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THE ASSAY OF SUBSTANCES FROM THE ADRENAL MEDULLA

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Much recent work has been devoted to *nor*-adrenaline. There is evidence that this substance is present in extracts of adrenergic nerves (Euler, 1946, 1948) and liberated when the nerves to the cat's spleen are stimulated (Peart, 1949). The discovery by Holton (1949) that large amounts are present in extracts of adrenal medullary tumours directed attention to the adrenal glands themselves. The present investigation was undertaken with the object of applying the methods described by Gaddum, Peart, and Vogt (1949) to blood obtained from the adrenal veins. While it has been in progress evidence has been obtained by others that *nor*adrenaline is present in extracts of the adrenal glands (Holtz and Schumann, 1948; Euler and Hamberg, 1949; Goldenberg, Faber, Alston, and Chargaff, 1949; Tullar, 1949), and liberated from these glands when the splanchic nerves are stimulated (Bülbring and Burn, 1949). The results recorded below are in agreement with these conclusions.

METHODS

Collection of blood.—Cats were anaesthetized with chloralose (40 mg. per kg.) after preliminary ether. Blood pressure was recorded with a mercury manometer. Most of the cats were eviscerated. The nerves in the mesentery were stimulated after the mesenteric arteries had been tied, but while the portal vein was still open; this transferred blood into the rest of the cat, so that the blood pressure usually remained high in spite of the removal of samples for testing. The kidneys were removed or the renal vessels tied. The adrenolumbar veins were tied just peripherally to the adrenal glands. All other branches of the inferior vena cava below the liver were tied. Both the aorta and the inferior vena cava were tied below the renal vessels, and a cannula inserted in the vena cava just above the ligature. When the vena cava was closed by a clip near this cannula adrenal blood flowed to the heart. When this clip was moved to a point above the origin of the adrenolumbar veins, blood flowed out through the cannula and was collected.

Various precautions were taken to prevent clotting and the release of interfering substances. The cannulae were made unwettable with Teddol, obtained from the British Thomson-Houston Co. This was diluted to 5 per cent with CCl_4, and the cannulae were rinsed in this fluid and then washed with water. Centrifuge tubes were coated with paraffin wax and cooled in ice-water. Heparin (3,000 units per kg.) was injected into the cat, and 10 units per ml. of blood were placed in each centrifuge tube. Blood was run into these tubes and rapidly centrifuged. Pharmacological tests were applied to the plasma, which was kept in the refrigerator until tested.

Stimulation.—The splanchnic nerves were divided early in the experiment. They were stimulated with an alternating current (50 cycles, 5–10 volts).

Preparation of extracts.—At the end of some experiments extracts of glands were prepared. After careful dissection the glands were weighed, immersed in 10 ml. 0.15*N* HCl, ground with sand, and kept in the refrigerator. Shortly before the assay an aliquot of the supernatant fluid was diluted with saline and the *p*H was adjusted to 5 with solid $NaHCO_3$. After standing, this fluid was filtered and the filtrate used for the test, further dilutions being made if necessary with saline containing ascorbic acid (10^{-5}).

Assay.—Samples of plasma extracts were compared with solutions of synthetic *l*-adrenaline (B.D.H.) and *dl-nor*adrenaline hydrochloride (Sterling Winthrop). The results for *nor*adrenaline are expressed in terms of *l-nor*adrenaline, the activity of the *d-nor*adrenaline being neglected.

The method used was based on that of Jalon, Bayo, and Jalon (1945). Rat's uterus, or colon, is suspended in a solution of the following composition (g./l.): NaCl 9, KCl 0.42, $CaCl_2$ 0.06, $NaHCO_3$ 0.2, glucose 0.5 at 30° C. Contractions are produced every 2 min. by a choline ester, and the assay depends on the inhibition of these contractions by adrenaline or *nor*-adrenaline.

The addition of the choline ester was made automatically by means of the apparatus shown in Fig. 1; it is based on that of Schild (1946, 1947). The uterus or colon is suspended in a small bath (2 ml.). Contractions are produced at intervals of 125 sec. by replacing the solution in the bath by a similar solution

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containing carbachol. This substance is more suitable than acetylcholine for this purpose since it is stable even at *p*H 8 for days, whereas acetylcholine is hydrolysed rapidly in the reservoir. The carbachol solution is allowed to act for 30 sec. and is then replaced by the original solution. The concentration of carbachol is 0.5–1.0 μg. per ml. for the uterus and 0.05–0.1 μg. per ml. for the colon.

The changes are made in the following way. A cog wheel, which is rotated by clockwork, makes and breaks a contact every 5 sec. At each break a telephone uniselector moves on one place. At suitable intervals this makes contacts which activate telephone relays and these release the compression of rubber tubing and allow solutions to run in at the bottom of the organ bath. A capillary tube permanently connected with a filter pump sucks off excess fluid near the top of the bath, which is wider than the lower part. Each time that the contents of the bath are changed the fluid flows for 5 sec. The height of the reservoir (about 30–40 cm.) is adjusted so that the amount of fluid which flows in 5 sec. is about 5 times the volume of the bath.

When left to itself this apparatus produces a very uniform series of contractions. Solutions of adrenaline or other substances are added to the bath in a volume of 0.02–0.2 ml. when a signal lights up 1 min. before the inflow of carbachol. The adrenaline thus acts on the muscle for 1 min. and is not present in the bath when the carbachol is acting; this causes no serious loss in the sensitivity of the test. In the method used previously the addition of plasma to the bath was apt to cause a variable loss of fluid in froth, so that the addition of a constant amount of cholineester produced a variable concentration in the bath. With the method described here the concentration is constant, and the froth is washed away so that it does not interfere with the contraction.

It has been found convenient to conduct tests on the uterus and colon simultaneously with the arrangement shown in Fig. 1.

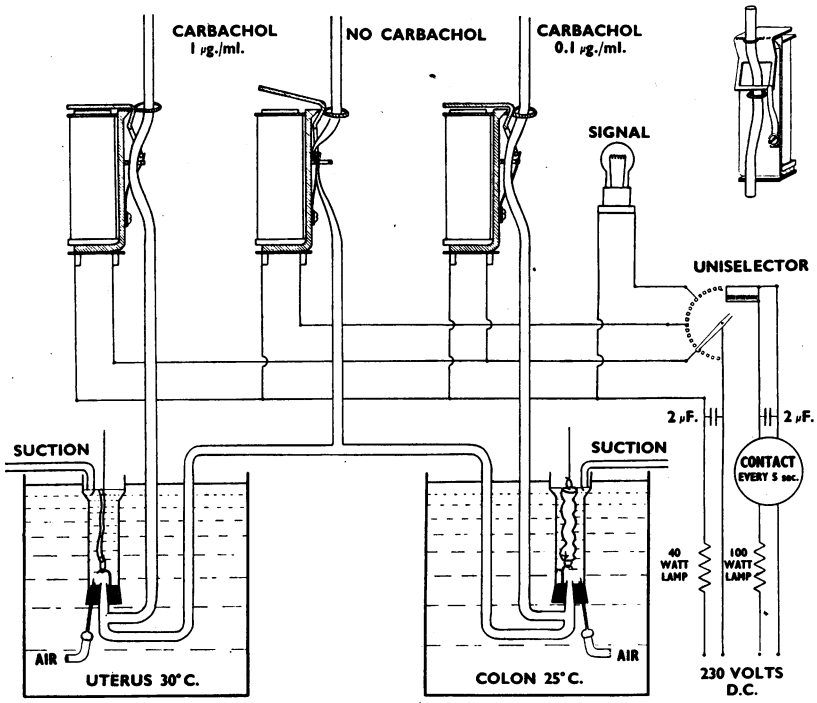

FIG. 1.—Automatic apparatus. Replaces fluid by carbachol solution for 30 sec. every 2 min.

Uteri from old rats often react slowly and it is sometimes desirable to alter the setting of the apparatus so that the carbachol is in the bath for more than 30 sec. Uteri from young rats (4–5 weeks) are generally preferable but may be too thin and weak. Satisfactory results have been obtained with such uteri by using both horns tied together, side by side.

RESULTS

In a number of experiments blood from the adrenal veins and extracts of glands were tested by the comparatively simple methods used by Gaddum, Peart, and Vogt (1949). It was easy to show in this way that stimulation of the splanchnic nerves, or the injection of acetylcholine, histamine, or KCl into the arteries, caused the release of large amounts of active substances into the plasma. When the plasma was compared with adrenaline by means of the uterus and colon the results were usually discrepant, and the discrepancy could usually be accounted for on the theory that *nor*adrenaline was present as well as adrenaline. Similar evidence was obtained with extracts, but none of this evidence was really convincing. When the percentage methylation of the mixture is small (i.e., when there is a large excess of *nor*adrenaline) the method works well, as it did in the experiments of Peart (1949). When methylation is more than 50 per cent, the method becomes inaccurate. These earlier results were considered

unsatisfactory and will not be presented in detail.

It became clear that better methods were needed, and various methods were tried. King (1949) has described a test for adrenaline in which the rat's uterus is bathed in a solution containing more than the usual amount of potassium. We have confirmed the fact that this provides a sensitive test which has the advantage of simplicity. On the other hand, it is slower than de Jalon's method, and did not appear to be more sensitive. In a small bath, solutions with a low potassium content inhibit the uterus even when they contain no adrenaline. It is not easy to avoid effects due to the potassium content of the solutions tested, and the use of this method was therefore abandoned.

The purpose of this paper is to describe experiments by de Jalon's method using the semi-automatic bath and statistics. The result depended on the measurement of the effects of two doses of the standard preparation (S_1 and S_2) and two doses of the unknown preparation (U_1 and U_2) and the use of the formulae proposed by Gaddum (1933). The application of this technique to isolated organs was first discussed by Schild (1942); our calculations of the errors were made by the simplified method described by Bliss (1944) and Noel (1945). The results are calculated from differences between effects obtained at about the same time and are thus independent of slow changes in the size of the response.

The validity of the calculations depends on the assumption that the effect is linearly related to the logarithm of the dose. Various methods of measuring the effect were tried. The response to adrenaline depends on the difference between the effect produced under its influence and the effect which would have been produced without it. An estimate of the latter quantity can be obtained by measuring on the drum the last contraction, before the addition of the adrenaline (or other substances) in each of a set of four doses. The average of these four figures is taken as the effect of carbachol alone during this time. The heights of the contractions in the presence of S and U are calculated as a percentage of this quantity. This method of measurement makes allowance for the fact that the response of the uterus to carbachol tends to diminish in the course of an hour or two. Fig. 2 shows the results of an experiment calculated in this way. It also shows the results of a similar experiment with the colon. Since the base-line is unstable with this organ the measurements were made from an artificial base-line fixed at an arbitrary level through the bottom of the tracing.

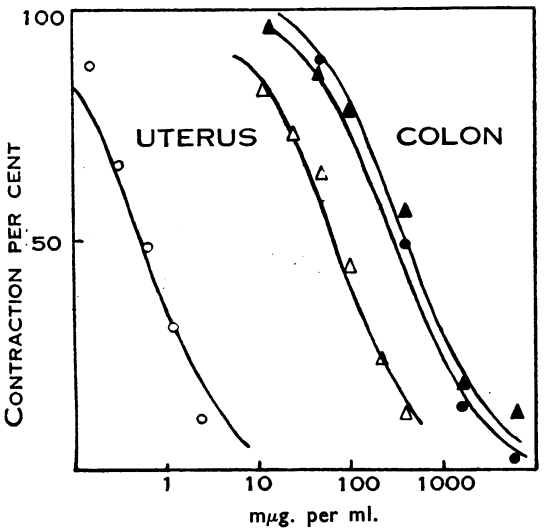

FIG. 2.—Vertical scale: contraction per cent, due to carbachol. Horizontal scale: concentration of drugs in bath. Circles: adrenaline. Triangles: *nor*adrenaline; white: rat's uterus; black: rat's colon. Theoretical curves (see text).

Clark (1937) has drawn attention to the fact that the relation between the dose of acetylcholine or adrenaline and some of their effects on plain muscle can be explained in terms of the mass laws. In the simplest case the curve connecting these quantities is a hyperbola, and the S-shaped curve connecting the effect with log dose has a characteristic slope. The curves drawn in Fig. 2 have been calculated on this theory. It will be seen that the observations fit the curves fairly well except for those showing the effect of adrenaline on the uterus, which would be better fitted by a steeper curve. The calculations might perhaps be based on the assumption that the points lie on

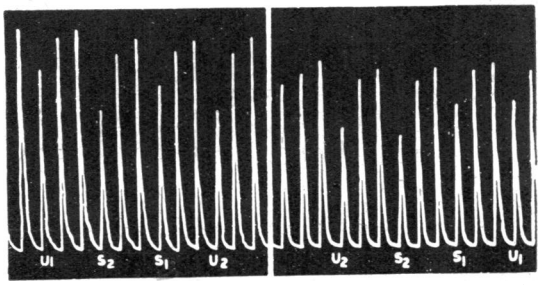

FIG. 3.—Rat's uterus. Exp. 50. Carbachol every 2 min. Effects of standard adrenaline (1 and 2 mμg.) and unknown solution. Ratio of doses $S_2/S_1 = U_2/U_1 = 2$.

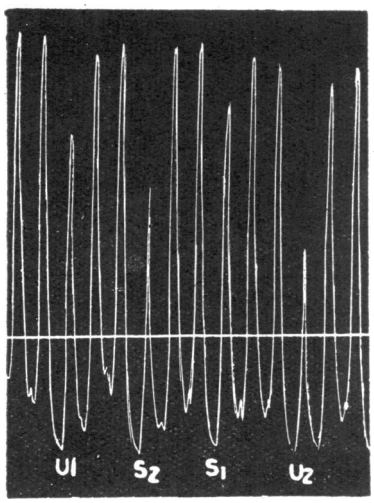

FIG. 4.—Rat's colon. As Fig. 3, but doses of adrenaline 400 and 800 mμg.

these curves, but in the present state of knowledge it seems safer to adopt the simpler approximation that the curves are straight lines; this seems to be nearly enough true for effects between 20 and 80 per cent. It is fortunate that the curves for adrenaline and *nor*adrenaline have about the same slope. If this had not been so, the results would have been very difficult to interpret.

TABLE I

EFFECTS ON UTERUS (EXP. 50)

Sets	Doses	Contraction before *U* or *S*		Contraction after *U* or *S*	
		mm.	Mean	mm.	Per cent of mean
1	U_1	74		60	84
	S_2	73		46	64
	S_1	70		54	76
	U_2	69	71.5	46	64
2	U_2	70		46	70
	U_1	65		54	82
	S_2	66		44	67
	S_1	63	66	50	76
3	U_2	62		41	67
	U_1	61		50	82
	S_1	61		46	75
	S_2	61	61	37	61
4	U_2	62		39	65
	S_2	59		37	62
	S_1	59		47	78
	U_1	60	60	48	80

Less elaborate methods of measuring the effect can be used in routine tests. The results with the uterus discussed below were actually calculated as percentages, but direct calculations from the heights of the records in mm. would probably be equally satisfactory, since the method of calcula-

TABLE II

CALCULATIONS FOR ASSAY ON UTERUS (EXP. 50)

N =	4 Sets of results	1	2	3	4
S_1 }	Effects of standard	76	76	75	78
S_2 }		64	67	61	62
U_1 }	Effects of unknown	84	82	82	80
U_2 }		64	70	67	65
D_1	U_2-S_2	0	3	6	3
D_2	U_1-S_1	8	6	7	2
D_3	U_2-U_1	−20	−12	−15	−15
D_4	S_2-S_1	−12	−9	−14	−16
y_1	D_1+D_2	8	9	13	5
y_2	D_3+D_4	−32	−21	−29	−31
y_3	D_1-D_2	−8	−3	−1	1

T_1	$S(y_1)$	Sample difference	35
T_2	$S(y_2)$	Dose difference	−113
T_3	$S(y_3)$	Slope difference	−11
I	Log ratio of doses	Log 2	0.3010
M	T_1I/T_2	Log potency of U/S	−0.09323
R	100 antilog M ...	Potency of U (% of S)	80.68
b	$T_2/2IN$	Slope	−46.927
A	$S(y_1)^2+S(y_2)^2+S(y_3)^2$	—	3,681
B	$S(T_1^2+T_2^2+T_3^2)$	—	14,115
s	$\sqrt{(A-B/N)/12(N-1)}$	S.D. of effect	2.0566
λ	s/b	S.D. of log dose	0.04383
λ_M	$\lambda\sqrt{(1+T_1^2/T_2^2)/N}$ (1-antilog λ_M)R or 2.303 λ_M R	S.D. of M	0.02294
		S.D. of R	4.3
t	$T_3/2s\sqrt{N}$	—	1.34

tion used is designed to eliminate errors due to a slow change in the response of the tissue. In the experiments with the colon the calculations actually were made directly from measurements of the height of the response above an artificial base-line. For this purpose, it is unnecessary that this base-line should be at the bottom of the record and it was placed at any convenient height (cf. Fig. 4). The response of the colon to carbachol was well maintained for six to eight hours, though there was a tendency for the muscle to lengthen gradually. The effect of this change is eliminated by the method of calculation.

TABLE III

ESTIMATES OF ERROR

Exp.	Uterus			Colon		
	λ	λ_M	Coefficient of variation per cent	λ	λ_M	Coefficient of variation per cent
40x	0.0778	0.0403	9.7	0.145	0.0744	18.7
40y	0.0425	0.0217	5.2	0.112	0.0572	14.1
48	0.0615	0.0540	11.3	0.179	0.0950	24.5
49	0.0597	0.0314	7.5	0.101	0.0505	12.3
51	0.0519	0.0251	5.9	0.093	0.0470	11.4
50	0.0438	0.0229	5.4	0.219	0.1125	29.6
52	0.0447	0.0270	6.4	0.0717	0.0423	10.3
53(2)	0.0653	0.0382	9.1	0.0529	0.0265	6.3
53(3)	0.1097	0.0567	14.0	0.164	0.0864	22.0
Mean	0.06188	0.03527	8.3	0.1264	0.0058	16.6

The appropriate doses are chosen as the result of preliminary tests. In experiments with the uterus the ratio of the larger doses (S_2 and U_2) to the smaller doses (S_1 and U_1) is 2; with the colon this ratio is 2 or 4. These doses are given in random order; this whole set of results is repeated four times using different random orders of doses. The paper is then varnished, and the contractions measured and the calculations made. Fig. 3 shows the first and last sets of results in experiment 50 with the uterus. Fig. 4 shows one of the four sets of results in experiment 50 with the colon.

Table I shows the measurements made from the tracing shown in Fig. 3 and the calculation of the percentage effect, which is shown in the last column.

Table II shows the calculation of the result of an assay and its error. In this assay the potency of *U* was estimated as 80.68 ± 4.3 per cent of that of *S*. The quantity *t* is used to calculate whether the slopes of the two curves are significantly different. A standard table of *t* shows that the value obtained here corresponds to a probability between 0.2 and 0.3, when there are $3(N-1)$ or 9 degrees of freedom. There is thus no evidence that the lines are not parallel.

Table III shows estimates of the error in 9 assays on uterus and colon. The quantity λ gives the best measure of the general accuracy of the test, being independent of the number of sets of results. The mean values were about 0.062 for the uterus and 0.126 for the colon. These figures may be compared with 0.043 for the assay of histamine on guinea-pig's ileum (Schild, 1942) and 0.033 for the assay of adrenaline on dog's blood pressure (Noel, 1945). The standard error of the actual assays was about 8.3 per cent for the uterus and twice as large for the colon.

The test for parallelism of the dose-effect curves was made in all 18 assays. *P* was greater than 0.05 in every assay except one, and in that one it was only slightly less. There was thus no evidence of lack of parallelism.

The calculation of concentrations in mixtures

If a solution containing a mixture of adrenaline and *nor*adrenaline is assayed against pure solutions of the two drugs using both uterus and colon, the four results may be used to provide estimates of the concentrations in the mixture, if it is assumed that the effects of the two drugs are simply additive and that no other active substances are present. The logical basis of the formulae used for this purpose is given below. Similar formulae have been devised independently by Euler (1950) and Bülbring (1949).

Let *Au*, *Ac*, *Nu*, and *Nc* denote the adrenaline-equivalents and the *nor*adrenaline-equivalents, on the uterus and colon respectively, as determined by assay, and let *A* and *N* denote the actual concentrations of the two drugs.

Let *Ru* and *Rc* denote the ratios of doses of *nor*-adrenaline to the equivalent doses of adrenaline, so that $Ru = Nu/Au$ and $Rc = Nc/Ac$.

The adrenaline equivalent of the mixture should be equal to the sum of the adrenaline equivalents

of the two drugs present. We thus have the two equations

$$Au = A + \frac{N}{Ru} \qquad Ac = A + \frac{N}{Rc}$$

Whence $N = RcRu\,\frac{(Ac - Au)}{(Ru - Rc)}$

and $A = \frac{AuRu - AcRc}{Ru - Rc}$

Actually Rc is negligibly small compared with Ru, therefore

$$N = (Ac - Au)\ Rc \qquad (1)$$

and $$A = Au - Ac\frac{Rc}{Ru} \qquad (2)$$

When the percentage methylation is high it is best to use adrenaline as the main standard preparation, and the equations are given above in a form which is convenient when this is done.

The evidence that *nor*adrenaline is present in the mixture depends on the difference between the two estimates of the adrenaline equivalent (Ac and Au) and is only significant when these estimates are significantly different from one another. When this is so, formula (1) gives a convenient estimate of the concentration of *nor*adrenaline. The result with the uterus (Au) can sometimes be taken as a direct estimate of the concentration of adrenaline; equation (2) provides a correction which is negligible when the percentage methylation is high, but becomes important when this percentage falls below 20.

When the percentage methylation falls below 10, it is theoretically better to use *nor*adrenaline as the standard preparation and the following equations will then be more convenient. They are really the same as those given above.

$$A = \frac{(Nu - Nc)}{Ru} \qquad (3)$$

$$N = Nc - Nu\frac{Rc}{Ru} \qquad (4)$$

The discussion on equations (1) and (2) also applies *mutatis mutandis* to equations (3) and (4).

Table IV shows the results by this technique with known mixtures of adrenaline and *nor*adrenaline. All these assays were made by the statistical procedure outlined above, and their errors have already been discussed. It will be seen that the calculations give estimates of the concentrations of adrenaline and *nor*adrenaline in the mixture which are likely to be accurate enough for some purposes.

Table V shows the results of the application of this method to extracts of adrenal glands and to blood obtained from the adrenal veins during

TABLE IV

ASSAYS OF KNOWN MIXTURES

No. of experiment:	40x	40y	48	49	51
Adrenaline equivalent:					
Uterus	240	432	1,438	2,234	2,030
Colon	1,177	1,160	6,508	4,860	3,796
Ratio (*Nor.*/Adr.)					
Uterus (*Ru*) ...	80	80	500	37	180
Colon (*Rc*) ...	1	1	0.5	0.73	1
Adrenaline concentration:					
Calculated ...	225	417	1,431	2,138	2,009
Actual	200	500	2,000	2,000	2,000
Error per cent ...	+12.5	−16.6	−28	+11	+0.4
*Nor*adrenaline concentration:					
Calculated ...	937	728	2,530	1,917	1,766
Actual	800	500	2,000	2,000	2,000
Error per cent ...	+17.1	+45.6	+26.5	−4.1	−11.7
Total amine:					
Calculated ...	1,162	1,145	3,961	4,055	3,775
Actual	1,000	1,000	4,000	4,000	4,000
Error per cent ...	+16.2	+14.5	−1	+1.4	−5.4
Methylation per cent					
Calculated ...	19	36	36	53	53
Actual	20	50	50	50	50

stimulation of the splanchnic nerves. In all these assays the estimated percentage of methylation was high, and it was necessary to test whether the results could be accounted for on the theory that adrenaline was the only active substance present. The evidence against this theory lies in the fact that $Ac > Au$. The fourth line gives estimates of the probability that the observed differences would occur by chance owing to the error of the test. The method by which these figures were calculated may be illustrated by an example. In experiment 50 the difference between the logarithms of the two estimates = log 938/556 = 0.2201. The standard errors of the logarithms of the two estimates are 0.0229 and 0.1125 (Table III). The standard error of 0.2201 is thus $\sqrt{(0.0229)^2 + (0.1125)^2} = 0.1148$. Therefore $t = 0.2201/0.1148 = 1.92$, and P lies between 0.05 and 0.1 (9 degrees of freedom). This experiment thus provides no significant evidence of the presence of any active substance in the solution besides adrenaline. The error of the test on the colon was unusually large in this assay. On the other hand, experiment 52 provides significant evidence of the presence of some other substance in the extract of

TABLE V

ESTIMATES OF CONCENTRATIONS IN ADRENAL GLANDS AND ADRENAL VENOUS PLASMA

	Adrenal glands μg. per g.		Plasma μg. per ml.	
No. of experiment:	50	52	53(2)	53(3)
Adrenaline equivalent:				
Uterus	556	618	2.62	1.66
Colon	938	877	4.78	2.32
Probability of difference ...	0.05–0.1	ca 0.01	<0.001	0.1–0.2
Ratio (*Nor.*/Adr.):				
Uterus (R_u) ...	180	150	75	75
Colon (R_c) ...	0.21	0.9	0.33	0.43
Calculated concentration:				
Adrenaline ...	555	613	2.61	1.65
*Nor*adrenaline ...	80	236	0.71	0.28
Methylation per cent	87	72	78.5	85.5

the gland. It would clearly not be possible to identify this substance in a mixture by tests of this kind, but, if it is justifiable to assume that it is *nor*adrenaline, then the results give an estimate of the amount present.

In experiment 53 a control sample of plasma from the adrenal veins was roughly equivalent on the uterus to 0.005 μg. of adrenaline per ml. On stimulation of the splanchnic nerves, the figure rose immediately to 2.62 μg. per ml. (sample 2). Stimulation was continued without intermission for 64 min. and sample 3 was collected during the last 16 min. of this time. The results of assays on these two samples are shown in Table V. In sample 2 there was significant evidence that adrenaline was not the only active substance present. The results with sample 3 were similar, but the evidence was not significant; such results might occur with adrenaline alone. The percentage methylation appeared to rise slightly. There was no evidence in this experiment of a loss of the adrenals' powers of methylation even when the nerves were continuously stimulated for over 45 min.

Discussion

Straightforward pharmacological methods can be used to identify adrenaline or *nor*adrenaline when either is present alone or in overwhelming excess. When the percentage methylation of a mixture of these substances is 10 or more, *Au* gives a direct estimate of *A*; when it is 10 or less, *Nc* gives a direct estimate of *N*. When the percentage is about 10 both these quantities can thus be estimated directly with fair accuracy. In other cases, more complicated methods must be used. If there is doubt about the nature of the active substances present in the mixture the methods described here are not well adapted for their identification. If evidence from other sources justifies the assumption that these substances are adrenaline and *nor*adrenaline the formulae give some indication of the percentage methylation.

The presence of *nor*adrenaline in extracts of adrenals is already proved, and the percentage methylation has been estimated by chemical methods. The pharmacological methods have the advantage of sensitivity and may be useful in problems where the concentrations are low. The fact that the methods described here have given similar results to the chemical methods when applied to extracts is evidence in favour of their value when applied to lower concentrations.

The results of experiment 53 show that adrenaline was not the only substance liberated by the nerves. Acetylcholine is known to be liberated, but would be hydrolysed in the conditions of these experiments and would not produce the observed effects. It would either cause a contraction of the colon within a few seconds of being added to the bath or have no effect at all. Histamine is unlikely to be the cause of the discrepancy, since it is comparatively inactive in these tests. The release of *nor*adrenaline is the most likely explanation of this result, which thus confirms the conclusions of Bülbring and Burn (1949). It is, however, clearly desirable to separate the two substances before tests are made; a method of doing this is in course of development.

Summary

1. The concentrations of both adrenaline and *nor*adrenaline in a mixture of these drugs can be roughly determined by parallel quantitative assays on the rat's uterus and colon.

2. The results may be misleading unless they are analysed statistically.

3. Extracts of cats' adrenals and plasma collected from the cat's adrenal veins during stimulation of the splanchnic nerves both contain some other substance besides adrenaline. The results confirm the view that this is *nor*adrenaline and give an estimate of the amount.

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