responses of each animal were within normal limits. Another 3 guinea-pigs received 100 mg./kg. of hydrocortisone sodium succinate intravenously. Five minutes after treatment, bronchiolar responses both to histamine and bradykinin were reduced (Fig. 2), but only for about 15 min.

We investigated how far acetylsalicylate and amidopyrine, given intravenously, antagonized

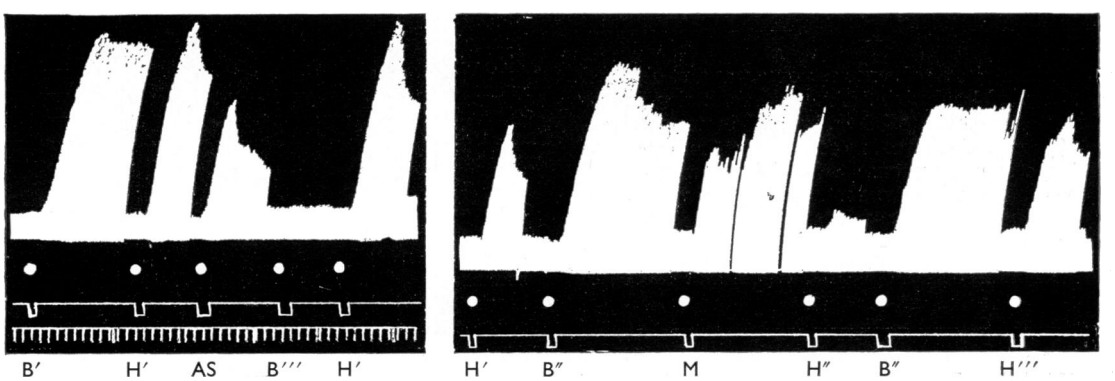

FIG. 3.—Resistance to inflation of guinea-pig lungs *in vivo*. Failure of calcium acetylsalicylate to suppress response to histamine and of mepyramine to suppress response to bradykinin. B′, 0.4 μg., B″, 0.8 μg., and B‴, 1.6 μg. of bradykinin. H′, 1 μg., H″, 1 mg., and H‴, 2 mg. of histamine. AS, calcium acetylsalicylate, 100 mg./kg. M, mepyramine maleate, 10 mg./kg. All doses were given intravenously. Guinea-pig weights: left, 460 g.; right, 500 g. Time, 10 sec.

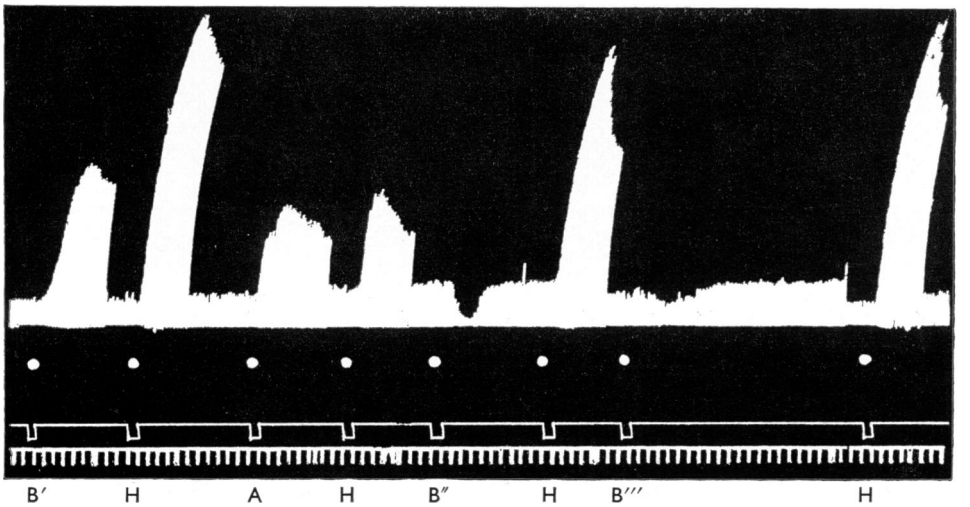

FIG. 4.—Resistance to inflation of guinea-pig lungs *in vivo*. Degree of specificity of amidopyrine towards bradykinin. B′, 0.4 μg., B″, 3.2 μg., and B‴, 6.4 μg. of bradykinin. H, histamine, 2 μg. A, amidopyrine, 100 mg./kg. All doses were given intravenously. Weight of guinea-pig, 580 g. Time, 10 sec.

bradykinin specifically. While 2 mg./kg. of acetylsalicylate effectively reduced the response to bradykinin, 100 mg./kg. did not alter that to 1 μg. histamine (Fig. 3). In other experiments, 100 mg./kg. of acetylsalicylate did not reduce the responses to 2 μg. of 5-hydroxytryptamine or 2 μg. of acetylcholine. Fig. 3 also shows that as much as 10 mg./kg. mepyramine intravenously failed to affect the response to 0.8 μg. of bradykinin. Lysergic acid diethylamide (1 mg./kg.) and atropine (1 mg./kg.), both given intravenously, also failed to affect the response to bradykinin. At 8 mg./kg., amidopyrine effectively reduced the bradykinin response (Table III), while 50 mg./kg. lowered by one-quarter and 100 mg./kg. by two-thirds the response to 2 μg. of histamine (Fig. 4). Figs. 3 and 4 show that high doses of acetylsalicylate, amidopyrine, and mepyramine themselves caused bronchoconstriction. After giving amidopyrine (Fig. 4), acetylsalicylate or phenylbutazone, an apparent bronchodilator action of bradykinin was also sometimes revealed.

Effect of Drugs on Response to Antigen.—De Schaepdryver (1950), using the preparation of Konzett and Rössler (1940), found that antigen

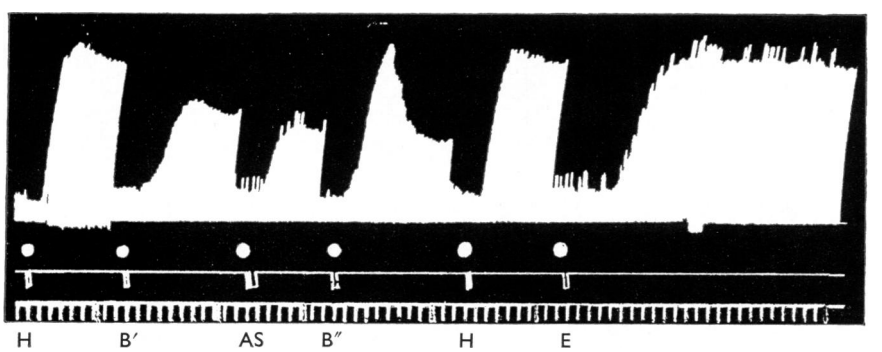

FIG. 5.—Resistance to inflation of guinea-pig lungs *in vivo* in an animal sensitized to egg albumen. Failure of a large dose of calcium acetylsalicylate to suppress bronchoconstriction due to antigen. B′, 0.4 μg., and B″, 12.8 μg. of bradykinin. H, histamine, 1 μg. AS, calcium acetylsalicylate, 100 mg./kg. E, egg albumen, 1 mg. All doses were given intravenously. Weight of guinea-pig, 340 g. Time, 10 sec.

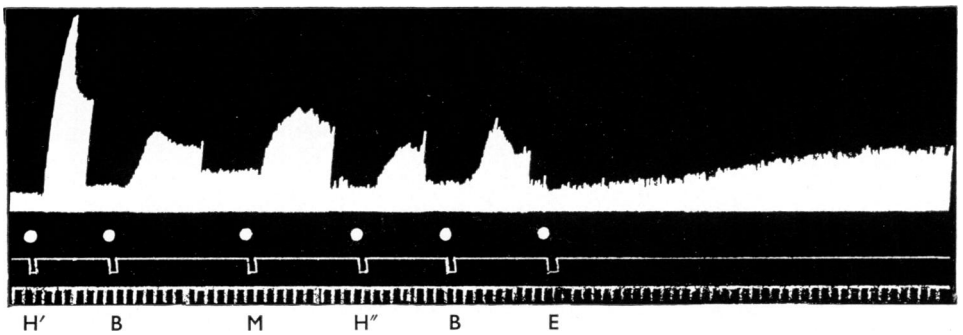

FIG. 6.—Resistance to inflation of guinea-pig lungs *in vivo* in an animal sensitized to egg albumen. Partial suppression by mepyramine of response to antigen. B, bradykinin, 0.4 μg. H′, 1 μg., and H″, 0.5 mg. of histamine. M, mepyramine, 5 mg./kg. E, egg albumen, 1 mg. All doses were given intravenously. Weight of guinea-pig, 420 g. Time, 10 sec.

injected intravenously produced a powerful bronchospasm in guinea-pigs sensitized to egg albumen. We have confirmed this. In 34 sensitized guinea-pigs, prepared for recording resistance of the lungs to inflation, antagonists of known bronchoconstrictor agents were given intravenously before egg albumen. Although acetylsalicylate (100 mg./kg.) markedly reduced the response to a large dose of bradykinin, it did not affect that to antigen (Fig. 5). Lysergic acid diethylamide (1 mg./kg.) and atropine (1 mg./kg.) were likewise ineffective against anaphylactic bronchospasm, although they readily suppressed respectively the responses to 5-hydroxytryptamine and acetylcholine. Mepyramine (0.5 to 5 mg./kg.) greatly lessened the response to antigen, but a small residual bronchoconstriction still remained (Fig. 6).

Skin Blueing in Guinea-pigs

In control experiments, we found that the curve relating area of skin blueing to intradermal dose of bradykinin had a flat slope, while that of

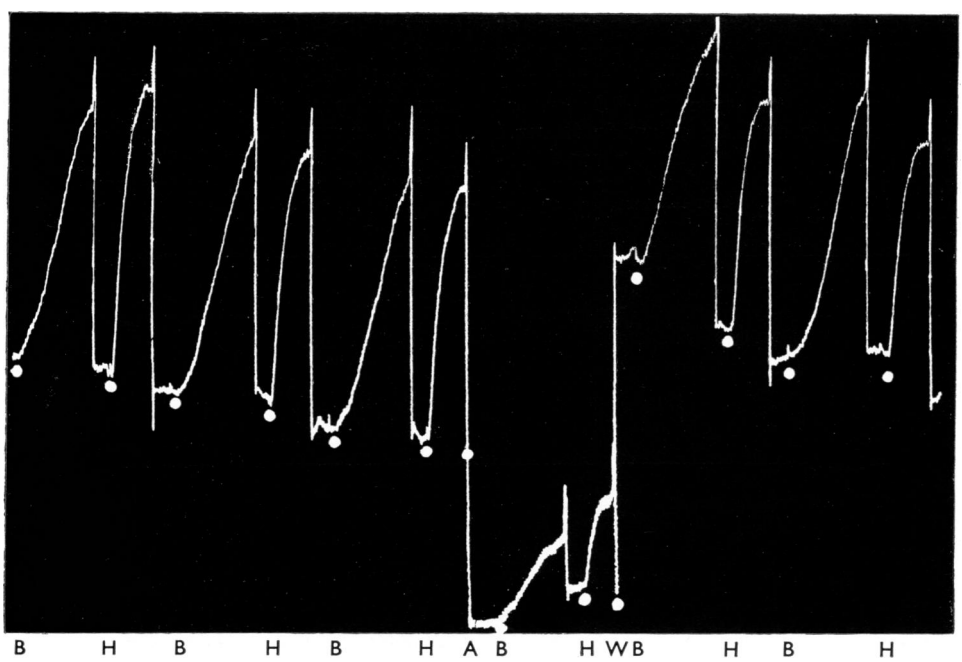

FIG. 7.—Isolated guinea-pig ileum in 15 ml. bath. Suppression by amidopyrine of responses to bradykinin and histamine. B, bradykinin, 0.24 μg. H, histamine, 0.2 μg. A, amidopyrine, 100 μg./ml. W, amidopyrine washed out.

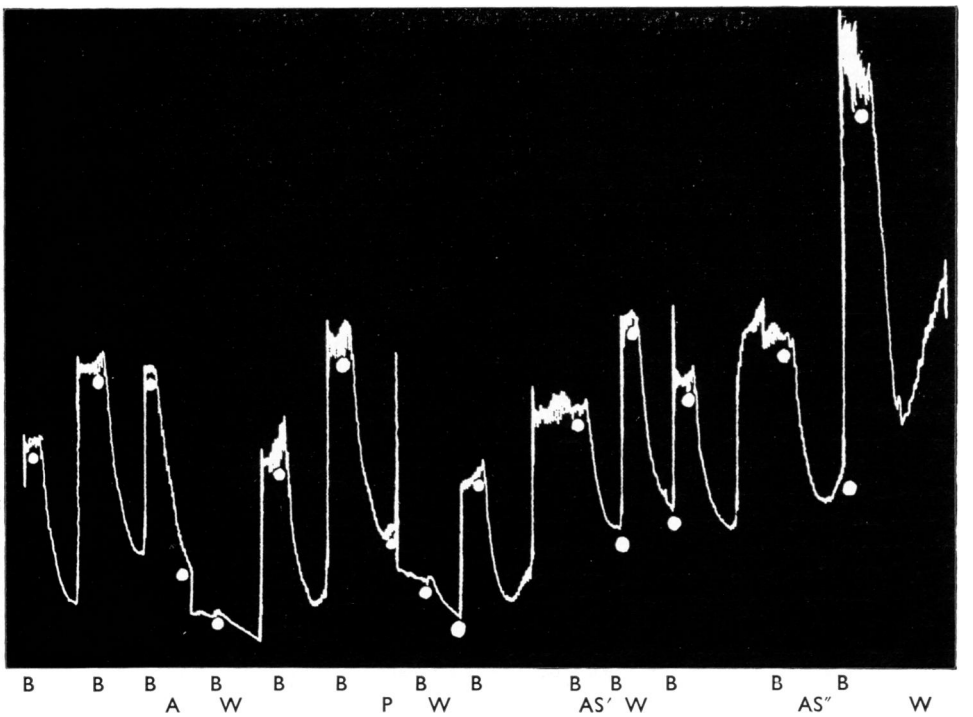

FIG. 8.—Isolated rat duodenum in 15 ml. bath. Failure of calcium acetylsalicylate to suppress response to bradykinin. B, bradykinin, 0.04 μg. A, amidopyrine, 50 μg./ml. P, phenylbutazone, 200 μg./ml. AS′, 200 μg./ml., and AS″, 800 μg./ml. of calcium acetylsalicylate. W, wash.

histamine had a steeper slope. This agrees with the observations of Bhoola, Calle, and Schachter (1960). Acetylsalicylic acid (150 and 200 mg./kg.) did not lessen the response of the capillaries to bradykinin or histamine, while mepyramine (0.5 mg./kg.) abolished the response to histamine, but left that to bradykinin unaffected (Table IV). Phenylbutazone (100 and 200 mg./kg.) and amidopyrine (75 and 150 mg./kg.) lessened but did not abolish responses to bradykinin and histamine, the effects on the histamine response appearing somewhat greater.

Intestinal Preparations in vitro

Phenylbutazone (200–400 μg./ml.), amidopyrine (20–100 μg./ml.), phenazone (200–400 μg./ml.) and cinchophen (200 μg./ml.) reduced the response of the isolated guinea-pig ileum to bradykinin. However, the same concentrations of these drugs also lessened the response to histamine, as will be seen for amidopyrine in Fig. 7. Acetylsalicylate (200 μg./ml.) failed to affect responses of the ileum to either agent.

TABLE IV

FAILURE OF ACETYLSALICYLIC ACID TO REDUCE AREAS OF SKIN BLUEING OF GUINEA-PIGS BY BRADYKININ

Animals were treated intraperitoneally with 200 mg./kg. of acetylsalicylic acid or with 0.5 mg./kg. of mepyramine after measurement of control areas of blueing.

Agent	Intradermal Dose (μg.)	Mean Area of Blueing (sq. mm.)			
		Before Acetylsalicylic Acid	After Acetylsalicylic Acid	Before Mepyramine	After Mepyramine
Histamine	0·025	22·5	27·5	13·5	0
	1·0	40	40	60·5	0
Bradykinin	0·002	27	22	31·0	31·5
	0·2	32	37·5	35·5	26·0

Amidopyrine (50 μg./ml.) and phenylbutazone (200 μg./ml.) relaxed the isolated rat duodenum, but did not prevent a further small relaxation after bradykinin (Fig. 8). Acetylsalicylate (200 and 800 μg./ml.) increased the tone of the rat duodenum, but did not suppress the relaxation due to bradykinin (Fig. 8). These drugs affected the response to vasopressin in the same way as that to bradykinin.

Discussion

We have used the term dose-ratio (Gaddum *et al.*, 1955) to describe the ratio of doses of agonist giving equal effects before and after antagonist.

We have studied the effects of acetylsalicylic acid, phenylbutazone and amidopyrine on four different biological responses to bradykinin. In one of these—bronchoconstriction in the guinea-pig *in vivo* — these drugs showed towards bradykinin several features of pharmacological antagonism. (1) Non-toxic doses of antagonist suppressed the response to the agonist; (2) larger doses of agonist restored the response; (3) when the dose of agonist was increased, larger doses of antagonist were effective; and (4) effective doses of antagonist did not reduce responses to other bronchoconstrictor agents. In another response—contraction of the guinea-pig ileum *in vitro*—phenylbutazone and amidopyrine decreased the effect of bradykinin, but affected equally the response to histamine. In two biological systems—the skin capillaries of blued guinea-pigs *in vivo* and rat duodenum *in vitro*—large doses of these drugs failed to suppress the response to bradykinin. In three of these preparations which responded to histamine, mepyramine specifically antagonized this substance.

The fact that some drugs specifically antagonized bradykinin in its bronchoconstrictor but not in its other actions has parallels in the antagonism of acetylcholine, histamine, 5-hydroxytryptamine and adrenaline. For example, different types of antagonists block acetylcholine at different sites of action, such as at the skeletal neuromuscular junction, at synapses in autonomic ganglia and at the nerve effector cell junctions in intestinal muscle. Such differences are generally ascribed to the existence of several types of receptor for acetylcholine. The same explanation may well apply to our observations on bradykinin.

The apparent bronchodilator action of bradykinin, which is unmasked in the presence of antagonists of its bronchoconstrictor action (Fig. 4), might be attributed to an impurity in the bradykinin preparations used. Alternatively and more probably it may be due to the presence in the bronchioles of a second type of receptor for bradykinin. This supposition would be consistent with the recent observation of Waaler (1960) that bradykinin does not constrict but sometimes slightly dilates the bronchioles of the isolated lung of the dog. This second type of receptor might perhaps correspond to that involved in the effects of bradykinin other than those blocked by antagonists of its bronchoconstrictor action.

It seems relevant to ask how far the degree of the antagonism we have observed is related to the other pharmacological actions of the antagonists. Winder, Wax, Burr, Been, and Rosiere (1958), Adams (1960), and Winder (personal communication) determined the degree to which orally administered drugs suppressed the skin erythema caused by ultra-violet irradiation of guinea-pigs. They showed that phenylbutazone, acetylsalicylic acid, amidopyrine and phenazone exhibit high to moderate anti-inflammatory potency in this test (Table V). Sodium salicylate, cinchophen and amodiaquine had low but detectable activity, while salicylamide, 4-hydroxyisophthalic acid, acetanilide, phenacetin, paracetamol, cortisone, cortisone acetate and hydrocortisone had little or no action. These results show some parallelism with our find-

Table V

POTENCIES OF VARIOUS AGENTS IN SUPPRESSING SKIN ERYTHEMA OF GUINEA-PIGS DUE TO ULTRA-VIOLET IRRADIATION

Approximate "effective doses" in mg./kg. orally are derived from Winder *et al.* (1958), Adams (1960), and Winder (personal communication). Values in column headed Winder *et al.* are total doses. An asterisk indicates subcutaneous route; — indicates not tested.

Drug	Winder *et al.*	Adams
Acetylsalicylic acid ..	100 to 200	80
Sodium salicylate	200	120
Salicylamide	>400	>320
4-Hydroxyisophthalic acid	>400	>320
Cinchophen	200	—
Phenylbutazone	18	10
Amidopyrine	130	80
Phenazone	100	—
Acetanilide	>400	>240
Phenacetin	400	>240
Paracetamol	>400	>240
Amodiaquine	200	—
Chloroquine	>400	—
Cortisone	>25	—
,, acetate	>75	—
Hydrocortisone acetate ..	>75*	—

ings in antagonizing bradykinin. Marked activity in both tests is restricted to drugs of the analgesic-antipyretic type, the most potent, apart from acetylsalicylic acid, being pyrazoline or pyrazolidine derivatives which have several chemical features in common. For the eleven agents of this type that we examined, a Spearman rank correlation coefficient between the potencies obtained by Winder *et al.* (1958) (Table V) and those in our test after intraduodenal administration had a value of 0.761, showing a probability of <0.01 that the two sets of potencies are independent. Since, however, intradermal bradykinin did not produce erythema in guinea-pigs and the skin blueing response did not show comparable antagonism by the drugs used, the interpretation of this correlation needs further study.

Winder *et al.* (1958) have called attention to a parallelism between the performance of analgesic-antipyretic drugs in suppressing ultra-violet erythema in guinea-pigs and the clinical value of these drugs in rheumatism. Since our results correlate well with those Winder *et al.* (1958), it is not surprising that parallelism can also be discerned between suppression of bradykinin bronchoconstriction in guinea-pigs and anti-rheumatic activity of these drugs in man. Paracetamol, which is not active in the test of Winder *et al.* (1958) and which is not generally used in rheumatism, provides an exception. With this drug, however, recovery of the bradykinin response was evident within one hour of its intraduodenal administration. This brevity of action may account for the ineffectiveness of the drug in the ultra-violet light erythema test.

In animals, 4-hydroxyisophthalic acid has somewhat stronger analgesic and antipyretic properties than acetylsalicylic acid (Collier and Chesher, 1956); but Adams (1960) has called attention to the difference between these drugs in their abilities to suppress ultra-violet light erythema. The contrast in the potencies of these two drugs as antagonists of bradykinin supports the conclusion of Adams (1960) as does also the failure of 4-hydroxyisophthalic acid to benefit rheumatoid arthritis (Hajnal, Sharp, and Popert, 1959).

Outside the group of analgesic antipyretic drugs discussed above, there was no correlation between anti-rheumatic and anti-bradykinin action. The corticosteroids failed to antagonize bradykinin and so did chloroquine and amodiaquine, which are reported to be useful in rheumatoid arthritis (Haydu, 1953; Freedman, 1956; Pomeroy, Warren, Mills, and Clark, 1959; Bepler, Baier, McCracken, Rentschler, Rogers, and Lansbury, 1959; Kersley and Palin, 1959).

Chen and Weston (1960) have reported that 1-(1-phenylcyclohexyl) piperidine causes analgesia in the monkey; but this compound and morphine were inactive in our test. There was, therefore, no correlation between analgesic action as such and antagonism to bradykinin.

The failure of acetylsalicylate to lessen anaphylactic bronchospasm suggests that bradykinin does not play an important part in this form of bronchoconstriction. This finding is consistent with that of Herxheimer (1955) that intramuscular doses of phenylbutazone and of salicylic acid do not protect sensitized guinea-pigs against antigen given by aerosol. In our experiments mepyramine greatly reduced anaphylactic bronchospasm, as might have been expected from the observation of Armitage, Herxheimer, and Rosa (1952) that this drug protected sensitized guinea-pigs against antigen administered by aerosol.

Note Added in Proof

We examined how far acetylsalicylate antagonized a specimen of the bradykinin-like synthetic nonapeptide H-L-Arg-L-Pro-L-Pro-Gly-L-Phe-L-Ser-L-Pro-L-Phe-L-Arg-OH (Boissonnas, Guttmann, Jaquenoud, Konzett, and Stürmer; *Experientia*, 1960, **16**, 326), which Dr. E. D. Nicolaides and Dr. H. A. De Wald prepared. Assays of this sample against purified specimens of bradykinin (supplied by Dr. D. F. Elliott, Dr. G. P. Lewis, and Dr. E. W. Horton) gave comparable results on guinea-pig ileum, rabbit blood pressure and rat uterus, showing the sample contained approximately 70–90% active nonapeptide. Acetylsalicylate (2 mg./kg. intravenously) suppressed the bronchoconstrictor effect of the nonapeptide to a comparable extent to that of an equiactive dose of bradykinin. Larger doses of the nonapeptide overcame the suppression as did bradykinin in the same preparation. After acetylsalicylate both nonapeptide and nearly pure bradykinin showed some bronchodilator action.

We wish to thank Dr. I. M. Lockhart and Mr. W. A. Jones for supplies of bradykinin, Dr. D. F. Elliott, Dr. G. P. Lewis and Dr. E. W. Horton for a specimen of pure bradykinin and Ciba Ltd., Basle, for aldosterone. We are indebted to Dr. B. T. Warner for advice on statistics, and to Miss M. D. Nicholas and Miss G. C. Clarke for technical assistance. We are also grateful to Dr. M. Schachter for helpful advice and discussions.

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MEMBRANE POTENTIAL CHANGES ASSOCIATED WITH TACHYPHYLAXIS AND POTENTIATION OF THE RESPONSE TO STIMULATING DRUGS IN SMOOTH MUSCLE

BY

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(RECEIVED OCTOBER 3, 1960)

Conditions which affect the response of smooth muscle to repeated application of stimulating drugs have been investigated. In guinea-pig taenia coli, tension changes were recorded simultaneously with membrane potential changes using the sucrose gap technique. Acetylcholine, histamine, and 5-hydroxytryptamine caused depolarization and, after removal of the drug, hyperpolarization which was followed by a sequence of damped oscillations of the membrane potential. The average rate of depolarization decreased in the order acetylcholine > histamine > 5-hydroxytryptamine. The readiness with which tachyphylaxis occurred increased in the order acetylcholine < histamine < 5-hydroxytryptamine. When a dose of a stimulating drug was repeated, the response obtained depended on the phase of the oscillatory potential changes during which it was applied. In general the effect was depressed during a phase of polarization and enhanced during a phase of depolarization. The degree of tachyphylaxis—or potentiation—depended not only on the direction in which the membrane potential changed at the moment of drug application, but also on the relation between the rate at which this potential change took place and the rate of depolarization caused by the drug. The results observed are consistent with the hypothesis that the fluctuating excitability and polarization of the smooth muscle membrane is brought about by periodical changes in the rate of active ion transport and other stabilizing processes in the cell membrane which depend on the rate of metabolic energy supply. The muscle was sensitized to acetylcholine and histamine by previous treatment with, or in the presence of, 5-hydroxytryptamine.

Barsoum and Gaddum (1935) pointed out that the desensitization of smooth muscle preparations by repeated exposure to a stimulating drug, known as tachyphylaxis, represents a difficulty as well as a help in biological assay. A difficulty, because a previously effective dose may not produce a comparable contraction on repetition; a help, because the action of another drug may not be reduced to the same extent and it can thus be detected and identified if the two drugs are present in a mixture. Cantoni and Eastman (1946) investigated the possibility that, in the course of repeated drug application, the muscle contraction was reduced by exhaustion due to a previous maximal contraction. They recorded the response to a weak concentration of acetylcholine at regular intervals and found that it was depressed if the muscle was at one time exposed to a 100 times stronger concentration which caused a maximal contraction. This temporary depression was also observed after maximal contractions in response to large concentrations of histamine, barium chloride, pilocarpine and mecholyl. If, however, the maximal contraction was obtained by a large dose of potassium chloride, the acetylcholine contractions were not diminished, instead they were increased. Rand (1957), who repeated these observations, advanced the hypothesis that the responsiveness of the smooth muscle depended on the ratio of intracellular to extracellular potassium. While this ratio would be increased when normal solution was admitted after prolonged exposure to excess potassium, it would be reduced when the muscle had lost potassium after treatment with acetylcholine. Hughes, McDowall and Soliman (1956) found that changes in the sodium chloride concentration affected the response to repeated doses of histamine; tachyphylaxis was abolished by low sodium, but intensified by high sodium in the bathing solution.

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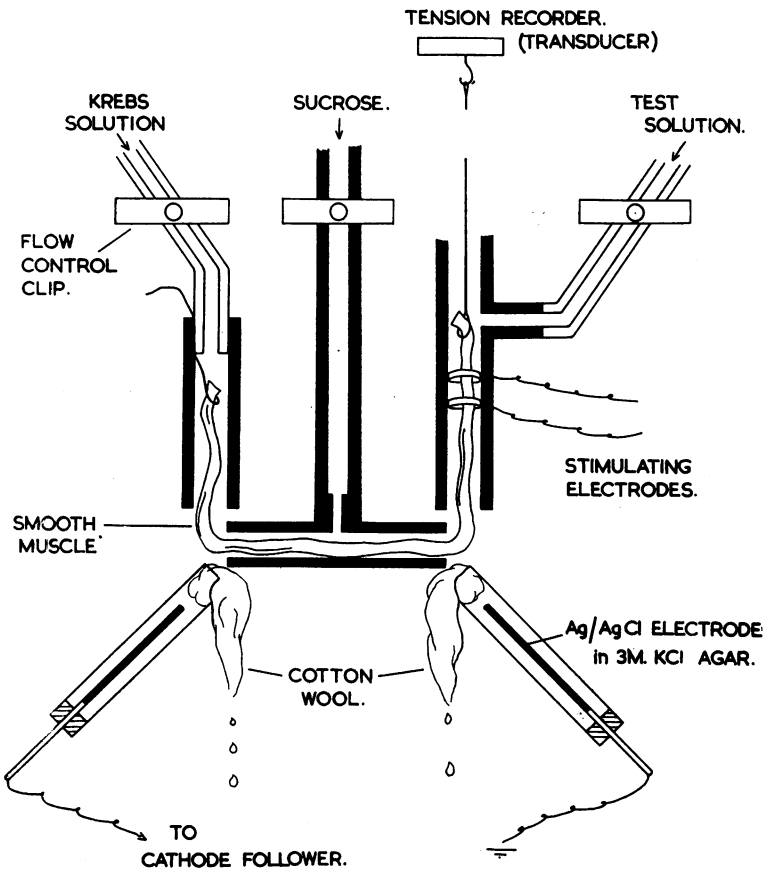

FIG. 1.—Diagram of apparatus used for extracellular recording by sucrose-gap method, modified to include stimulating electrodes and means for recording the tension.

The authors interpreted their observations by assuming that tachyphylaxis was due to a difficulty in sodium extrusion.

The present work was undertaken to investigate further the conditions leading to a change of the response to a stimulating drug by applying acetylcholine, histamine and 5-hydroxytryptamine for short periods in rapid succession and recording the mechanical responses as well as the changes in membrane potential and electrical activity.

A short account of the results was given at the meeting of the British Pharmacological Society on January 11, 1959.

METHODS

Ganglion-free intestinal smooth muscle taken from the taenia coli of the guinea-pig was used in all experiments and bathed by a modified Krebs' solution (Bülbring, 1953).

Extracellular recording from smooth muscle by the "sucrose gap" method (Stämpfli, 1954) has been described previously (Burnstock, 1958a; Burnstock and Straub, 1958). A modification of the chamber so that the muscle could be stimulated electrically has also been described (Bülbring, Burnstock, and Holman, 1958). In the present experiments a further alteration of the apparatus was made so that tension changes could be recorded electrically simultaneously with those of the membrane potential. The test side of the chamber was constructed so that solutions flowed through a side tube while the end of the muscle was attached by a vertical thread to a mechano-electronic transducer valve (RCA 5734) mounted in the manner described by Bülbring (1955). Care was taken to ensure that the flow of solutions was constant. The modified chamber is shown in Fig. 1.

RESULTS

Changes During Brief Exposure and Following the Removal of Stimulating Drugs

The changes of membrane potential, spike discharge and tension produced by short application of acetylcholine or histamine in intestinal smooth muscle are known to be qualitatively similar (Bülbring, 1955). The course of events is represented diagrammatically in Fig. 2*a*. Initially, after administration of the drug, there is a period of depolarization during which there is no spike discharge (or no increase in rate if spikes are already present) and no change of tension. This period, from A to B, will be called "lag." At a critical level (marked by an asterisk) spikes appear and consequently the tension rises. The period taken to reach the maximum tension, from A to C, will be called the "maximum response time." The maximum depolarization often coincides but sometimes occurs a few seconds before or later. After the maximum values are reached during the presence of the drug there is usually a slight repolarization and loss of tension. When the drug is washed out (at D)

the spike discharge is slowed and it soon stops (marked by asterisk), at which point the tension falls to the original level or slightly below. The membrane potential recovers more slowly and usually it also reaches a higher value than that recorded initially. The period taken from the time of washing out the drug to the maximum polarization of the membrane, from D to E, will be called the "maximum polarization time." The membrane potential then fluctuates in the pattern of a damped oscillation (see Fig. 4, to be discussed later) before it returns to the original value. Thus full recovery is not reached within the time course shown in the diagram of Fig. 2*a*. A typical record of the events following the administration of a drug (histamine 1×10^{-7}) for 45 sec. and the first stage of recovery after its removal is shown in Fig. 2*b*.

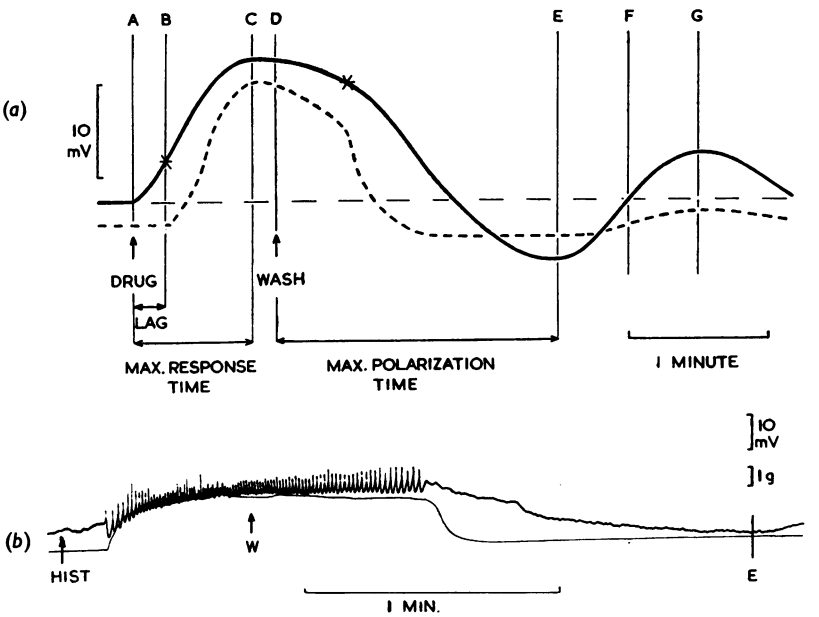

FIG. 2.—(*a*) Diagram representing the course of events following administration of a stimulating drug. Continuous line, membrane potential; broken line, tension. For description see text. (*b*) Records of electrical changes (upper trace) and tension changes (lower trace) produced by histamine 10^{-7}.

Comparison of the Responses to Acetylcholine, Histamine and 5-Hydroxytryptamine

The taenia coli was found to be most sensitive to 5-hydroxytryptamine and least sensitive to acetylcholine, for which 10^{-7} was sometimes the threshold concentration. Histamine 10^{-7} produced the most constant change in tension and this was, in different experiments, about equal to that caused by acetylcholine 10^{-7} to 10^{-6} and 5-hydroxytryptamine 2×10^{-9} to 5×10^{-8}. Equiactive concentrations of the three drugs, judged by the tension response, were applied to compare the periods defined above. The "lag" was shortest for acetylcholine and longest for 5-hydroxytryptamine. This was due to the difference in the rate of depolarization which decreased in the order acetylcholine > histamine > 5-hydroxytryptamine, as shown in Fig. 3. The period taken to reach the maximum tension was also shortest for acetylcholine and longest for 5-hydroxytryptamine. In Fig. 3, for example, the maximum response to acetylcholine occurred after 24 sec., to histamine after 61 sec., and to 5-hydroxytryptamine after 81 sec. (not shown in the record).

Whereas both the lag and the maximum response time showed consistent differences between the three drugs tested, the maximum polarization time after removal of the drug did not. In general, this period was prolonged when the dose and the duration of application of any given drug was greater. These results are summarized in Table I.

Comparison of the Responses to Different Concentrations of the Same Drug

With increasing concentrations of acetylcholine and histamine the lag became progressively shorter and the maximum response was reached earlier. However, as may be seen from Table I, the lag of the response to the greatest concentration of histamine (10^{-5}) was only about that of the weakest concentration of acetylcholine (10^{-7}). Furthermore, with a hundredfold increase in concentration, the average rate of depolarization caused by acetylcholine was increased four times, whereas the rate of depolarization caused by histamine was only increased by 50%. Thus the peak of the response to acetylcholine was reached between 35.9 and 27.7 sec., while that to histamine occurred much later, between 61.4 and 49.6 sec. Also for 5-hydroxytryptamine the lag became

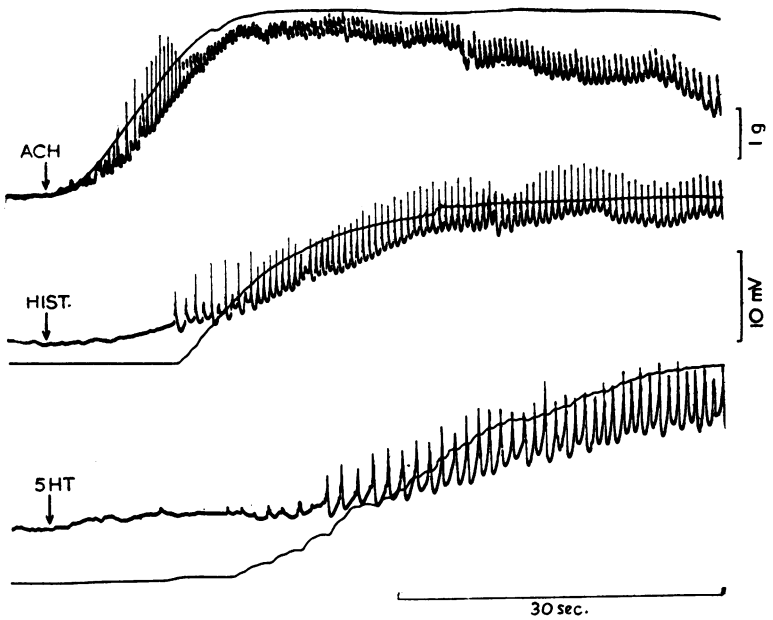

FIG. 3.—Records as in Fig. 2*b*. Comparison of the lag, of the rate of depolarization and of the maximum response time of equiactive concentrations of acetylcholine (ACH: 10^{-6}), histamine (10^{-7}) and 5-hydroxytryptamine (5HT: 5×10^{-8}). For description see text.

shorter with increasing concentrations, but the time taken to reach the maximum response became longer as the doses increased. With excessive concentrations of 5-hydroxytryptamine (such as 10^{-6}) the depolarization was usually preceded by a period of hyperpolarization. This was also observed when lower concentrations of 5-hydroxytryptamine were applied repeatedly for periods longer than 90 sec. A similar initial hyperpolarization was seen when high concentrations of histamine (namely 10^{-5}) were used.

We also compared the mean rates of repolarization following application of different concentrations of the same drug (Table I). After acetylcholine the rate of repolarization was about half that of depolarization. The

TABLE I

MAXIMUM RESPONSE TIME, AVERAGE RATES OF DEPOLARIZATION AND OF REPOLARIZATION IN THE TAENIA COLI OF THE GUINEA-PIG

The numerals in brackets are standard errors of the mean.

Drug and Conc.		Lag (sec.)	Maximum Response Time (sec.)	Average Rate of Depolarization (mV/sec.)	Average Rate of Repolarization (mV/sec.)	No. of Observations
Acetylcholine	10^{-7}	6·2 (±0·95)	35·9 (±2·08)	0·174 (±0·027)	0·097 (±0·021)	20
	5×10^{-7}	5·3 (±0·77)	35·7 (±1·05)	0·313 (±0·032)	0·106 (±0·016)	9
	10^{-6}	4·2 (±0·68)	31·9 (±2·50)	0·531 (±0·069)	0·323 (±0·143)	6
	10^{-5}	2·3 (±1·13)	27·7 (±3·25)	0·716 (±0·103)	0·173 (±0·012)	5
Histamine	10^{-7}	19·0 (±2·16)	61·4 (±2·23)	0·219 (±0·019)	0·173 (±0·026)	18
	10^{-6}	11·7 (±1·41)	60·6 (±5·74)	0·212 (±0·014)	0·214 (±0·044)	4
	10^{-5}	5·8 (±0·79)	49·6 (±7·09)	0·327 (±0·057)	0·158 (±0·028)	5
5-Hydroxy-tryptamine	2×10^{-9}	22·8 (±2·2)	62·0 (±5·94)	0·156 (±0·012)	0·161 (±0·023)	7
	1×10^{-8}	22·9 (±5·38)	72·0 (±7·42)	0·151 (±0·022)	0·100 (±0·011)	7
	5×10^{-8}	15·8 (±2·46)	72·5 (±9·49)	0·299 (±0·071)	0·242 (±0·050)	13
	1×10^{-7}	14·0 (±5·08)	86·0 (±10·0)	0·124 (±0·037)	0·084 (±0·020)	3
	5×10^{-7}	8·3 (±2·50)	140·0 (±20·0)	0·079 (±0·017)	0·372 (±0·175)	3
	1×10^{-6}	115·0 (±13·2)	280·0 (±20·0)	0·029 (±0·005)	0·043 (±0·009)	3

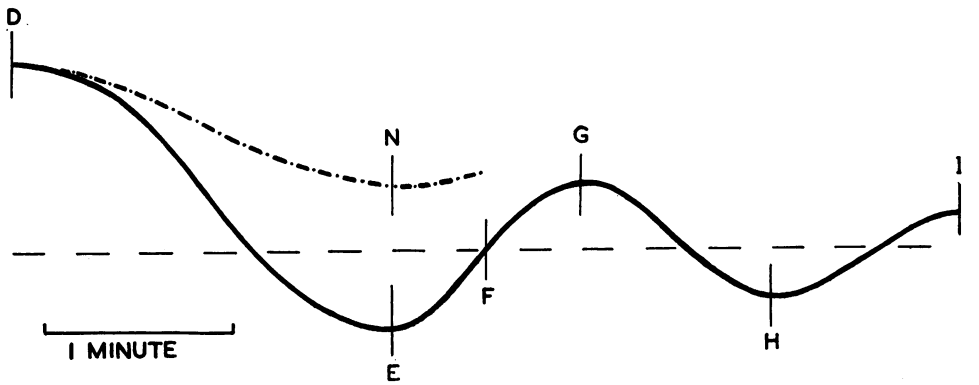

FIG. 4.—Diagram showing the oscillations of the membrane potential following removal of a stimulating drug at *D*. For description see text.

greater the depolarization caused by increasing acetylcholine concentrations the faster was the repolarization. After histamine and 5-hydroxytryptamine the rate of repolarization was very similar to the rate of depolarization. After exposure to excessive concentrations of 5-hydroxytryptamine (5×10^{-7} to 10^{-6}) repolarization was faster than depolarization.

Tachyphylaxis

The pattern of changes during recovery as already mentioned consisted of a phase of repolarization usually beyond the initial membane potential, and a subsequent period in which the membrane potential fluctuated in the manner of a damped oscillation. This period is shown diagrammatically in Fig. 4 (continuous line), where it has been divided into sections so that the time of application of the second dose could be specified in relation to the membrane oscillation. (Occasionally the membrane remained depolarized for a longer time; this condition is represented by the broken line DN.)

The time taken for recovery and the pattern of changes following exposure of the tissue to a drug depended both on the concentration used and on the duration of its application. Thus in experiments designed to determine the conditions causing tachyphylaxis these two factors were varied as well as the interval between doses.

Impairment of the response to a second dose of a drug could be detected by two criteria, namely the magnitude of the response and the delay before the response occurred. Thus tachyphylaxis was either evident from a decreased tension change, or it could be detected by a long lag, or it was shown by both criteria.

All three drugs, when applied during the first phase of polarization (period DE), showed tachyphylaxis which was evident, in nearly every experiment, from the greatly increased lag. With 5-hydroxytryptamine the tension was always correspondingly reduced; but in a number of experiments with histamine, and especially with repeated application of low concentrations of acetylcholine, the tension response was as great as before despite the increased lag. This was due to the fact that the "maximum response time" for acetylcholine was short. Consequently, though the onset of the response was delayed, the peak was reached before the drug was removed.

During the period EF, when the membrane potential was still hyperpolarized in relation to the initial level but had passed the peak of polarization, there was usually no reduced mechanical response to acetylcholine and rarely to histamine, but the lag was still prolonged. With 5-hydroxytryptamine tachyphylaxis was evident both from the longer lag and from the reduction of the mechanical response.

After this phase the tension change produced by acetylcholine and histamine was no longer impaired. Indeed, during FG or HI, when the membrane was depolarizing, a potentiation of the acetylcholine and histamine response often occurred. In contrast, the response to 5-hydroxytryptamine was even then diminished.

During the second polarizing phase of the recovery oscillations, GH, a longer lag was often observed once more. But while the mechanical responses to acetylcholine and histamine were not reduced, the response to 5-hydroxytryptamine was usually diminished even in the later stages of the oscillations, suggesting that some more permanent change had taken place. Typical examples of the progressive change of the responses to repeated doses are shown in Table II.

TABLE II

RESPONSES TO DRUGS ADMINISTERED IN CERTAIN OF THE PHASES OF OSCILLATION SHOWN IN FIG. 2

Expt. No.	Conc.	Duration of		Stage of Oscillation	Lag (sec.)	Tension (g.)
		Exposure (sec.)	Interval (sec.)			
		Acetylcholine				
1	10^{-7}	45	195	—	3·9	3·4
	10^{-7}	45	195	FG	4·3	3·2
	10^{-7}	45	195	FG	7·5	3·3
	10^{-5}	120	60		0·5	5·0
	10^{-7}	45	135	DE	18·0	2·1
	10^{-7}	45	135	FG	5·7	3·1
	10^{-7}	45	135	FG	3·8	3·5
	10^{-7}	45		HI	2·0	4·0
2	10^{-7}	45	195	—	9·5	2·3
	10^{-7}	45	195	DE	10·0	3·2
	10^{-5}	90	150		0·25	4·6
	10^{-7}	45	195	DE	38·0	1·5
	10^{-7}	45	195	DE	32·0	2·5
	10^{-7}	45		EF	7·0	2·8
		Histamine				
3	10^{-7}	60	180	—	30·5	1·2
	10^{-7}	60	180	FG	26·5	1·5
	10^{-5}	60	180	FG	5·0	2·7
	10^{-7}	60	180	EF	32·0	1·3
	10^{-7}	60	180	EF	27·5	1·3
	10^{-5}	120	120	FG	5·0	3·0
	10^{-7}	60		DE	44·0	1·0
4	10^{-7}	60	150	—	4·3	2·2
	10^{-5}	60	150		2·1	4·6
	10^{-7}	60		DE	33·0	1·2
5	10^{-7}	60	150	—	18·0	2·2
	10^{-5}	60	210		6·2	4·0
	10^{-7}	60		FG	10·0	2·2
		5-Hydroxytryptamine				
6	2×10^{-9}	150	150	—	19·6	1·8
	2×10^{-9}	150	150	DE	28·0	1·6
	2×10^{-9}	150	150	DE	35·0	1·3
	2×10^{-9}	150		EF	55·0	0·9
7	5×10^{-8}	60	180	—	2·0	3·5
	5×10^{-8}	60	180	EF	10·5	2·3
	5×10^{-8}	60		EF	30·0	2·2
8	5×10^{-8}	45	195	—	14·6	1·0
	5×10^{-8}	45	195	FG	11·9	1·1
	5×10^{-8}	45	195	FG	16·2	0·9
	5×10^{-8}	90	150	N	33·0	0·7
	5×10^{-8}	45	240	EF	—	0
	5×10^{-8}	45	195	EF	4·0	1·5
	5×10^{-8}	45	150	FG	9·5	1·6
	5×10^{-8}	45		FG	13·5	1·7

We found that a reduction of the mechanical response to acetylcholine occurred most readily if a large dose was interposed in a series of smaller test doses. This was the condition described by Cantoni and Eastman (1946) and by Rand (1957). From our electrical records it appeared that, in order to observe this effect, the small dose had to be given during the period DE or near the time of maximal polarization. The fast rate at which the membrane repolarized after the removal of a large dose of acetylcholine delayed the response to a subsequent small dose considerably and reduced the mechanical response. (This is illus-

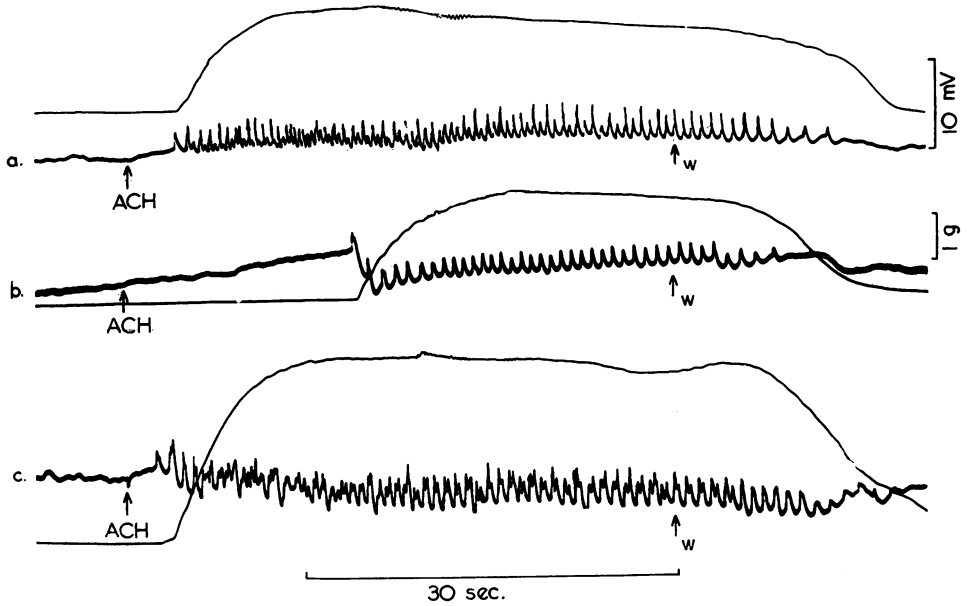

FIG. 5.—Tachyphylaxis produced by acetylcholine (ACH). Records as in Fig. 2*b*. Effects of acetylcholine 10^{-7}: (*a*) Control before exposure for 2 min. to acetylcholine 10^{-5}; (*b*) 1 min. after removal of the high concentration, when the membrane potential was 5 mV higher than in (*a*); and (*c*) 9 min. later when the membrane potential was 5 mV lower than in (*a*). Note longer lag in *b*, shorter lag in *c*. For description see text.

trated by Expts. 1 and 2 in Table II, and in Figs. 5 and 8.) On the other hand, the slow rate of repolarization following the removal of a weak concentration of acetylcholine might still prolong the lag of the next response, but the delay was insufficient to impair the production of tension. Fig. 5 shows three records from a series of responses to acetylcholine 10^{-7}: (*a*) is one of the controls obtained before, and (*b*) is the first response obtained after the removal of acetylcholine 10^{-5} at the time when the membrane was hyperpolarized with respect to the control. The lag was prolonged and the tension response was reduced; (*c*) is the fourth response obtained during a phase of the recovery oscillations when the membrane was depolarized with respect to the control. The lag was shortened and a greater tension was produced. This will be referred to later.

As the rate of depolarization due to histamine was slow, tachyphylaxis readily occurred even with repeated application of low doses, particularly if the exposure was somewhat longer or if the intervals between doses were short. For example, an interval of only 2 min. prolonged the lag while an interval of 3 min. shortened it (see Fig. 8*b*). Again tachyphylaxis was most severe when the test dose was applied during period DE, especially if a high concentration of histamine was interposed. This affected the responses differently according to the time of exposure and the interval until the test dose was applied, as shown by Expts. 3, 4, and 5 in Table II. Fig. 6 shows typical records of (*a*) the response to histamine 10^{-7} and (*b*) to histamine 10^{-5} applied for 1 min. After removal of the high concentration, while the membrane was rapidly repolarizing, the application of histamine 10^{-7} was repeated (*c*). Two spikes were seen, but further discharge stopped as the membrane continued to hyperpolarize. Indeed, a burst of spikes and the associated production of tension barely appeared before the histamine was due to be washed out. It can also be seen that, when the spikes finally appeared, the membrane polarization was still increasing, that is, it was moving in a direction which effectively counteracted depolarization and the response was diminished. This divorce of initiation of spike activity from membrane depolarization was characteristic of tachyphylaxis (it was especially pronounced with 5-hydroxytryptamine).

From the experiments described so far there appeared to be a clear relation between the point

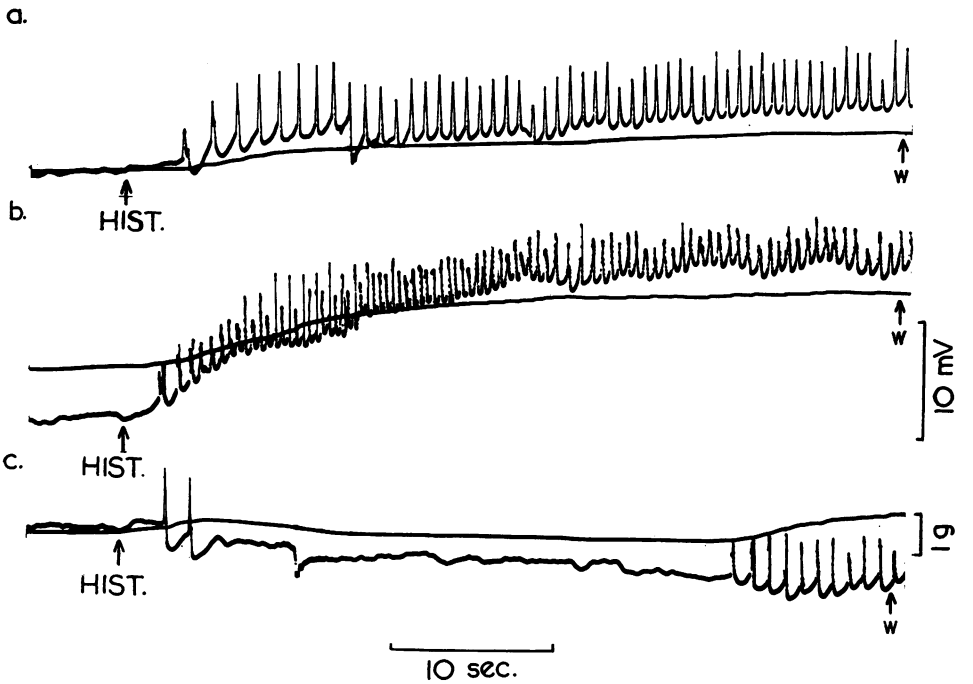

FIG. 6.—Tachyphylaxis produced by histamine. Records as in Fig. 2*b*. (*a*) Control 10^{-7}, (*b*) 10^{-5}, (*c*) 10^{-7}. The interval between the removal (*W*) and the next exposure to histamine was 2.5 min. each time.

during the recovery oscillations at which the application of a stimulating drug was repeated and the degree of tachyphylaxis which was observed. This relation held for 5-hydroxytryptamine only if low doses were applied for short periods. As the maximum response time for 5-hydroxytryptamine was, however, longer than 1 min., it had to be applied for longer periods if the full effect was to be obtained. It was then observed that tachyphylaxis occurred in almost every instance. An example is shown in Fig. 7. Three experiments in which different periods of exposure and different intervals between doses were used are shown in Table II. If the duration of application was longer, or the intervals shorter, or when stronger concentrations were used, the conditions became complicated. The degree of tachyphylaxis was no longer correlated with the membrane oscillations. It was also found that oscillations occurred already in the presence of 5-hydroxytryptamine and persisted, often accentuated, after its removal, so that it was impossible to decide whether successive doses produced an effect of their own or whether they merely coincided with the oscillation.

Potentiation

Potentiation by Repeated Application of the Same Drug.—In many experiments it was observed that the response to a weak concentration of acetylcholine, after having been impaired by a strong concentration, was subsequently potentiated. This is illustrated in Fig. 5*c*. The suppression of the first response after the strong concentration, as recorded in (*b*), effectively increased the interval between doses. Furthermore the response recorded in (*c*) occurred in the phase FG, when the membrane was depolarized. Another experiment of this kind is shown graphically in Fig. 8*a*, in which the depression of the response during the phase DE was followed later by a potentiation of the response during phase EF.

Potentiation occurred also with histamine when weak concentrations were applied repeatedly. Fig. 8*b* shows a graph of an experiment in which histamine 10^{-7} was used. It can be seen that the second dose, applied at E, produced a response with a prolonged lag but an unimpaired tension. The third dose, applied after a longer interval, so that it was introduced during the phase FG, had a shorter lag and produced a greater tension

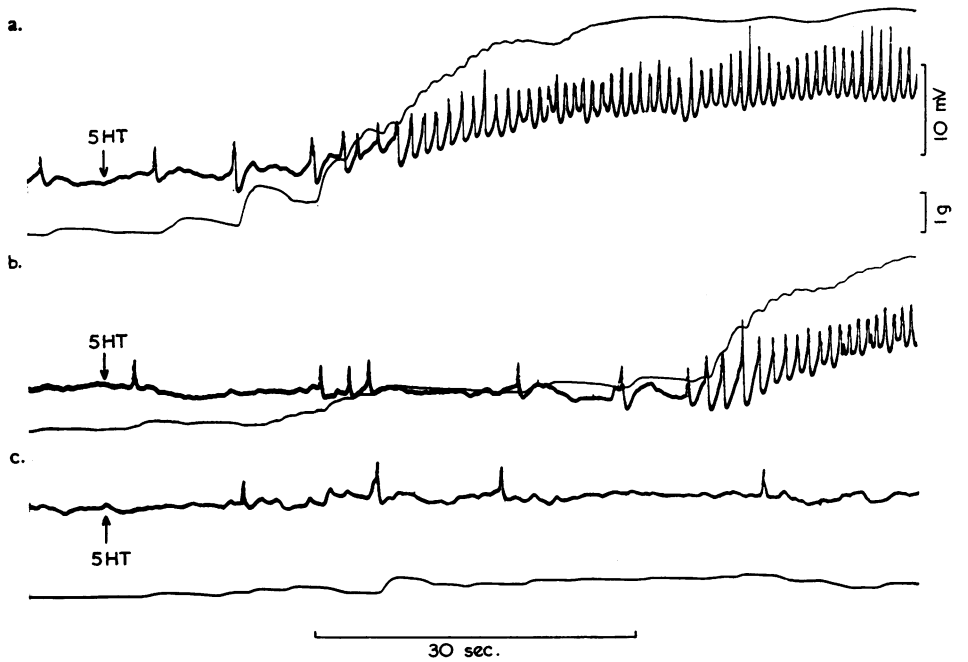

FIG. 7.—Tachyphylaxis produced by 5-hydroxytryptamine (5HT). Records as in Fig. 2*b*. Three successive applications of 5-hydroxytryptamine 5×10^{-8} applied for 2 min. every 4 min. Note the increased lag in (*b*) compared with (*a*) and the absence of any effect in (*c*).

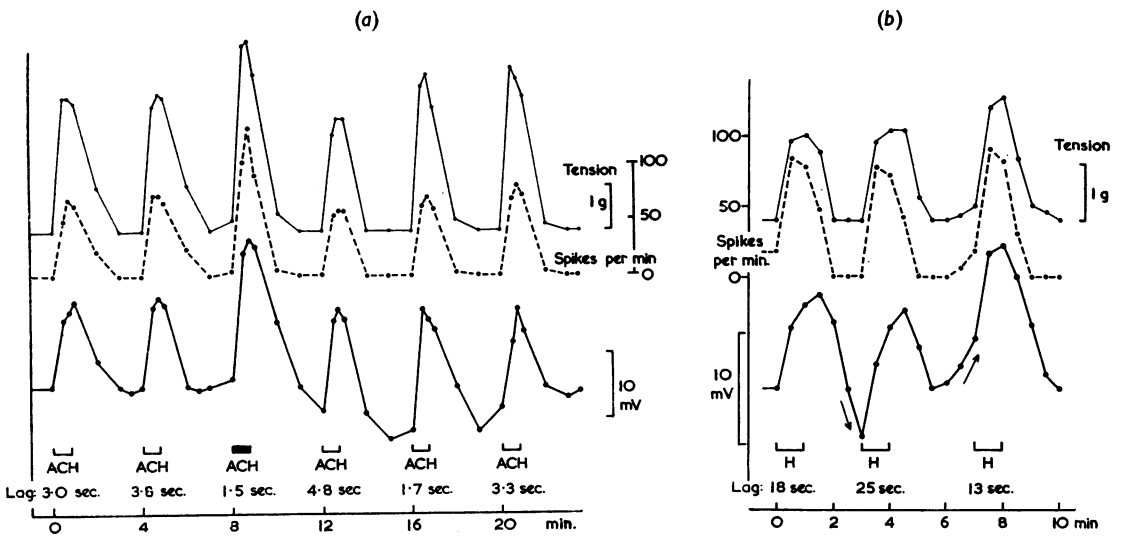

FIG. 8.—Potentiation following tachyphylaxis after repeated drug application. Ordinates: Tension (upper line); spike frequency (broken line); membrane potential (lower line). Abscissa: time in min. In (*a*) acetylcholine (ACH) 10^{-7} was given repeatedly, but once acetylcholine 10^{-5} (black bar) was interposed. In (*b*) the first interval between doses of histamine 10^{-7} was 2 min., the second interval 3 min. Note longer lag when drug was applied while membrane polarized, and shorter lag when drug was applied while membrane depolarized. For detailed description see text.

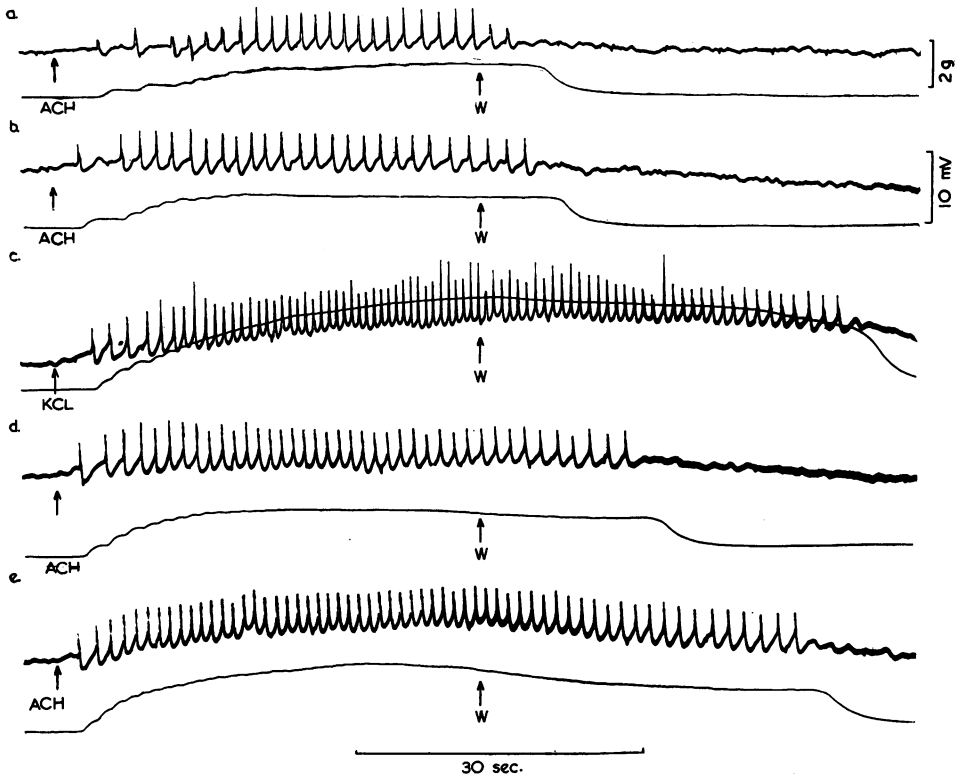

FIG. 9.—Potentiation of acetylcholine (ACH) effect after exposure to 20 mM potassium chloride (KCl). Records as in Fig. 2*b*. Doses were applied for 45 sec. every 4 min.; (*a*), (*b*), (*d*), and (*e*) acetylcholine 10^{-7}, (*c*) 20 mM potassium chloride. In (*d*) the membrane was depolarized by 5 mV, in (*e*) by 4 mV, with respect to the initial potential.

change. With 5-hydroxytryptamine we observed potentiation only in one experiment.

Potentiation after KCl.—Cantoni and Eastman (1946) observed that the interposition of a strong concentration of potassium chloride in a series of small doses of histamine or acetylcholine did not depress the response but enhanced it. It has been shown by Burnstock and Straub (1958) and by Holman (1958) that exposure to 20 mM potassium chloride caused a depolarization of 16 to 20 mV, and that after washing in normal solution the membrane potential slowly returned to the initial value over a period of several minutes, during which time there was no phase of hyperpolarization. We have applied acetylcholine or histamine during the recovery from exposure to potassium chloride while the membrane was still depolarized. We always found an augmentation of the response (Cantoni and

TABLE III

EFFECT OF 5-HYDROXYTRYPTAMINE ON THE RESPONSE TO ACETYLCHOLINE

Expt. No.	Conc. of Acetylcholine	Control		In the Presence of 5-Hydroxytryptamine (5×10^{-8})		1 min. after Removal of the 5-Hydroxytryptamine	
		Lag (sec.)	Tension (g.)	Lag (sec.)	Tension (g.)	Lag (sec.)	Tension (g.)
1	10^{-7}	16·2	1·1	4·0	2·7	—	
2	10^{-6}	4·7	2·9	0·5	4·6	—	
3	10^{-6}	12·4	1·5	—		0·7	1·8
4	10^{-6}	5·5	2·6	—		4·3	3·2

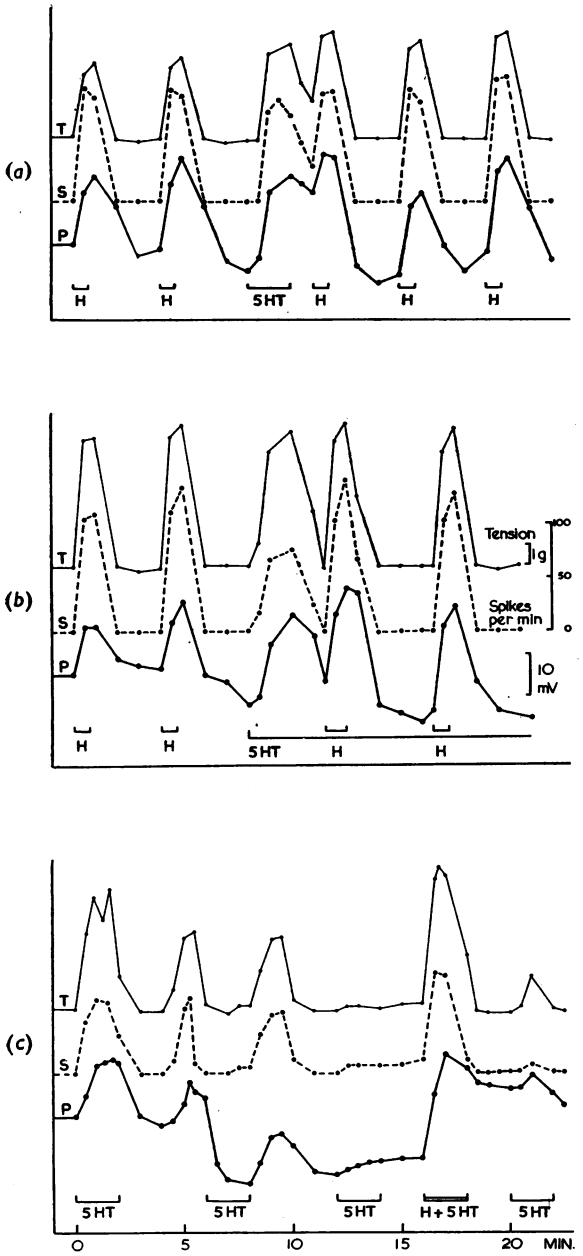

FIG. 10.—Potentiation of the effect of histamine (H) by 5-hydroxytryptamine (5HT). Symbols as in Fig. 8. The response to a constant dose of histamine (10^{-7}) was enhanced (*a*) by the interposition of a dose of 5×10^{-8} of 5-hydroxytryptamine. It was further enhanced (*b*) in the presence of 5-hydroxytryptamine, and still larger (*c*) though the muscle had been desensitized to 5-hydroxytryptamine.

Eastman, 1946; Rand, 1957). This augmentation was associated with a shorter lag. A typical experiment is shown in Fig. 9.

Potentiation of the Acetylcholine and Histamine Response by 5-Hydroxytryptamine.—The responses to acetylcholine and to histamine were potentiated by 5-hydroxytryptamine (Bülbring and Crema, 1958; Draškoci and Haranath, 1959). This potentiation was independent of the time of application during recovery from the 5-hydroxytryptamine, and was also seen in the presence of 5-hydroxytryptamine (see Table III).

Fig. 10 shows an experiment in which the potentiation of the response to histamine by 5-hydroxytryptamine was demonstrated in three different ways. In (*a*) the response to histamine was increased by interposition of 5-hydroxytryptamine. The potentiation was still evident 13 min. later (*b*) and was undiminished in the presence of 5-hydroxytryptamine. In (*c*) the preparation was desensitized to 5-hydroxytryptamine by repeated exposure. Yet, when histamine was applied together with 5-hydroxytryptamine, its effect was greater still.

DISCUSSION

The depolarization which occurs during exposure of intestinal smooth muscle to a stimulating drug is followed, after its removal, by a phase of hyperpolarization and then by a period during which the membrane potential fluctuates. This sequence of events had been noticed by Bülbring (1955) using intracellular recording, but in the present work it could be followed more accurately by continuous recording using the sucrose gap technique. Recent evidence (Burnstock, 1958a) suggested that the depolarization caused by acetylcholine (and possibly by other stimulating drugs) might be the result of a simultaneous increase in permeability to K^+, Na^+, and perhaps other ions. The observations of Durbin and Jenkinson (1959), who found that carbachol increased the permeability of the smooth muscle membrane to both potassium and chloride, are consistent with this view.

The events which follow during the recovery may be understood after considering some recent observations concerning the factors which determine the excitability of the taenia coli. It appears that the membrane permeability to sodium is much greater than that of other excitable tissues (Goodford and Hermansen, 1960). This may be

one of the reasons for the tendency to fire spontaneously and for the relatively low membrane potential. The rate of energy supply to the membrane would therefore be expected to be of great importance for the maintenance of membrane polarization, as energy is required for active ion transport (Hodgkin and Keynes, 1955) and probably also for the reduction of the inward leakage of sodium (Shanes, 1958). In taenia coli metabolic inhibitors cause depolarization and increase the spontaneous discharge (Born and Bülbring, 1955; Bülbring and Lüllmann, 1957) as well as excitability (Axelsson and Bülbring, unpublished observations). Removal of glucose from the medium or sudden cooling, for example from 37° to 26°, has the same effect (Axelsson and Bülbring, 1960a and b). On the other hand, a rise in temperature after a period of cooling, or the restoration of the normal glucose concentration after a period of glucose depletion, stabilizes the membrane. The cessation of spontaneous discharge, the loss of excitability and the hyperpolarization produced by a sudden increase of metabolic rate are phenomena which are also characteristic for the recovery after drug stimulation and, to a larger extent, for the effect produced by adrenaline. Axelsson, Bueding and Bülbring (1959) have recently shown that the inhibitory effect of adrenaline coincides with an activation of phosphorylase. This stimulation of one reaction in a chain of metabolic processes supplying energy is likely to have a causal connexion with the membrane stabilization. Born and Bülbring (1956) observed an increased rate of potassium uptake in the presence of adrenaline, which was similar to that observed after the removal of stimulating drugs. They concluded that adrenaline accelerated active processes in the cell membrane which operate also normally during recovery from activity. Burnstock (1958b) suggested that the hyperpolarization produced by adrenaline might be due to a transitory shift in the balance of ionic fluxes through the membrane involving an electrogenic extrusion of sodium. There is evidence (Bülbring and Goodford, unpublished observations) that adrenaline reduces the rate of uptake and increases the rate of loss of radioactive sodium in taenia coli.

In the experiments described in the present paper we observe a transient depression of excitability during those phases of the recovery period when the polarization of the membrane is increasing. We may assume that the loss of excitability is associated with an accelerated energy supply to the membrane causing an acceleration of the sodium pump and possibly a reduction of the inward leakage of sodium. However, as soon as the membrane becomes hyperpolarized—or rather when the potential approaches its true resting level—the rate of active processes would slow. This, with the relatively high membrane permeability to sodium, would lead to depolarization which, in turn, would provoke a repetition of the initial process. The oscillations of excitability and of membrane polarization can then be explained as being due to a periodically changing rate of active processes at the membrane affecting its stability and causing periodical shifts in the balance of ion fluxes. It may well be that also in normal conditions in which we find spontaneous fluctuations of the membrane potential a cycle of the type described operates, in which metabolic rate and excitability are linked (see Bozler, 1948).

The action of the three stimulating drugs, acetylcholine, histamine and 5-hydroxytryptamine, was studied by applying them for relatively short periods and at short intervals, that is, in conditions which might prevail during a biological assay. The magnitude of the response to repeated doses was found to depend on the time of application during the recovery oscillations of the membrane potential which followed the previous dose. During the first period of hyperpolarization, while the membrane potential was actually increasing (DE), tachyphylaxis was most pronounced. But, as a result of the differences in the rates of depolarization, which was fastest with acetylcholine, tachyphylaxis was less readily observed with small doses of acetylcholine than with histamine and 5-hydroxytryptamine. However, if a small dose of acetylcholine was given immediately after a large dose, tachyphylaxis was readily seen. In this condition the rate of repolarization following removal of a high concentration was equal or faster than the rate of depolarization caused by the weak concentration; and thus the response to the small dose was reduced. During the subsequent fluctuations, if the drug was applied while the membrane was hyperpolarizing, the lag was prolonged; but if it was applied during phases of depolarization, the response was facilitated, that is, the lag was shorter. The mechanical effect of acetylcholine and histamine was then potentiated.

From these observations it became evident that the excitability of the tissue by the stimulating drug was not determined by the absolute value of the membrane potential but by the direction into which the potential moved and by the rate at which this occurred at the moment when the

drug was applied. Furthermore, whenever the membrane was in the process of depolarization the threshold at which spikes appeared was at a much higher absolute level of membrane potential than that at which they stopped when the membrane was in the process of repolarization (Fig. 2*b*), indicating that there was no absolute firing level. Thus the degree of tachyphylaxis depended on the ratio between the rate of the spontaneous change in membrane potential and the rate at which the drug depolarized. As this rate was fastest with acetylcholine, slower with histamine, and slowest with 5-hydroxytryptamine, tachyphylaxis occurred least with acetylcholine and more with the other two drugs.

The observation by Cantoni and Eastman (1946) and by Rand (1957) that pretreatment with excess potassium chloride enhanced the response to subsequent doses of acetylcholine and histamine falls into line with our interpretation. As the recovery of the membrane potential after exposure to excess potassium chloride consisted of a simple, slow return to the initial level (Burnstock and Straub, 1958; Holman, 1958), the membrane was still depolarized when the next dose of acetylcholine was applied. The decreased lag was therefore not surprising and the response to acetylcholine was enhanced.

It is possible to interpret the observation by Hughes, McDowall and Soliman (1956) along the same lines. They found that tachyphylaxis depended on the external sodium concentration. If this was high there would be a larger increase of the internal sodium concentration during exposure to the stimulating drug. This would cause a greater acceleration of the sodium pump (Hodgkin and Keynes, 1956) and in the light of the present hypothesis favour tachyphylaxis.

Our observations suggest that 5-hydroxytryptamine, unlike acetylcholine and histamine, produced some more permanent alteration of the membrane properties. Whereas, with acetylcholine and histamine, the larger the concentration applied (up to a limit) the greater was the rate of depolarization produced, this was not so for 5-hydroxytryptamine, where increasing concentrations caused a progressively slower rate of depolarization. Tachyphylaxis often occurred when 5-hydroxytryptamine was introduced at a stage during which acetylcholine and histamine responses would have been potentiated.

The changes of the membrane properties produced by 5-hydroxytryptamine may be the result of absorption of the non-ionized form of the molecule on to the membrane, thereby impairing the depolarizing action of the ionized form. Another explanation for the ready occurrence of tachyphylaxis to 5-hydroxytryptamine is provided by the finding of Born (unpublished) that 5-hydroxytryptamine is taken up by the smooth muscle cells.

The action of acetylcholine and histamine was potentiated both after treatment and in the presence of 5-hydroxytryptamine (see also Bülbring and Crema, 1958; Draškoci and Haranath, 1959). That acetylcholine and histamine were still active when the muscle had been made insensitive to 5-hydroxytryptamine could be explained partly by the fact that both acetylcholine and histamine depolarize at a much faster rate than 5-hydroxytryptamine. Secondly, they are likely to act on different receptors (Gaddum, 1953). The potentiation is, however, difficult to explain. It might be due to the fixation of 5-hydroxytryptamine within the tissue leading to a change in membrane properties.

With the exception of the specific effects produced by 5-hydroxytryptamine, the results described are consistent with the hypothesis that the response obtained by a stimulating drug, applied for short periods in rapid succession, depends firstly on the rate at which it depolarizes. Secondly, the response depends on the state of the membrane and its excitability. This appears to be determined by the fluctuating rates of active ion transport and of metabolism. Experiments are in progress to test this hypothesis further.

Our thanks are due to the Medical Research Council for a grant to one of us (G. B.). We also wish to thank Mr. O. B. Saxby and Mr. D. Groves for their technical assistance.

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A COMPARATIVE STUDY OF HYDROXYINDOLE OXIDASES

BY

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(RECEIVED JULY 4, 1960)

A comparative study has been carried out of the oxidation of 5-hydroxytryptamine and related compounds by the oxidase present in the gill plates of *Mytilus edulis* and of caeruloplasmin, the copper containing oxidase of mammalian plasma. Both preparations oxidized indole derivatives carrying a hydroxyl group in the 4-, 5-, 6-, or 7- position. The oxidation of bufotenine was compared with that of its 4- and 6-hydroxy analogues; the 4-hydroxy analogue is psilocine, a naturally occurring hallucinogenic compound. Bufotenine and the 6-hydroxy analogue were oxidized by both preparations with the formation of brown pigments; psilocine was more rapidly oxidized with the appearance of a blue colour. 4-Hydroxytryptamine and 7-hydroxytryptamine were also oxidized, the former with the formation of a blue compound. The N-1-methyl derivatives of both bufotenine and psilocine were also oxidized. The *Mytilus* preparation acted also on 4-, 5-, and 7-hydroxytryptophan and on 5-hydroxyindole, none of which was oxidized by caeruloplasmin. The *Mytilus* enzyme also oxidized 5-hydroxyindoleacetic acid. Paraphenylenediamine, a very good substrate of caeruloplasmin, was much more slowly oxidized by the gill plate enzyme. The evidence suggests that the two enzymes catalyse the same reactions, but that the substrate specificity of the mammalian oxidase is somewhat more restricted. Both enzymes are " hydroxyindole oxidases," not specific for 5-hydroxyindoles alone. Inhibitors of the *Mytilus* oxidase included inhibitors of copper enzymes but not edetate or carbon monoxide. The action of pig serum on 5-hydroxytryptamine was due to caeruloplasmin and not to amine oxidase.

The oxidation of 5-hydroxytryptamine by amine oxidase and the subsequent formation of 5-hydroxyindoleacetic acid are reactions that play an important part in the inactivation of 5-hydroxytryptamine in mammals (see Udenfriend, 1958; Blaschko, 1958). However, it is also known that other pathways of breakdown of 5-hydroxytryptamine must exist. Erspamer (1955) has shown that in man and dog less than one-half of the amine administered is excreted as 5-hydroxyindoleacetic acid in the urine, and he has found that in a number of herbivorous species the urine contains only insignificant amounts of 5-hydroxyindoleacetic acid. It is not known to what extent this deficit is accounted for by known metabolites of 5-hydroxytryptamine, for example, the O-glucuronate and the O-sulphate, the N-acetyl derivative or 5-hydroxyindoleaceturic acid (Curzon, 1957; Chadwick and Wilkinson, 1958; McIsaac and Page, 1959).

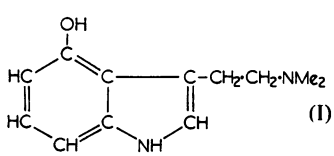
Little is known of the biological inactivation of 5-hydroxytryptamine in invertebrates. In molluscs, amine oxidase has been found in many species (Blaschko, Richter, and Schlossmann, 1937; Blaschko, 1941). Erspamer (1948) reported that enteramine was destroyed by the amine oxidase from Octopods, and after the identity of enteramine with 5-hydroxytryptamine had been established it was shown that the synthetic amine was oxidized by the molluscan enzyme (Blaschko, 1952a). In *Mytilus edulis* the digestive gland as well as the anterior retractor muscle of the byssus contain an amine oxidase that acts on 5-hydroxytryptamine (Blaschko and Hope, 1957).

In the gill plates of *Mytilus* there occurs another enzyme which oxidizes 5-hydroxytryptamine (Blaschko and Milton, 1959, 1960). This differs from amine oxidase. It acts also on other 5-hydroxyindoles, for example, 5-hydroxytryptophan and bufotenine. Unlike amine oxidase, this enzyme is cyanide-sensitive and it may be a kind of phenol oxidase, acting upon the indole ring with the formation of a quinone-imine, a reaction discussed some time ago (Blaschko and Philpot,

1953). Support for this formulation is seen in two observations: first, a brown pigment is formed in the enzymic reaction and, second, the gill plate preparation, even when somewhat purified, is still able to oxidize catechol derivatives, for example, dopa or dopamine.

It seemed probable that catalysts analogous to the gill plate enzyme occur in mammals. Caeruloplasmin, the copper containing protein of mammalian plasma, has been reported to act on 5-hydroxytryptamine (Porter, Titus, Sanders and Smith, 1957; Martin, Eriksen and Benditt, 1958; Nakajima and Thuillier, 1958; Siva Sankar, 1959; Zarafonetis and Kalas, 1960; Curzon and Vallet, 1960). Since caeruloplasmin is also able to oxidize catechol compounds and, as a copper enzyme, is cyanide-sensitive, it was decided to compare the action of the oxidase from *Mytilus* gill plates with that of pig plasma caeruloplasmin.

In order to obtain more information on the reaction catalysed by the two enzymes, the study has been extended to indole derivatives other than those carrying a hydroxyl group in position 5. The compounds tested were all hydroxyindoles. They include the 4-hydroxy and the 6-hydroxy analogue of bufotenine (I and II) and two *N*-methyl derivatives, that of bufotenine (III) and the corresponding 4-hydroxy compound (IV). In addition, 4-hydroxytryptamine, 7-hydroxytryptamine, 4-hydroxytryptophan, and 7-hydroxytryptophan were tested. The formula of 7-hydroxytryptamine is shown in (V).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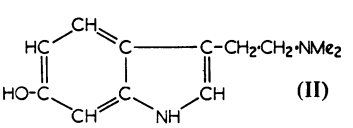
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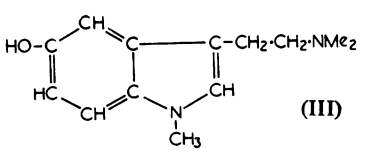
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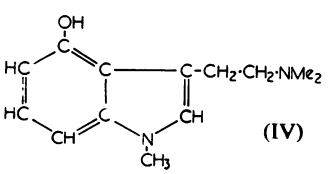
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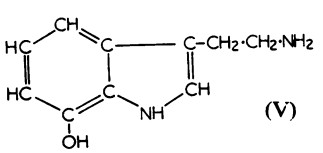

(V)

Of these compounds, substance (I) is known to be of pharmacological interest; it has been identified with psilocine, a hallucinogenic substance occurring in the fungus, *Psilocybe mexicana Heim* (Hofmann and Troxler, 1959; Hofmann, Heim, Brack, Kobel, Frey, Ott, Petrzilka and Troxler, 1959).

Methods

Enzyme Preparations.—The *Mytilus* gill plate preparations used were similar to those described by Blaschko and Milton (1960). Homogenates of the gill plates were prepared and the supernatant fluid, after centrifugation for 2 hr. at 5,000 *g*, was fractionated with ammonium sulphate, the material precipitating between 30 and 60% saturation being rich in oxidase activity. The precipitate was dissolved and dialysed first against running tap water and then in the cold room against distilled water. The dialysed preparation was freed from a slight precipitate by centrifugation for 30 min., at 100,000 *g*, and the supernatant fluid was used.

Caeruloplasmin was prepared from pig serum according to Holmberg and Laurell (1948). Material precipitating between 35 and 50% saturation with ammonium sulphate was collected and dialysed against running tap water overnight. Impurities were removed by two successive centrifugations, the first after adjusting the *p*H to 6.2 with 5% acetic acid, the second after adjustment to *p*H 5.5. The caeruloplasmin was then precipitated in 15% ethanol at 0°. The precipitate was resuspended in 0.9% sodium chloride, and after dialysis overnight the *p*H was adjusted to 6.5 and an equal volume of an ethanol-chloroform (9:1, v/v) mixture was added. The resulting precipitate was extracted with saline until the washings no longer had the blue colour characteristic of caeruloplasmin. The second ethanol-chloroform treatment used by Holmberg and Laurell (1948) was omitted. The extracts were combined and concentrated by precipitation with ammonium sulphate at 65% saturation. Dialysis against tap water overnight was followed by centrifugation for 30 min. at 5,000 *g*, in order to remove a turbid contaminant.

Measurement of Enzyme Activity. — Oxygen uptake was measured manometrically. The temperature of the manometer bath was 37.5°, with both the molluscan and mammalian preparations. The gas phase was air. The substrate concentrations were 5×10^{-3} M for the indole derivatives and 10^{-2} M for paraphenylenediamine. In the experiments with the *Mytilus* oxidase, 0.067 M sodium phosphate buffer of *p*H 7.4 was used. With caeruloplasmin, the *p*H of the buffer was 6.0; in the experiments with the indole derivatives 0.067 M sodium phosphate buffer was used, in experiments with paraphenylenediamine

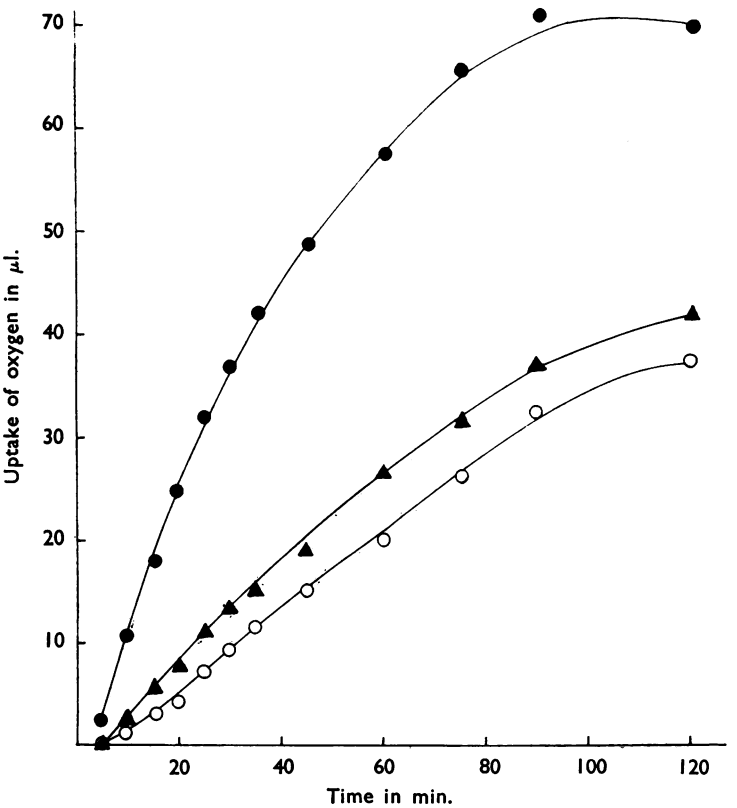


FIG. 1.—The rate of oxidation of bufotenine (O—O), psilocine (●—●), and 6-hydroxy-*N*-*N*-dimethyltryptamine (▲—▲) by *Mytilus* gill plate oxidase. The enzyme was incubated at 37.5° with 5×10^{-3} M substrate in phosphate buffer at *p*H 7.4. The gas phase was air.

0.01 M acetate buffer was used. The substrates were tipped in from the side arms of the conical manometer flasks.

Substances Used.—The 5-hydroxyindole was prepared by Dr. E. W. Gill, of this department, by catalytically debenzylating 5-benzyloxyindole (Ek and Witkop, 1954); it was purified by sublimation and recrystallization. The bufotenine was prepared by Dr. R. B. Barlow, of Edinburgh University; this was the sample used by Blaschko and Milton (1960). Compounds I, II, III and IV were given to us by Dr. A. Hofmann, of Sandoz A.-G., Basel.

Professor V. Erspamer, Parma, kindly gave us samples of 4-hydroxytryptamine creatinine sulphate, 7-hydroxytryptamine hydrochloride, 4-hydroxytryptophan, and 7-hydroxytryptophan; all these substances were prepared in the laboratories of Farmitalia, Milan.

We should like to record our gratitude for these valuable gifts.

RESULTS

Observations on the Mytilus Gill Plate Oxidase: Substrate Specificity

The observation that bufotenine is a substrate of the gill plate enzyme was readily confirmed. Bufotenine was therefore used together with the other indole derivatives. Fig. 1 shows an experiment in which the oxidation of bufotenine was compared with that of its 4-hydroxy and 6-hydroxy analogues. The first of these is psilocine. It can be seen that the rate of oxidation of the 6-hydroxy analogue was similar to that of bufotenine; psilocine was oxidized more rapidly. The experiment was terminated after 2 hr. when the uptake of oxygen with psilocine had ceased; with the 5- and 6-hydroxy analogues it still continued at a slow rate. The total uptake with psilocine was 70 μl. O_2; this

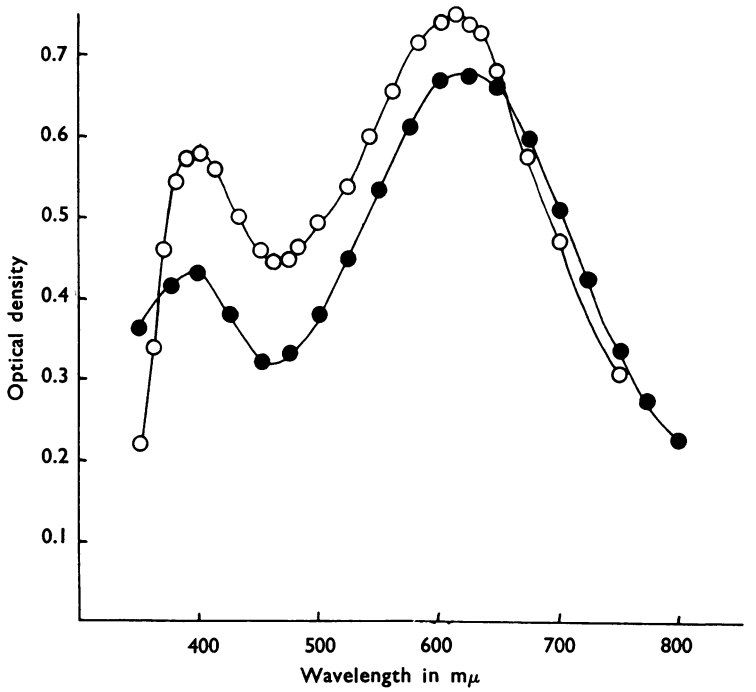


FIG. 2.—Absorption spectra of pigments formed in the oxidation of psilocine by the *Mytilus* enzyme (●—●) and by ferric sulphate (O—O).

represents 1.25 atoms of oxygen/mole of psilocine added. For 5-hydroxytryptamine, the total oxygen uptake is 0.9 atoms/mole of substrate (Blaschko and Milton, 1960).

During the oxidation of these three compounds, coloured products were formed. As observed by Blaschko and Milton (1960), the flask containing bufotenine acquired a brownish colour. With the 6-hydroxy analogue, a similar yellowish-brown colour developed. In the flask containing psilocine a deep blue colour became apparent. After centrifugation the blue pigment from psilocine remained in the supernatant fluid; any colour deposited with the sediment could be brought into solution by adding a few drops of N hydrochloric acid. The brown pigments formed from the other two isomers, on the other hand, remained entirely in the sediment and could not be similarly eluted.

The absorption spectrum of the blue pigment obtained from psilocine is shown in Fig. 2. The curve shows absorption maxima at 620 and at 400 mμ. That the blue material was an oxidation product was made likely by the observation that the colour was bleached upon adding sodium bisulphite, a reducing agent. Also, a blue compound was formed when a solution of psilocine in sodium phosphate buffer of *p*H 7.4 was oxidized by adding ferric sulphate. The time course of the development of the blue colour was followed spectrophotometrically by measuring the increase in absorption at 625 mμ. The reaction was complete within 20 min. The absorption was then measured throughout the visible range (see Fig. 2); the spectrum obtained was similar to that of the material formed when psilocine was oxidized by the gill plate enzyme, except for a shift of the absorption maximum at 620 mμ to 610 mμ and a relative increase of the peak at 400 mμ.

The *N*-methyl derivatives of both bufotenine and psilocine (III and IV) were also tested. They were also oxidized by the *Mytilus* oxidase. A comparison of the oxidation of the two *N*-methyl derivatives with that of the two parent compounds is shown in Fig. 3. Methylation of the ring nitrogen of psilocine depressed the initial rate of oxidation by 40%, but the rate of oxidation of *N*-methylbufotenine was about 30% higher than that of bufotenine. The oxidation product of *N*-methylpsilocine had a distinctly more greenish-blue hue, as compared to the deep

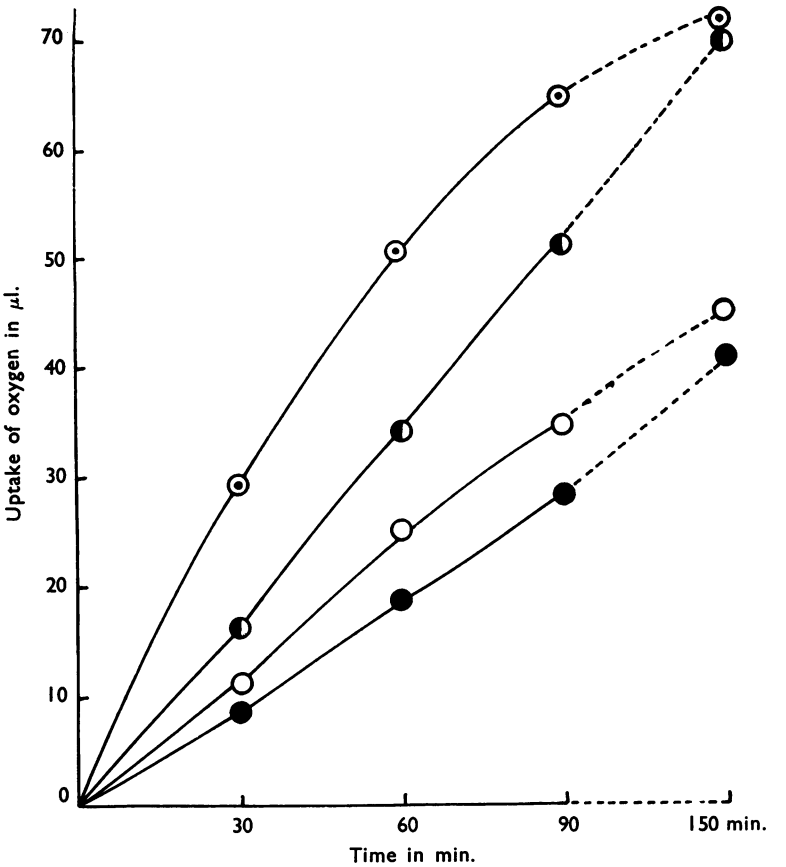


FIG. 3.—The rate of oxidation of bufotenine (●—●—●), psilocine (⊙—⊙), N-methylbufotenine (O—O), and N-methylpsilocine (◐—◐) by the *Mytilus* gill plate preparation. The enzyme was incubated at 37.5° with 5×10^{-3} M. Substrate in phosphate buffer at *p*H 7.4. The gas phase was air.

blue colour when psilocine was used as the substrate. This difference in colour is reflected in a difference in the absorption spectrum. The ratio, absorption at 400 mμ: absorption at 620 mμ, was increased from 0.685 for the oxidized psilocine to 1.14 for the oxidized N-1-methylpsilocine.

The gill plate preparation also oxidized 4- and 7-hydroxytryptamine as well as 4- and 7-hydroxytryptophan. In presence of one of the two 4-hydroxy compounds, oxygen uptake was rapid and was accompanied by appearance of a blue colour, similar to that of the oxidation product of psilocine. The oxidation of the two 7-hydroxy compounds was less rapid, being about equal to the rate of oxidation of 5-hydroxytryptamine; a brown pigment was formed when the 7-hydroxy compounds were oxidized.

In order to establish the role of the side chain in the enzymic oxidation, 5-hydroxyindole was also tested. It was oxidized, and its rate of oxidation was very similar to that of 5-hydroxytryptamine. However, in contrast to the brown pigment formed during the oxidation of 5-hydroxytryptamine, the product of oxidation of 5-hydroxyindole had a yellow-green colour.

Another hydroxyindole tested was 5-hydroxyindoleacetic acid. It was also rapidly oxidized by the gill plate enzyme, at about the same rate as 5-hydroxytryptamine. It is known that 5-hydroxyindoleacetic acid is oxidized by human caeruloplasmin (Curzon and Vallet, 1960).

Paraphenylenediamine was also oxidized by the gill plate preparation but at a rate only slightly greater than 5-hydroxytryptamine. This was in

sharp contrast to the more rapid rate of oxidation of paraphenylenediamine by caeruloplasmin.

Inhibitors of the Mytilus Oxidase

It has already been reported that the gill plate enzyme is cyanide-sensitive (Blaschko and Milton, 1960). Other substances that were tested as inhibitors of the oxidation of 5-hydroxytryptamine are listed in Table I. No inhibition was seen with

TABLE I

INHIBITORS OF THE HYDROXYINDOLE OXIDASE OF *MYTILUS* GILL PLATES

Substrate 5×10^{-3} M 5-hydroxytryptamine.

Inhibitor	Concentration	% Inhibition
Sodium diethyldithiocarbamate ..	10^{-3} M	47
Sodium azide ..	10^{-2} M	88
Salicylaldoxime ..	10^{-2} M	27
Diquinolyl ..	10^{-3} M	91
Edetate	10^{-2} M	0
Carbon monoxide	80% CO + 20% O_2	0

carbon monoxide and with 10^{-2} M edetate. It is of interest that not all copper containing enzymes are inhibited by carbon monoxide. Keilin and Mann (1939) found no inhibition of laccase; caeruloplasmin is also not inhibited (Holmberg and Laurell, 1951a).

Observations on Caeruloplasmin of Pig Plasma

Under the conditions of our experiments, 5-hydroxytryptamine was oxidized by the preparations of caeruloplasmin. Geller, Eiduson and Yuwiler (1959) did not see any oxidation of 5-hydroxytryptamine by caeruloplasmin, but observations of Porter *et al.* (1957) and other authors already quoted are in agreement with our findings. Using 10^{-2} M paraphenylenediamine as standard, the relative initial rate of oxidation of 5-hydroxytryptamine was one-sixtieth of that of paraphenylenediamine. It may be mentioned that this refers to experiments in which the 5-hydroxytryptamine was oxidized in phosphate buffer of *p*H 6.0; its rate of oxidation was less in acetate buffer of the same *p*H. The reverse is true for paraphenylenediamine: its oxidation is far more rapid in acetate than in phosphate buffer (Holmberg and Laurell, 1951b).

Oxidation of 5-hydroxytryptamine was accompanied by the appearance of a brown pigment, similar to what was seen when the amine was oxidized by the preparation from *Mytilus* gill plates.

The pig plasma preparation also oxidized bufotenine and its 6-hydroxy and 4-hydroxy analogues. Psilocine was oxidized more rapidly, bufotenine more slowly, than 5-hydroxytryptamine. The 6-hydroxy analogue was oxidized at about twice the rate of 5-hydroxytryptamine; this is different from what was observed with the *Mytilus* enzyme.

Brown pigments were formed from bufotenine and the 6-hydroxy analogue, but with psilocine a deep blue colour appeared during incubation; this is analogous to the observations with the *Mytilus* enzyme. Measurement of the absorption spectrum of the blue oxidation product showed the same two maxima seen with the *Mytilus* enzyme (see Fig. 2).

Neither 5-hydroxyindole nor 4-, 5- or 7-hydroxytryptamine was oxidized by caeruloplasmin. In order to ensure that the lack of oxidation of these amino-acids was not due to a difference in *p*H optimum, measurements with 5-hydroxytryptophan as substrate were carried out at *p*H 5.0, 6.0 and 7.4. The absence of oxidation of the amino-acids is in contrast to what is seen with the *Mytilus* enzyme. Thus, the nature of the side chain is of importance in determining whether or not an indole derivative is oxidized by caeruloplasmin.

Like the gill plate enzyme, caeruloplasmin readily attacked both 4- and 7-hydroxytryptamine. Again, a deep blue colour appeared during oxidation of the 4-hydroxy compound, and the reaction proceeded at a rate considerably more rapid than with 5-hydroxytryptamine. In contrast to the *Mytilus* preparation, caeruloplasmin oxidized 7-hydroxytryptamine at nearly 10 times the rate seen with 5-hydroxytryptamine; the pigment formed again had a dark brown colour.

Table II lists the initial rates of oxidation of all substances tested, in % of that of 5-hydroxytryptamine. The table contains the results obtained with the *Mytilus* enzyme as well as with the pig plasma preparation.

In the blood plasma of a number of mammalian species there occurs an amine oxidase that acts on benzylamine and other amines (Blaschko, Friedman, Hawes, and Nilsson, 1959); such an enzyme is found in pig plasma. Dialysed pig serum slowly oxidizes 5-hydroxytryptamine. In the work of Blaschko *et al.* (1959) it was assumed that the oxidation of 5-hydroxytryptamine was catalysed by the amine oxidase present. However, it has now been found that in the purification of

TABLE II

RELATIVE RATES OF OXIDATION OF DIFFERENT SUBSTRATES BY THE HYDROXYINDOLE OXIDASE OF *MYTILUS* GILL PLATES AND BY PIG PLASMA CAERULOPLASMIN

The initial rate of oxidation of 5-hydroxytryptamine is taken as 100.

Substrate	Hydroxyindole Oxidase from *Mytilus* Gill Plates	Pig Plasma Caeruloplasmin
4-Hydroxytryptamine	520	700
5-Hydroxytryptamine	100	100
7-Hydroxytryptamine	100	1,100
Psilocine	145	230
Bufotenine	60	44
6-Hydroxy analogue of bufotenine	55	170
N-Methyl psilocine	120	—
N-Methyl bufotenine	78	—
4-Hydroxytryptophan	200	0
5-Hydroxytryptophan	50	0
7-Hydroxytryptophan	100	0
5-Hydroxyindoleacetic acid	82	—
5-Hydroxyindole ..	100	0
Paraphenylenediamine	150	6,000

caeruloplasmin the ability to oxidize 5-hydroxytryptamine could be separated from the benzylamine oxidase action; activity with 5-hydroxytryptamine always ran parallel to the ability of the fractions to oxidize paraphenylenediamine. In other words, the experimental evidence, to be described in detail elsewhere, suggests that the oxidation of 5-hydroxytryptamine by pig serum is brought about by the caeruloplasmin present, not by the amine oxidase.

Attempts were also made to study the effect of 5-hydroxytryptamine on the oxidation of paraphenylenediamine by caeruloplasmin. However, it was noted that a rapid oxygen uptake occurred when 5-hydroxytryptamine was added to paraphenylenediamine in the absence of caeruloplasmin; at the same time a deep purple colour appeared in the flask. This observation made it impossible to find out if the enzymic oxidation of paraphenylenediamine was affected by 5-hydroxytryptamine.

DISCUSSION

The experiments described have shown many similarities between the two enzyme preparations studied, but there are also minor differences. In a preliminary note, in which the action of *Mytilus* oxidase on the derivatives of 4-hydroxy and 6-hydroxyindole was described, the name "hydroxyindole oxidase" was proposed for the gill plate enzyme (Blaschko and Levine, 1960), because oxidation was not restricted to indole derivatives carrying a hydroxyl group in the 5-position. We can now say that both the *Mytilus* enzyme and caeruloplasmin will act on derivatives of 4-, 5-, 6- and 7-hydroxyindoles. There are some differences in substrate specificity: the *Mytilus* oxidase acted also on 4-, 5- and 7-hydroxytryptophan and on 5-hydroxyindole, none of which was attacked by the pig plasma enzyme. One is left with the impression that the range of substrates of the mammalian oxidase is narrower, a difference from the *Mytilus* oxidase probably due to specific differences in the two enzyme proteins.

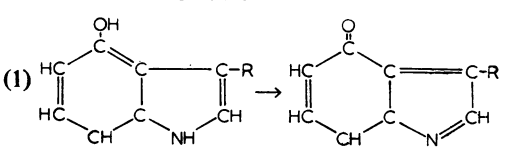
The reactions catalysed by the two enzymes lead to the formation of the same coloured products. The chemical composition of these compounds has not been studied, but the fact that a similar blue colour is obtained when psilocine is oxidized by ferric ions suggests that the reaction involves an oxidation at the phenolic hydroxyl group. The colour reaction of psilocine with Keller's reagent (Hofmann *et al.*, 1959) may be similarly explained.

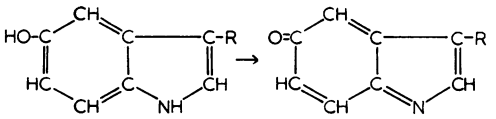
The possibility has already been discussed that the first attack of the *Mytilus* enzyme on the 5-hydroxyindoles results in the formation of a quinone-imine (Blaschko and Milton, 1960). It was therefore of interest to study the hydroxyindole derivatives which contain a phenolic hydroxyl in different positions of the indole nucleus. We have now found that 4-, 6- and 7-hydroxyindole derivatives are oxidized by the two oxidases.

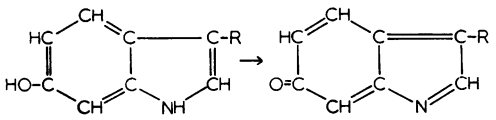
A quinone-imine could also be formed from 7-hydroxyindoles, since in these compounds the phenolic hydroxyl group is in position ortho to the indole nitrogen, but the question arises whether the oxidation of substances carrying a hydroxyl group in positions 4- or 6- can be reconciled with the formulation previously given.

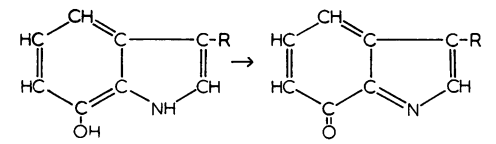
It is possible that the conjugated systems formed in the primary attack of the oxidases on 4- and 6-hydroxyindoles are different from those formed in the oxidation of the 5- and 7-hydroxyindoles. The reactions believed to occur are shown below:

(1)

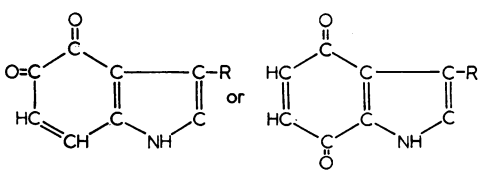


(2) 

(3) 

(4) 

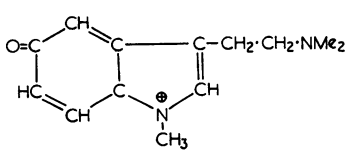
The formulation given for the 5-hydroxy compounds is that already discussed (Blaschko and Philpot, 1953; Blaschko and Milton, 1960). The oxidation of the 6-hydroxyindole derivative would also lead to a paraquinonoid structure, but the oxidation of the 4-hydroxyindoles would lead to an orthoquinonoid oxidation product. However, the reaction proposed under (1) is not the only one that has been considered. The blue oxidation product of psilocine may have a structure like this:



In this connexion it is of interest to note that Teuber and Staiger (1956) have prepared blue 4:5-diquinones by the action of potassium nitrosodisulphonate (Frémy's reagent) on hydroxyindoles.

The oxidation of 7-hydroxytryptamine (or of 7-hydroxytryptophan) would lead to the formation of an orthoquinone-imine.

The oxidation of the *N*-methyl derivatives of bufotenine and psilocine is of interest. If the formulation given under (2) is correct for bufotenine, the oxidation of the *N*-methyl derivative of bufotenine would be expected to lead to this product:



The structure of the oxidation product of *N*-methylpsilocine would probably be similar to the product of the enzymic oxidation of psilocine.

The evidence suggests that the mammalian and the molluscan enzymes catalyse the same reactions. Holmberg and Laurell (1951a) did not see any action of caeruloplasmin on monophenolic compounds. However, it has previously been pointed out that 5-hydroxytryptamine cannot be expected to behave like a typical monophenolic compound (Blaschko and Philpot, 1953; Blaschko and Milton, 1960); the presence of the indole nitrogen in position para to the phenolic hydroxyl group makes it comparable to a paradiphenol.

Caeruloplasmin is a copper-protein compound, and it is of interest that the properties of the molluscan oxidase are such that it too could be a metal-protein. This is an aspect that will have to await further purification of the *Mytilus* enzyme.

In *Psilocybe mexicana Heim* and related species of fungi, psilocine contributes only a small fraction of the total indole derivatives present (Hofmann and Troxler, 1959). Most of it is present as psilocybine, the phosphate ester of psilocine. Both psilocine and psilocybine are said to be hallucinogenic, but it seems worth while to consider the possibility that the phosphate ester exerts its central activity after being hydrolysed to psilocine and inorganic phosphate. At any rate, the biological inactivation of both substances may proceed *via* the free phenol.

This raises the question as to the physiological significance of the oxidation reactions here described. No precise answer to this question can at present be given. The catalytic action of caeruloplasmin on the catechol amines can easily be demonstrated *in vitro*, but it is still uncertain if such a reaction plays a part in their inactivation in the living animal. This has been fully discussed elsewhere (Blaschko, 1952b). The uncertainty arises from the fact that reducing agents, for example, ascorbic acid or sulphydryl compounds, immediately reduce the primary product of oxidation with regeneration of the free phenol. A similar mechanism might be operative in the oxidation of the hydroxyindoles. However, we do know that in both vertebrates and invertebrates oxidases of the type here studied are active on phenolic substances, in the formation of melanin. Enzymic processes of this kind may therefore occur in the living animal. It has been suggested that the indole derivatives might be possible pigment precursors in invertebrates (Blaschko and Milton, 1960), and similar considerations can be applied to the mammalian enzyme.

The oxidation of psilocine is so rapid that it is tempting to assume that the reaction studied is of importance in the biological inactivation of this compound. Whether the reactions here studied constitute an alternative pathway of catabolism of the 5-hydroxyindoles remains to be elucidated.

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ERRATUM

In the paper by D. Mackay and D. M. Sheppard in the December 1960 issue, page 553, line 20, " 30 ml." should read " 3 ml."