

A COMPARATIVE STUDY OF 6-SELENOPURINE AND 6-MERCAPTOPURINE IN THE *LACTOBACILLUS CASEI* AND EHRlich ASCITES TUMOR SYSTEMS*

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Abstract—6-Selenopurine has antimicrobial activity exceeding that of 6-mercaptopurine. In an Ehrlich ascites tumor cell system selenopurine, like mercaptopurine, inhibits the incorporation of formate into guanine and adenine, but not into thymine, and (except at high levels) does not inhibit the uptake of adenine.

RECENTLY, 6-selenopurine was synthesized¹ as a member of a series of selenium analogs of various sulfur compounds with physiological activity. Selenopurine inhibits the growth of the mouse lymphoma derived from leukemia L-1210 to the same extent as does 6-mercaptopurine,² the latter being a clinically useful antileukemic compound.^{3, 4} Against two other mouse tumors, selenopurine exerted less activity than did its sulfur analog. Since the selenium compound was considerably less stable than mercaptopurine, the half-life of the former agent being only six hours in phosphate buffer (pH 7 or 8) at 37°C (under these conditions 6-mercaptopurine exhibited no degradation), it seemed likely that much lower blood levels were achieved with selenopurine than with equimolar amounts of mercaptopurine.

The observation that equimolar amounts of these two compounds yielded equal effects against at least one tumor implied that the intrinsic activity of selenopurine

TABLE 1. MINIMUM INHIBITORY CONCENTRATIONS OF 6-SELENOPURINE AND 6-MERCAPTOPURINE

Organism	Mercaptopurine μg/ml	Selenopurine μg/ml
<i>Micrococcus pyogenes</i> (var. aureus)	20	0.4
<i>Klebsiella pneumoniae</i>	> 50	20
<i>Bacille Calmette Guérin</i>	50	3
<i>Aspergillus fumigatus</i>	> 100	100
<i>Botrytis cinerea</i>	> 100	1.6
<i>Candida albicans</i>	> 100	> 100
<i>Microsporium audouini</i>	> 100	25
<i>Saccharomyces cerevisiae</i>	> 100	> 100
<i>Trichophyton mentagrophytes</i>	> 100	25
<i>Fusarium bulbigenum</i>	> 100	25
<i>Ceratostomella ulmi</i>	> 100	25

* This material was presented before the Division of Biological Chemistry at the 133 Meeting of the American Chemical Society, April 1958, San Francisco, California. The work was supported, in part, by a grant (C-2817) from the National Cancer Institute, U.S. Public Health Service.

must be very high, and suggested further comparisons of these compounds in other biological systems.

When the inhibitory activity of selenopurine on the growth of a fairly wide range of micro-organisms was tested, the substance proved to be considerably more active than did the corresponding thio compound. A similar relationship had been found previously in comparing the antifungal actions of seleno- and thio-semicarbazones and ureas.⁵

Mercaptopurine and selenopurine were examined particularly with respect to their relative ability to inhibit the growth of *Lactobacillus casei* (ATCC-7469) an organism widely used in assaying "antipurines" and in gathering clues regarding their mechanism of action.⁶ Selenopurine is an inhibitor of the growth of *L. casei* whether the organism is grown on optimal amounts of folic acid, adenine, hypoxanthine, or guanine (Table 2).

TABLE 2. CONCENTRATIONS OF 6-SELENOPURINE AND 6-MERCAPTOPURINE REQUIRED TO INHIBIT BY 50 PER CENT THE GROWTH OF *Lactobacillus casei* (ATCC-7469)

Growth factor* ($\mu\text{g/ml}$)	Mercaptopurine $\mu\text{g/ml}$	Selenopurine $\mu\text{g/ml}$
Folic acid (0.002)	93	9
Adenine Sulfate (20.0) + Thymine (1)	460	10
Hypoxanthine (5.4) + Thymine (1)	82	9
Guanine · HCl (15) + Thymine (1)	32	9

* The amounts of growth factor used were those giving rise to optimal growth, as determined by means of growth curves.

Reversal of the inhibition caused by either compound could be obtained with adenine, hypoxanthine, guanine, or xanthine; on the basis of previous work with mercaptopurine⁷ this result would have been expected. Partial reversal also was seen with glutathione, cysteamine, or cysteine.

Selenopurine inhibited a mercaptopurine-resistant strain of *L. casei* as efficiently as it did the wild strain, in contrast to the finding with a mercaptopurine-resistant strain of the mouse leukemia L-1210 which showed full cross-resistance to selenopurine.⁸ However, it should be pointed out that because of the low water-solubility of mercaptopurine the amounts of purine thiol to which the resistant organism can be exposed are comparatively small. Furthermore, decomposition of the selenium compound during the long incubation periods required for growth made it difficult to evaluate the lack of cross-resistance observed here.

Clearly, it was desirable to use a system capable of yielding results during incubation periods short enough to be compatible with the low order of stability of selenopurine, but yet productive of data concerning its mechanism of action. Ehrlich ascites tumor cells appeared to fulfill these requirements and a study of the abilities of mercaptopurine and selenopurine to block the uptake of formate and adenine was undertaken.

Incubation of mercaptopurine or selenopurine with mouse Ehrlich ascites tumor cells in the presence of either formate- C^{14} or adenine-8- C^{14} , separation of ribonucleic and deoxyribonucleic acids, and isolation and determination of the specific activity

of adenine, guanine, and thymine, yielded the data shown in Table 3. All figures are related to uptake in the absence of inhibitor expressed as 100.

TABLE 3. INCORPORATION OF FORMATE-C¹⁴ AND ADENINE-8-C¹⁴ IN THE PRESENCE OF MERCAPTOPURINE OR SELENOPURINE IN EHRlich ASCITES TUMOR CELLS

Inhibitor	μ moles/ml	DNA-Gu	RNA-Gu	DNA-Ad	RNA-Ad	Th
Uptake of adenine Mercaptopurine	0.25	90	90	150	100	
	2.5	120	130	140	140	
Selenopurine	0.25	90	100	110	110	
	2.5	50	40	50	40	
Uptake of formate Mercaptopurine	0.25	60	50	70	40	120
	2.5	80	60	60	40	100
Selenopurine	0.25	40	40	40	30	120
	2.5	10	10	5	5	40

It can be seen that mercaptopurine inhibits the uptake of formate into adenine and guanine but not into thymine. However, the uptake of adenine into RNA and DNA adenine and guanine are actually increased at the higher levels of mercaptopurine, and that of adenine into DNA-adenine at even the lower level; presumably this is attributable to interference with synthesis *de novo* of purines^{10, 11} and to a resultant increase in the utilization of exogenous purines.

Selenopurine at the higher level (2.5 μ moles/ml) caused some blockage of the incorporation of adenine into purines and of formate into thymine, possibly due to some general inhibition of cell processes; however, the blockage of incorporation of formate into purines was much more extensive. At the lower level, 0.25 μ moles/ml, selenopurine caused a pattern of inhibition which was almost identical with that resulting from equimolar amounts of mercaptopurine.

Therefore, it appears that selenopurine and mercaptopurine have very similar mechanisms of action, and that these compounds, or their metabolic derivatives, interfere to a greater extent with a reaction concerned with the synthesis of the purine ring than with the utilization of adenine or its derivatives. Since in some bacterial, tissue culture, and enzymatic systems² the activity of selenopurine exceeds that of the sulfur analog, and since the only difference between the two essentially isosteric compounds consists of replacing one atom with another only one step down in the periodic table, it is hoped that eventually it may be possible to express the differences between mercaptopurine and selenopurine in terms of basic physicochemical phenomena.

EXPERIMENTAL

Antimicrobial screening of mercaptopurine and selenopurine. *M. pyogenes* (var. *aureus*) and *K. pneumoniae* were grown on Difco Penassay broth. Six-hour cultures were diluted 1:1,000,000 in the broth giving an inoculum of *circa* 1,000 organisms/ml. To tubes containing 2 ml of inoculated broth, different amounts of test sample were added by means of a Kahn pipette to give dilutions ranging from 1:20 to 1:5120. The test tubes were shaken and, together with inoculated control tubes, were incubated

at 37°C for 17 hr. Minimum inhibitory concentration was obtained by determining the presence or absence of turbidity comparable to that of the controls.

For the antifungal assays, different amounts of test compound were dissolved in F₄ agar medium (5.0 g tryptone, 1.0 g malt extract, 10.0 g glucose, 3.0 g yeast extract, 15.0 g agar, diluted to 1,000 ml with distilled water). With the aid of pipettes three different fungi were streaked across each plate and these were incubated for 3 to 4 days at room temperature. Minimum inhibitory concentrations were determined by lack of visible growth. When no level of the compounds tested caused complete inhibition, the minimum inhibitory concentration was reported as $> 100 \mu\text{g/ml}$.

L. casei studies. The organisms were maintained on agar (beef extract, yeast extract, peptone, tryptone, agar, dextrose, sodium acetate) and transferred every three weeks to the medium of Sauberlich and Baumann¹⁷ as modified by Welch *et al.*¹² The medium used for the mercaptopurine-resistant strain contained 500 μg of mercaptopurine/ml.

For assays, the above medium^{12, 17} was used, excluding purines and uracil. Standard washed inocula were added to duplicate tubes containing 5 ml of double strength medium enriched with growth factor. Appropriate amounts of test solution were added and the final volume was adjusted to 10 ml. Solutions of selenopurine were sterile-filtered, since the lack of stability of this compound does not permit autoclaving. It should be noted that selenopurine (in a concentration of 20 $\mu\text{g/ml}$) which had been permitted to decompose in a 37°C incubator for 48 hr did not inhibit the growth of *L. casei*.

An incubation period of 48 hr at 37°C was used for both the wild and the resistant strain. Growth was followed turbidimetrically using a Klett-Summerson photoelectric colorimeter (filter 66). Good correlation was obtained between Klett readings and potentiometric titrations of the acid produced. At selenopurine concentrations exceeding 0.2 $\mu\text{mole/ml}$, the latter method had to be used because of the precipitation of colloidal selenium.

For reversal studies, the metabolites tested were added prior to inoculation.

Ehrlich ascites tumor cell studies. Mouse Ehrlich ascites tumor cells were obtained by the method of Prusoff, Lajtha, and Welch.¹³ Sodium formate-C¹⁴ (specific activity, 1.5 $\mu\text{c/mole}$) was obtained from the California Foundation for Biochemical Research, as was adenine-8-C¹⁴ (specific activity, 0.9 $\mu\text{c/mole}$). In 20-ml beakers were placed 0.25 ml of packed cells, 5 μc of formate-C¹⁴ or adenine-8-C¹⁴, 0.2 ml of pig serum, 1 ml of inhibitor solution, and the volume was adjusted to 2.5 ml with a modified Chamber's solution.¹³ The beakers were placed in a Dubnoff metabolic shaker for 4 hr at 37°C under an atmosphere of air.

The reaction was stopped by pouring the cell-suspensions into 20–30 ml of 95 per cent ethanol. After centrifugation, fats and acid-soluble fractions were removed in the usual fashion¹⁴ and the residue was dissolved in 0.5 N KOH. Following neutralization with Dowex-50-H⁺ resin the mixture was filtered. To the filtrate ice-cold trichloroacetic acid (TCA) was added to give a pH of 1. The precipitate, which contained the deoxyribonucleic acid (DNA), was separated from the soluble ribonucleotides by centrifugation. The DNA-fraction was heated with 5 per cent TCA for 30 min at 90°C and the residue discarded. TCA was removed from the supernatant fluid with ether and the residual solution was evaporated to dryness on a steam bath, as was the solution containing the ribonucleotides. Treatment with 0.2 ml of 12 N perchloric acid

on a boiling water bath was used to liberate the free bases. After neutralization with 6 N KOH and centrifugation the supernatant solutions were evaporated to 0.5 ml. The bases were separated by paper chromatography using an isopropanol-HCl system.¹⁵ The individual spots were eluted with water and chromatographed in a butanol-ammonia system,¹⁶ except for that of guanine which was rechromatographed in isopropanol and HCl. The concentrations of the individual bases were determined spectrophotometrically and their radioactivity was measured, subsequent to plating aliquots on stainless steel planchets, by counting in a windowless flow-counter. Determinations were made in duplicate.

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INFLUENCE DE L'ACIDE MÉTHYL-3 PENTÈNE-3 OÏQUE SUR LA BIOSYNTHÈSE DE L'ERGOSTÉROL PAR LA LEVURE (*SACCHAROMYCES CEREVISIAE*)

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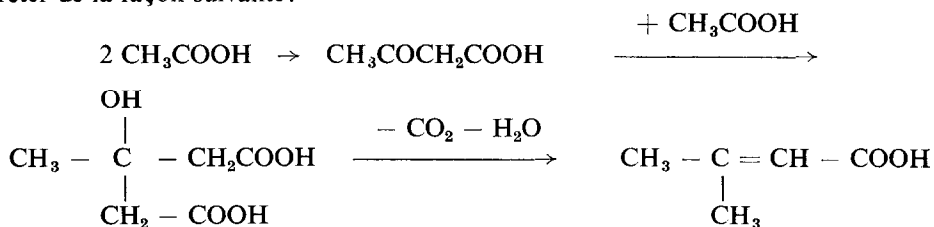
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Résumé—L'acide méthyl-3 pentène-3 oïque, administré à de la levure de biere vivante, à la concentration de 2×10^{-5} , permet d'exalter chez ce micro-organisme la biogenèse de l'ergostérol dont la teneur peut passer du simple au double dans ces conditions.

Abstract—When living brewer's yeast (*Saccharomyces cerevisiae*) is treated with 3-methyl pent-3-enoic acid, at a concentration of 2×10^{-5} , the rate of biosynthesis of ergostérol is increased, so that the ergostérol content of the cells can become more than double the normal amount.

AU COURS de ces dernières années, des recherches très importantes ont profondément modifié nos conceptions sur la synthèse des stérols chez les êtres vivants. Si le rôle de l'acide acétique comme précurseur de ce groupe de composés terpéniques est connu depuis longtemps déjà,¹ la nature des autres substances intermédiaires intervenant dans cette chaîne de réactions est à l'étude depuis peu de temps seulement. Parmi les molécules susceptibles de s'intégrer dans le cholestérol, après marquage au ^{14}C , l'acide diméthyl-acrylique² présente un intérêt particulier, car c'est une unité structurale à 5 atomes de carbone, du type isoprénique dont l'élaboration *in vivo* est facile à interpréter de la façon suivante:



Dans ce mécanisme, l'acide acétique se fixerait sur l'acide acétylacétique sous forme d'acétyl-coenzyme A, comme dans d'autres réactions d'un type analogue (cycle citrique, etc. . .). Il y a lieu de noter cependant qu'un composé très voisin, l'acide "mévalonique", à 6 atomes de carbone, s'intègre dans les stérols avec un rendement bien supérieur et semble être un précurseur plus direct des édifices isopréniques que l'acide diméthyl-acrylique. Alors que la plupart des recherches dans ce domaine ont porté sur la biosynthèse du cholestérol lui-même, l'ergostérol, constituant fondamental de la levure de bière, n'a été que très peu étudié sous cet angle.

Au cours de ses investigations sur l'inhibition de la biosynthèse du cholestérol chez

les animaux, l'un d'entre nous, en collaboration avec G. Zwingelstein et J. Jouanneau³, a eu l'occasion d'étudier un certain nombre d'analogues structuraux de l'acide diméthyl-acrylique en vue de vérifier dans quelle mesure de tels analogues peuvent exercer un effet de blocage sur la chaîne biogénétique en question. On sait en effet que des modifications structurales bien déterminées peuvent conférer à certains métabolites des propriétés antimétabolites correspondantes, en vertu d'un phénomène de compétition dont nous connaissons actuellement de très nombreux exemples. C'est ainsi que, parmi plusieurs composés étudiés, l'acide méthyl-3 pentène-3 oïque manifeste effectivement un pouvoir hypocholestérolémiant quand on l'administre à de jeunes rats soumis à un régime donné.

Partant de ces faits, nous nous sommes demandé quel allait être l'effet de cet acide vis à vis de la biosynthèse de l'ergostérol par la levure de bière (*Saccharomyces cerevisiae*). La parenté structurale entre ce phytostérol et le cholestérol permettait en effet de s'attendre à un mécanisme biogénétique identique en ce qui concerne l'édification de ces deux substances.

PARTIE EXPÉRIMENTALE

Nous avons utilisé pour cette étude *Saccharomyces cerevisiae*, souche Hansen, du C.B.S. de Baarn.

Les micro-organismes sont cultivés sur le milieu liquide de Eaton et Klein⁴ de la façon suivante: Dans des erlens de 100 cm³, munis sur le côté d'une cuve à faces parallèles, contenant 30 cm³ du milieu nutritif, et préalablement autoclavés, on ajoute stérilement le produit à essayer en solution dans du bicarbonate de soude à 1 pour cent. Puis on ensemence chaque cuve avec des cellules âgées de 24 h ayant subi un passage sur milieu liquide. Les cultures sont maintenues à l'étuve à 34°. La croissance est suivie par des mesures d'opacité faites à l'électrophotomètre de Meunier, à l'aide de la cuve à faces parallèles soudée sur les erlens.

Après 60 h, on effectue le dosage de l'ergostérol formé dans les cellules. Il n'est pas nécessaire d'agiter les fioles de culture car il n'y a aucune différence sensible dans la teneur en ergostérol des cellules ayant été agitées pendant leur croissance ou non. D'ailleurs, dans nos cuves, la surface est très grande par rapport à l'épaisseur du liquide et les conditions d'aération sont suffisantes.

Technique du dosage d'ergostérol

Nous utilisons la mesure colorimétrique de la teinte verte donnée par l'ergostérol en solution chloroformique avec l'anhydride acétique et quelques gouttes d'acide sulfurique concentré (réaction de Liebermann-Burchard), seule méthode suffisamment sensible pour les faibles quantités d'ergostérol que nous avons à doser.

Les meilleurs résultats ont pu être obtenus conformément à la technique suivante:

Le contenu de la fiole de culture est centrifugé. Les cellules récoltées sont lavées à l'eau distillée puis centrifugées à nouveau. Le culot de centrifugation est repris par un peu d'eau; le volume de cette suspension est amené à une valeur connue, en général 2 cm³. On prélève 0,25 cm³ pour faire la mesure du poids sec des cellules et on effectue le dosage sur le reste de la suspension. A celle-ci, on ajoute 10 cm³ du mélange de solvants: méthylal-méthanol (4:1). (Le méthylal est un excellent agent précipitant des protéines, ainsi qu'un excellent solvant des stérols et des lipides. Préconisé pour la première fois par Delsal⁵, en vue de l'analyse du sérum sanguin, son emploi n'avait

pas encore été étendu jusqu'ici aux dosages de stérols autres que le cholestérol, à notre connaissance tout au moins).

Après agitation et centrifugation, le liquide est recueilli dans un petit ballon. Le précipité de nature protéique est rincé avec 5 cm³ du solvant. Après une nouvelle centrifugation, les liquides sont réunis et le solvant est chassé au bain-marie. Le résidu est desséché à l'étuve à 105°. (Il doit être absolument incolore et non jaunâtre).

Après refoidissement, le résidu sec est additionné de 3,5 cm³ de chloroforme qui dissout les stérols, éliminant ainsi les sels qui gênent la réaction colorée. Après addition de 3,5 cm³ d'anhydride acétique et de 2 gouttes d'acide sulfurique concentré, il y a apparition d'une teinte rose qui vire au vert très rapidement. Après 5 minutes, la teinte s'est stabilisée à son maximum d'intensité (pour le cholestérol, 30 minutes sont nécessaires). La lecture est effectuée à l'électrophotomètre Meunier, écran rouge. $D = 10$. La courbe d'étalonnage de l'appareil en ergostérol donne la quantité d'ergostérol de l'échantillon. La mesure du poids sec permet d'en déduire la concentration dans 100 g de levure sèche.

Il est à remarquer que cette méthode ne fait pas intervenir la saponification de l'extrait lipidique. En effet Delsal démontre que, par sa technique les esters de cholestéryle donnent dans ces conditions une coloration d'intensité identique à celle fournie par le cholestérol pur. Aussi, il juge inutile une saponification préalable au dosage. Dans notre cas, nous avons fait ce même dosage sans saponifier et après saponification avec des résultats identiques. De plus, la saponification a pour effet parfois de jaunir le milieu, ce qui rend le dosage moins précis. Pour cette raison, les résultats mentionnés dans ce travail ont été obtenus sans saponification préalable.

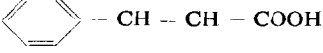
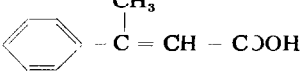
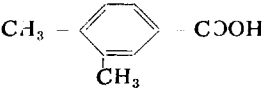
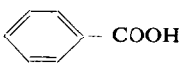
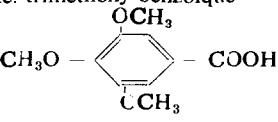
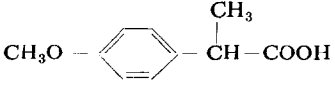
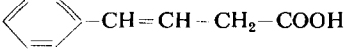
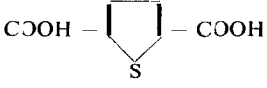
RÉSULTATS

Nous avons tout d'abord étudié l'action éventuelle sur la croissance de *Saccharomyces cerevisiae* et sur la synthèse de l'ergostérol chez ce micro-organisme d'un certain nombre d'acides organiques. Nous avons constaté que la plupart des produits étudiés gênaient peu la croissance de la levure mais, pour certains, diminuaient sa teneur en ergostérol. Seul parmi tous, l'acide méthyl-3 pentène-3 oïque augmentait du simple au double la concentration de l'ergostérol des cellules dans nos conditions expérimentales. Nous avons joint aux résultats du tableau 1 l'action du squalène, précurseur connu, sur la biosynthèse de l'ergostérol.

DISCUSSION

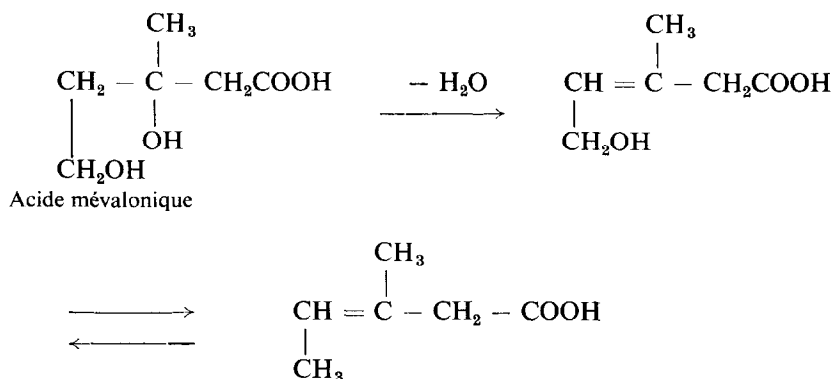
Les faits ainsi établis prouvent tout d'abord que l'acide méthyl-3 pentène-3 oïque peut se comporter d'une façon diamétralement opposée suivant l'espèce à laquelle on l'administre. Alors qu'il est capable d'inhiber la biogénèse du cholestérol chez le rat, il favorise au contraire la formation de l'ergostérol chez la levure. De plus, il est tout à fait remarquable de noter la grande spécificité de cette propriété activatrice. Parmi les divers composés étudiés, seul le squalène est doué d'une action analogue; les autres dérivés essayés pour diverses raisons se sont révélés inefficaces. Or le squalène se comporte comme un précurseur des stérols, ce qui signifie que les organismes chez lesquels il présente cette propriété ont le pouvoir d'élaborer des systèmes enzymatiques capables d'intégrer ce composé dans la chaîne métabolique conduisant au système isoprénique. On peut dès lors se demander si l'acide méthyl-3 pentène-3 oïque ne doit

TABLEAU 1

Produits ajoutés (conc. 2×10^{-5})	Croissance exprimée en degrés photométriques				Ergostérol g pour 100 g de matière sèche
	après 24h	après 39h	après 48h	après 63h	
Témoin		370	400	418	0,57
ac. cinnamique 	40			367	0,42
ac. β -méthyl-cinnamique 	34			368	0,44
ac. diméthyl-benzoïque 	46			385	0,50
ac. benzoïque 	50			376	0,51
ac. triméthoxy-benzoïque 		28	290	410	0,24
		28	390	420	0,61
ac. phényl-isocrotonique 		24	330	420	0,40
ac. thiofane dicarbonique 		28	330	380	0,25
ac. diméthyl-acrylique		360	400	420	0,36
ac. β -méthyl- β -isobutyl acrylique		370	400	420	0,47
ac. méthyl-3 pentène-3 oïque		370	400	420	(a) 1,33 (b) 1,16
squalène	comme le témoin				1,36

Influence de certains acides organiques et du squalène sur la croissance et la teneur en ergostérol de *Saccharomyces cerevisiae*.

pas lui aussi être considéré comme un tel précurseur. Sa filiation avec l'acide mévalonique et surtout avec son produit de déshydratation éthylénique est évidente; il suffit en effet de remplacer le radical méthyle en bout de chaîne par un groupement CH_2OH pour passer de l'un de ces composés à l'autre, comme le montrent les formules suivantes:



Des hydroxylations et déshydroxylations de ce type sont très fréquentes dans le domaine des terpènes et des stérols, à la fois chez les animaux et les plantes et surtout chez les micro-organismes, qui possèdent très souvent des enzymes capables de catalyser de telles réactions réversibles.

Dans le cas de l'acide méthyl-3 pentène-3 oïque, la double liaison en position allylique par rapport au méthyle terminal augmente d'ailleurs la mobilité des hydrogènes de ce méthyle et peut faciliter de ce fait la fixation d'un atome d'oxygène. Néanmoins, la présence d'un enzyme spécifique est nécessaire en vue d'une telle fixation et on peut alors admettre que la levure de bière fait justement partie du groupe des champignons inférieurs susceptibles d'élaborer un tel enzyme, alors que le rat n'en dispose probablement pas. Mais, pour le moment, ce n'est là qu'une hypothèse qui appelle de nouvelles expériences.

L'une des méthodes permettant d'apporter une solution au problème ainsi posé consiste à marquer l'acide méthyl-3 pentène-3 oïque au moyen de ^{14}C sur l'un de ses chaînons et à doser ensuite la radioactivité de l'ergostérol isolé des cellules traitées par un tel acide. Des recherches dans ce sens sont actuellement en cours et doivent faire l'objet d'un mémoire ultérieur.

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ANTIMITOTIC ACTIVITY OF SUBSTANCES SHOWING A HEPARIN-LIKE BEHAVIOR

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Abstract—Antimitotic activity of polysaccharide derivatives showing heparin-like action is described with regard to their pharmacological behaviour. The conclusions are discussed with reference to the possible parallelism between anticoagulant activity of the substances and their inhibiting capacity on protoplasmic gelation.

CHANGES in protoplasmic viscosity that occur during mitosis have been considered very important for a regular mitotic process.¹

Heilbrunn² assumes that a substance which prevents gelation during mitosis hinders the formation of the achromatic figure inhibiting the cleavage of cells. This author considers that this hypothesis is supported by the action of heparin on mitosis which is known to inhibit blood clotting and which is also able to prevent protoplasmic gelation.

The antimitotic activity of heparin was first observed in tissue cultures by Fischer^{3, 4} and by Heilbrunn in investigations using the eggs of the worm *Chaetopterus*.⁵ Some other mucopolysaccharides are also able to suppress the growth of embryonic tissue *in vitro* according to Balazs and Holmgren⁶. A heparin-like activity was observed also with bacterial polysaccharides.⁷

From the foregoing we particularly investigated the antimitotic activity of some polysaccharide derivatives showing a lower anticoagulant action than heparin.

The following substances have been tested:

PPE. Polylactose Phosphoric Ester (approximate mol. wt. 10,000; P 12 per cent).

IH. Inactivated Heparin, i.e. the product of heparin oxidation in alkaline media followed by acetic acid precipitation (mol. wt. approximately 17,000).

ACSE. Amino Cellulose Sulphuric Ester. Degradation product of cellulose (mol. wt. 15,000–20,000) containing an amino group and an ester group for each glucose unit. The product is fairly water soluble.

DS. Dextran Sulphate (mol. wt. 10,000–15,000; S 16 per cent). All these substances were prepared in the Laboratorix Ricerche Farmitalia by Dr. A. Gallo who kindly supplied us with the necessary samples. Details concerning preparation of these substances will be subject of a further report.

Heparin. "Liquemin" Roche (pure heparin).

EXPERIMENTS

Determination of anticoagulant and clearing action

(1) The "in vitro" anticoagulant action of the above substances on recalcified plasma, determined by Lewis and De Maria's method⁸ was found to be remarkably lower than that of heparin Roche.

The results are summarized in Table 1.

TABLE 1.

Time of clotting	Heparin (μ g)	I H (μ g)	ACSE (μ g)	D S (μ g)	PPE (μ g)
2' 30"	0	0	0	0	0
2' 30"	0.125	2.5	5		50 *
3'	0.25	10	15	0.5	100 †
3' 30"	0.5	25	25	2	
6'	1.0	100	75	5	500

* inactive dose.

† minimal active dose.

(2) The "in vitro" clearing action of the same substances has been examined by Seifter and Baeder's method.⁹

Plasma from rats injected with the different substances was added to a lipidic emulsion. The turbidity, expressed as percentage of the transmission (per cent T), was measured with Beckmann D.O. spectrophotometer at $\lambda = 700 \text{ m}\mu$. Readings were made at the beginning and after 3 and 20 hr respectively. The clearing activity was determined from the differences of turbidity (Δ) between 20 and 0 hr.

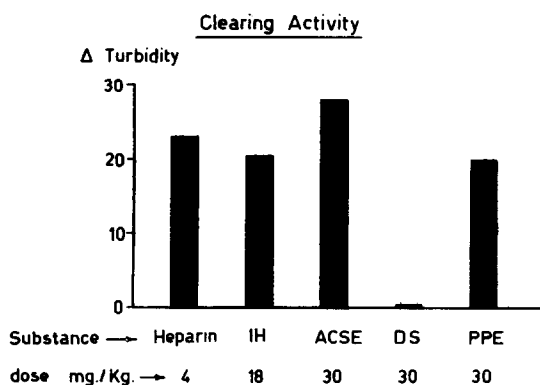


FIG. 1

The results, summarized in text Fig. 1, show that the clearing activity obtained with 4 mg/kg of heparin is equivalent to the one of 18–30 mg/kg of the substances under study.

DS shows no activity.

ANTIMITOTIC ACTIVITY

Our experiments were carried out on cultures of myocardial fibroblasts from 8 day-old chicken embryos, according to the following techniques:

- rollertubes with three mica coverslips inserted.
- observation under phase-contrast microscope of living cells after the application of the substances.

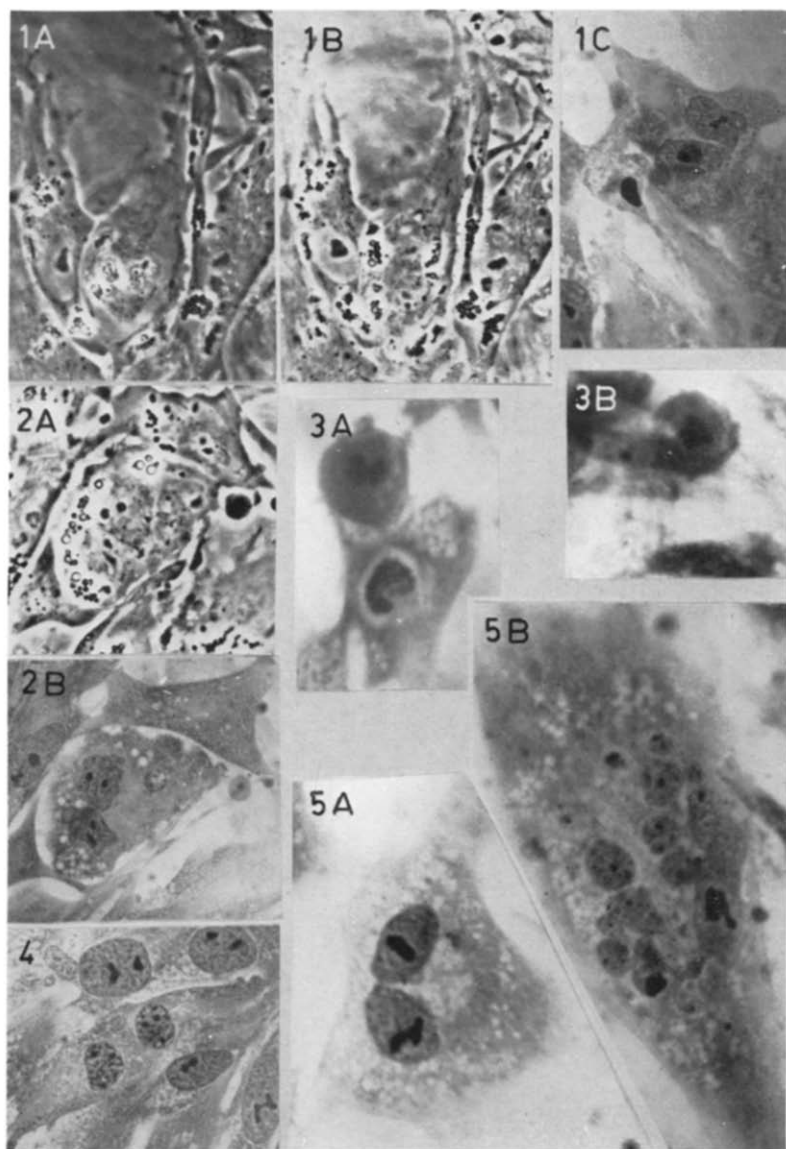


PLATE 1

- No. 1. Effect of 500 µg/ml heparin Roche on a cell in mitosis:
 - (a) 120 min Anaphase reconstruction has begun;
 - (b) 210 min Binucleated cell;
 - (c) Fixation after 285 min. The same Binucleated cell.
- No. 2. Effect of 500 µg/ml heparin Roche on a cell in mitosis:
 - (a) 210 min Binucleated cell;
 - (b) Fixation after 285 min. The same cell.
- No. 3. Effect of 250 µg/ml heparin Roche on roller tubes cultures.
 - (a) and (b) Fixation after 24 hr. Colchicine-like metaphase.
- No. 4. Effect of 500 µg/ml IH. Fixation after 285 min.

Reconstruction of daughter nuclei.
- No. 5. Effect of 250 µg/ml PPE on roller tubes cultures:
 - (a) Fixation after 24 hr. Binucleated cell.
 - (b) Fixation after 24 hr. Micronuclei.

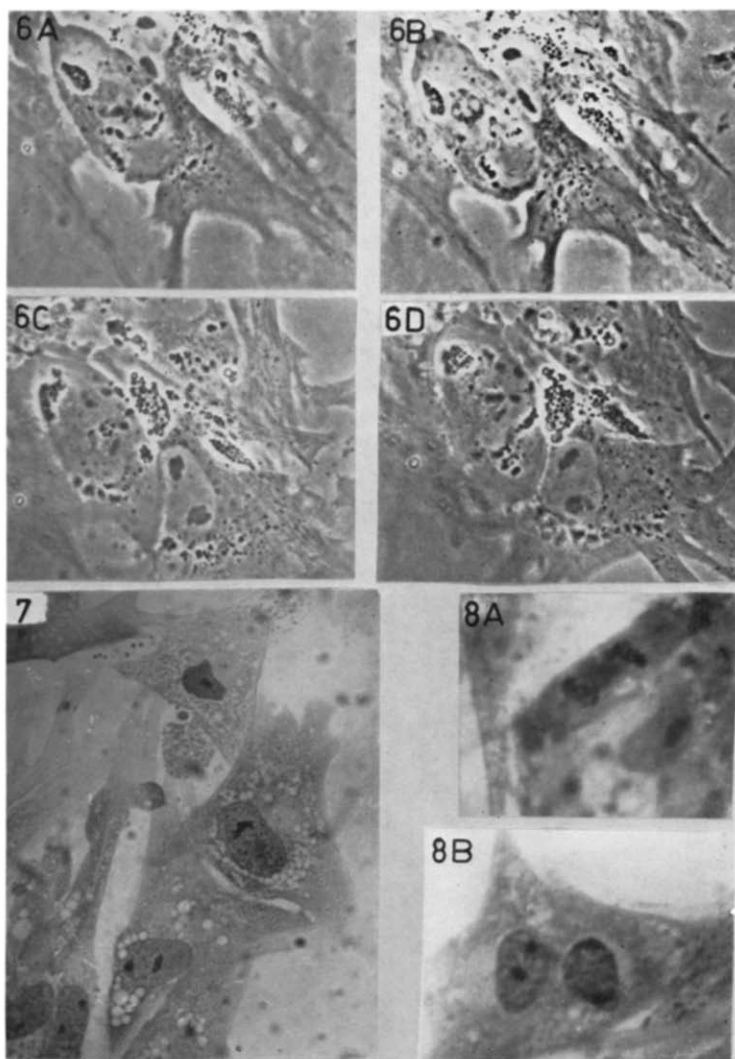


PLATE 2

- No. 6. Effect of 1000 $\mu\text{g}/\text{ml}$ ACSE on a cell in mitosis:
 (a) 52 min. Metaphase;
 (b) 55 min. Early anaphase;
 (c) 115 min. Binucleated cell;
 (d) 120 min. Binucleated cell.
- No. 7. Effect of 500 $\mu\text{g}/\text{ml}$ ACSE. Fixation after 285 min. Nuclear pyknosis, vacuolation, cytolysis.
- No. 8. Effect of 250 $\mu\text{g}/\text{ml}$ ACSE on roller tubes cultures:
 (a) Fixation after 24 hr. Anaphase reconstruction;
 (b) Fixation after 24 hr. Binucleated cell.

Substances were dissolved in Hanks' saline at concentrations of 250, 125, 25 and 5 $\mu\text{g/ml}$. Their activity is referred to that of heparin Roche at the same concentrations as control.

With the former technique, after 24 hr incubation at 38°C, the substances were placed in test tubes as follows: from 2 ml of a fluid medium, consisting of a mixture of Hanks' saline (two parts), horse serum (two parts), and chick embryo extract (one part), 0.2 ml were substituted with the same volume of Hanks' saline containing the respective substances. Cultures were further incubated for 24 hr and then fixed in Bouin's fluid and stained with Mayer's hematoxylin.

With the latter technique, 24-hr-old cultures were examined under the phase-contrast microscope. The effect of the addition of substances to the cells was photographically recorded for 3–4 hr. Cultures were then fixed and stained. The appearance of cells of permanent preparations was compared with the one observed during life.

RESULTS

By phase-contrast microscope it can be noticed that about one hr after the addition of: 500 $\mu\text{g/ml}$ heparin; 500 $\mu\text{g/ml}$ IH; 1000 $\mu\text{g/ml}$ ACSE and 2000 $\mu\text{g/ml}$ PPE to the cultures, cells entering mitosis have slowed down metaphase. While some of them stop in this phase after cytoplasmic somewhat turbulent movements, other undergo anaphase without dividing and a nuclear reconstruction begins (Plate 1, No. 1, 2, 4; Plate 2, No. 6, 7). Within the second hour no other cells enter mitosis and some unhealthy ones, that previously revealed themselves by a progressive stuffing of granules, undergo degeneration.

DS compound, at concentrations of 500, 1000 and 2000 $\mu\text{g/ml}$ does not influence either the duration or the course of the division process. No morphological alteration of nucleus or nucleolus is observed in fixed preparation after 4 hr from the addition of the substance.

Rollertubes cultures, after 24 hr treatment, show a different appearance.

An amount of 250 $\mu\text{g/ml}$ of heparin Roche delayed the growth and in the meantime colchicin-like metaphase and pyknotic ana-telophases appeared (Plate 1, No. 3). Cells lost its spindle-shape and underwent lysis. This picture attenuates when concentrations decrease (125–5 $\mu\text{g/ml}$).

Block of the growth and the mitotic process occurs with 250 $\mu\text{g/ml}$ of IH. Very many unhealthy cells with marked vacuolation and cytolysis are observed. These phenomena decrease parallel to the concentrations (125–5 $\mu\text{g/ml}$).

ACSE and PPE at concentration of 250 $\mu\text{g/ml}$ inhibit the cytodieresis in the same way. Several binucleated cells and anaphase reconstructions are found in the cultures (Plate 1, No. 5 and Plate 2, No. 8). This phenomenon is well evident also at lower concentrations (125–5 $\mu\text{g/ml}$).

The DS which proved to be inactive at 4 hr observation on living cells, at concentration of 250 $\mu\text{g/ml}$ prevents any growth with marked cytolysis and nuclear pyknosis. The picture returns to normal at concentration of 5 $\mu\text{g/ml}$. Prolonged contact of this substance with the cells gives evidence to a certain degree of toxicity that 4 hr observation did not reveal.

DISCUSSION AND CONCLUSIONS

The results reported may be summarized as follows:

- (a) substances endowed with a good clearing action, such as IH, ACSE and PPE (the anticoagulant action of which is 1/120 to 1/500 lower than that of heparin Roche) behave like antimitotic drugs.
- (b) DS, which is a good anticoagulant (1/2–1/5 heparin) but not a clearing substance, shows no antimitotic activity.

Therefore, according to our results, it seems possible to exclude a parallelism between anticoagulant activity of a substance and its inhibiting power of protoplasmic gelation.

It should be noticed that concentrations active on cell division are much higher than those active on coagulation. This may be ascribed to the slow penetration of these high molecular weight substances into the cells.

The observation that substances endowed with clearing action behave like antimitotic drugs, suggests that they may have a particular affinity for lipoproteins and that their possible link with lipophil groups of fibrous proteins of the spindle induces changes in the degree of folding of these polypeptide chains until more or less corpuscular shape.

The same hypothesis was advanced by Östergren¹⁰ to explain the colchicine activity on the spindle, since it is known that colchicine too is able to inhibit the process responsible for the viscosity changes that occur during the mitosis.

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THE ACTIVITY OF VARIOUS ESTERASE INHIBITORS TOWARDS ATYPICAL HUMAN SERUM CHOLINESTERASE

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Abstract—Guided by genetic studies, human sera were selected and postulated to contain either the usual type of serum cholinesterase (pseudocholinesterase), or an atypical variety of this enzyme, or a mixture of these two enzymes. The shape of concentration-inhibition curves obtained with sera of the last-named category permitted no other interpretation than the presence of a mixture of two enzymes. The concept of the existence of a usual and of an atypical form of human serum cholinesterase was thereby supported.

The values for I_{50} (concentration of inhibitor causing 50 per cent inhibition) of 16 inhibitors for normal and atypical esterase were compared under standard conditions. Both types of esterase were equally affected by the phosphate inhibitors DFP and TEPP. All other inhibitors blocked the atypical esterase less than the normal one. In relation to their usual blocking capacities, the bisquaternary compounds C_{10} and succinylcholine were particularly weak inhibitors of the atypical esterase. For the 12 remaining inhibitors, which included tetramethylammonium, procaine, and eserine, the normal and atypical I_{50} could be related by the exponential equation $I_{50}\text{-atypical} = (I_{50}\text{-usual})^{0.785}$; that is, the difference between corresponding I_{50} -values increased with increasing potency of the inhibitors.

Several compounds, e.g., decamethonium and chlorpromazine, were competitive inhibitors of atypical esterase, but they were not completely competitive inhibitors of the usual enzyme.

Every fourth molecule of C_{10} and of succinylcholine seemed to block two active centres of the normal esterase, but each of these molecules blocked only one active centre of the atypical enzyme.

THE susceptibility to inhibition of human serum cholinesterase (pseudocholinesterase) varies from person to person if the local anaesthetic, Dibucaine, is used as the inhibitor.¹ By testing a large population,² it was found that the sera could be divided into three groups according to the strength of inhibition exerted by Dibucaine.

Family studies revealed that this triple division of sera was genetically determined. There appeared to exist two autosomal alleles without dominance, which were responsible for esterase characteristics. The esterase of the homozygous normal or usual group was more sensitive to inhibition than the esterase of the homozygous atypical group, while the heterozygotes took an intermediate position.² The present report gives a survey of the action of several inhibitors upon representative samples of esterase.

METHODS

Esterase activity was measured by ultraviolet spectrophotometry, using a Beckman Recording Spectrophotometer Model DK2. The reactions were observed at 240 m μ

in absorption cells of either 10 mm or 50 mm light path. The experimental temperature was between 24° and 26°C. The solvent was M/15 phosphate buffer, pH 7.4. The substrate was benzoylcholine, 5×10^{-5} M. Details of the procedure have been previously reported.³

Source of enzyme was usually human serum diluted 1:100. The criterion for the selection of individual sera for this study was their "Dibucaine Number" (DN).² The DN equals the per cent inhibition produced by 10^{-5} M dibucaine at pH 7.4 and 26°C, with 5×10^{-5} M benzoylcholine as substrate and 100-fold diluted human serum as source of enzyme. Genetic studies had previously indicated that DN-values of about 79 are given by homozygotes with normal or usual esterase, while a DN of about 16 is typical of homozygotes with atypical esterase; and that of heterozygotes approximates 61 (with a range from 40 to 70). Altogether, sera of seven individuals were used: two of normal homozygotes, two of atypical homozygotes, and three of heterozygotes. In one series, purified serum cholinesterase (Cholase) was used in concentrations which had approximately the same esterase activity as diluted average serum. The DN of Cholase was 79.

The following inhibitors were used:

1. Tetramethylammonium bromide (TMA) (cf., B.P. 1958 for the hydroxide).⁴
 2. Choline chloride U.S.P. XV.⁴
 3. Decamethonium bromide (C_{10}) (cf., B.P. 1953 for the chloride).⁴
 4. Succinylcholine chloride B.P. 1958, U.S.P. XV.⁵
 5. Meperidine hydrochloride B.P. 1958, U.S.P. XV.⁶
 6. Chlorpromazine hydrochloride B.P. 1958.⁷
 7. Procaine hydrochloride B.P. 1958, U.S.P. XV.⁸
 8. Tetracaine hydrochloride B.P. 1958, U.S.P. XV.⁸
 9. Dibucaine hydrochloride U.S.P. XV.¹
 10. Win 5303 = 2-*n*-pentoxy, 4-amino-diethylaminoethylbenzoate hydrochloride.⁹
 11. Win 4510 = 2-*n*-hexoxy, 4-amino-diethylaminoethylthiobenzoate hydrochloride.⁹
 12. Neostigmine bromide B.P. 1958, U.S.P. XV.¹⁰
 13. Physostigmine salicylate B.P. 1958, U.S.P. XV.¹¹
 14. RO2-0683 = dimethylcarbamate of (2-hydroxy-5-phenylbenzyl)-trimethylammonium bromide.¹²
 15. Tetraethylpyrophosphonate (TEPP).¹³
 16. Diisopropylfluorophosphonate (DFP Isofluorophate) U.S.P. XV.¹³
- (Compounds No. 3 and No. 4 are skeletal muscle relaxants; No. 5 is a synthetic analgesic; No. 6 is known as a tranquillizer; Numbers 7–11 are local anaesthetics; Numbers 12–16 are known as inhibitors of cholinesterases, and, of those, No. 14 is a specific inhibitor of serum cholinesterase).

Each inhibitor was tested in several concentrations, with repetitions on at least two different days. Each time, every measurement was made in duplicate. For most purposes, only initial reaction rates were considered, i.e., the rates obtained in the interval between 30 sec and 210 sec after mixing enzyme and substrate. A deviation from this procedure was adopted for the purpose of determining the nature of inhibition. Tests for competitive inhibition consisted in the recording of complete hydrolysis curves in the presence and in the absence of inhibitors, and determining the rates of breakdown of benzoylcholine as the tangents to these curves. As pre-

viously shown, the error inherent in this method due to the accumulation of split products is negligible;¹⁴ this has been confirmed in this series on a few occasions when data obtained by continuous recording were compared with those obtained by measuring initial rates.

In studies designed for comparing strengths of inhibition, rapidly acting inhibitors were always added to the enzyme simultaneously with the substrate. Slowly acting inhibitors were pre-incubated at 26°C with the enzyme for a suitable length of time. This time was estimated in preliminary experiments by adding inhibitors to the enzyme prior to, and simultaneously with, the addition of substrate. During pre-incubation, enzyme and inhibitor were contained in 3 ml of buffer; addition of substrate solution increased the volume to 4 ml. The reported concentrations of inhibitor are the final concentrations, i.e., not those which prevailed during pre-incubation, but those which existed during the hydrolysis of benzoylcholine.

For the determination of I_{50} values (concentration of inhibitor causing 50 per cent inhibition), the data were analyzed graphically from plots of per cent inhibition *vs* log concentration of inhibitor. For control, the ratios of uninhibited reaction rates over inhibited ones were plotted against concentration of inhibitor whenever linearity of such a plot was postulated.

Enzyme activities are stated in units which are defined as micromoles of acetylcholine hydrolyzed per hour by 1 ml of serum at 37°C. These units were calculated from spectrophotometric measurements on benzoylcholine with the aid of an empirical conversion factor.³

RESULTS

A survey of the activities of all investigated inhibitors towards usual and atypical esterase is given in Table 1. The activities are expressed as pI_{50} , that is the negative

TABLE 1. INHIBITION OF NORMAL AND ATYPICAL CHOLINESTERASE*

No.	Inhibitor	Pre-incubation (min)	Normal esterase pI_{50} -normal	Atypical esterase pI_{50} -atypical	Ratio I_{50} -atyp/ I_{50} -norm
1	Choline	0	1.52	1.23	2.0
2	TMA	0	1.55	1.15	2.5
3	Meperidine	0	3.12	2.74	2.3
4	Succinylcholine	0	4.01	2.02	99
5	Procaine	0	4.36	3.23	14
6	C ₁₀	0	4.88	2.74	141
7	Chlorpromazine	0	5.36	4.13	17
8	Dibucaine	0	5.57	4.27	20
9	Win 5303	0	6.10	4.67	27
10	Tetracaine	0	6.56	5.20	23
11	Win 4510	0	6.81	5.27	35
12	Neostigmine	20	6.89	5.48	25
13	Physostigmine	20	7.84	6.57	18
14	DFP	30	8.70	8.70	1
15	RO2-0583	50	8.86	6.78	120
16	TEPP	30	9.19	9.19	1

* The inhibitors are listed in the order of their observed activity toward normal human serum cholinesterase. Indicated are the duration of incubation of enzyme and inhibitor prior to the addition of substrate, the negative logarithms of the measured concentrations of the inhibitors causing 50 per cent inhibition (pI_{50}) and the (non-logarithmic) ratio of the two I_{50} -values. The concentration of benzylcholine was 5×10^{-6} M in all cases.

logarithm of the concentration of inhibitor causing 50 per cent inhibition. For reasons which will be explained below, purified esterase in the form of cholinesterase was used to represent the normal or usual type of esterase. The data indicating the inhibition of atypical esterase were obtained with sera from two individuals; the susceptibility to inhibition of the esterase in each of these two sera was alike. In doing the experiments, attention was directed primarily towards comparing the actions of a given inhibitor upon the two enzymes. Each value for I_{50} resulted from the determination of concentration-inhibition curves, as will be described below. The inhibitors used without pre-incubation are compounds Numbers 1–11. The compounds listed under Numbers 12–16 were found to exert their inhibiting effects slowly upon both usual and atypical esterases. Therefore, they were pre-incubated with the enzymes prior to the addition of substrate. The durations of pre-incubation are stated in Table 1.

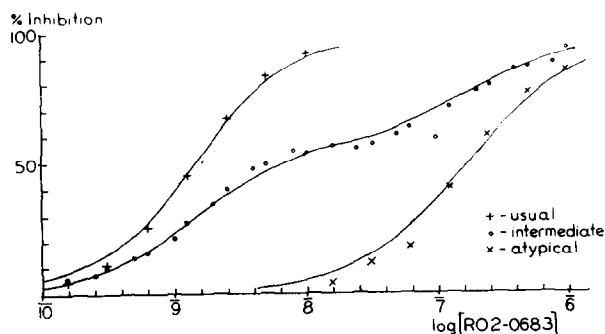


FIG. 1. Cholinesterase inhibition by RO2-0683 in sera from three different persons.

The abscissa indicates log molar concentration of the inhibitor. Each experimental point is an average of duplicate determinations. Theoretical curves are fitted to the data. The curve to the left indicates Zone B kinetics, as worked out for RO2-0683 by Myers¹². The right-hand curve represents Zone A kinetics. The intermediate curve is a combination of the two limiting curves, calculated under the assumption that 56 per cent of the uninhibited reaction velocity is due to the usual type of enzyme. The deviation of the data from the theoretical curves is presumably explained by the simultaneous dilution of, and addition of substrate to, the enzyme-inhibitor system.

Figure 1 pictures the inhibition of cholinesterase in the sera of three persons, selected to represent the usual and atypical homozygotes, and the heterozygotes, in regard to esterase. The inhibitor was the neostigmine derivative RO2-0683. Each serum was pre-incubated for 50 min¹³ with several concentrations of the inhibitor. The I_{50} , the concentration producing 50 per cent inhibition, differed more than a hundredfold between the two homozygous sera, which were designated as usual and atypical. The third serum, which was genetically heterozygous and labelled as intermediate, behaved like a mixture of these two. All three sera had characteristic activities in the absence of inhibitors; the usual serum had 206 units, the intermediate 153, and the atypical 91 units.

Figure 2 shows principally the same pattern as Fig. 1; however, the difference between the extreme curves is smaller, the intermediate curve is steeper than in Fig. 1, and it has no step. Similar investigations have been performed with neostigmine, dibucaine, Win 5310, meperidine, succinylcholine, and decamethonium. In

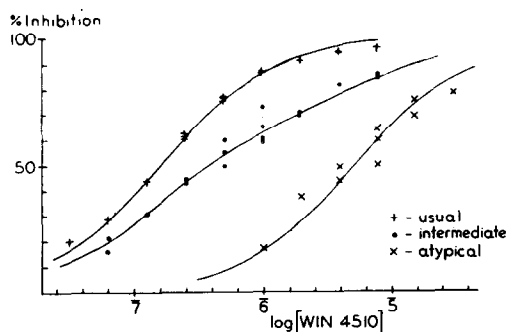


FIG. 2. Cholinesterase inhibition by Win 4510 in sera from three different persons.

The abscissa indicates log molar concentration of inhibitor. Theoretical curves are fitted to the data assuming Zone A kinetics to hold for all three sera. The intermediate curve is a combination of the two limiting curves, calculated under the assumption that 66 per cent of the uninhibited reaction velocity is due to the usual type of enzyme.

each case, there was an intermediate curve stretching from the base of the usual curve to the top of the atypical curve.

The two last named drugs, succinylcholine and decamethonium, differed from all other compounds in that the slopes of the inhibition data for the usual enzyme were less steep; that is, the slopes at the inflection point were about 46 if per cent inhibition was plotted against log concentration of inhibitor.

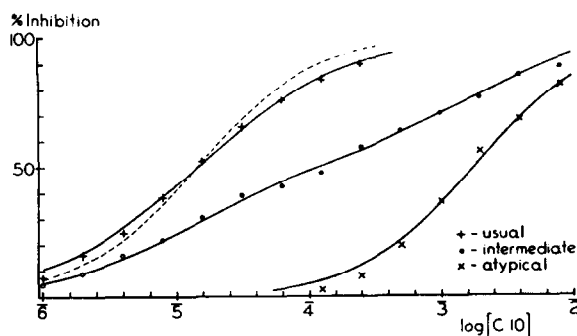


FIG. 3. Cholinesterase inhibition by decamethonium in sera from three different persons.

The right-hand curve and the dashed curve are parallel; their shape indicates the assumption that one mole of decamethonium blocks one active centre of the enzyme. The fully-drawn curve to the left, which fits the data obtained with the usual esterase, expresses the assumption that every fourth molecule of inhibitor blocks two active centres. The intermediate curve is a combination of the two limiting curves, calculated under the assumption that 56 per cent of the uninhibited reaction velocity is due to the usual type of esterase.

In the example shown in Fig. 3, the slope was 44.94 ± 2.25 , if calculated by the method of least squares, from three experimental points of the middle section of the usual curve (38.8, 52.5, and 65.9 per cent inhibition). The 99 per cent fiducial limits of this slope were 34.60 and 55.28. The theoretical curve shown by the dashed line in

Fig. 3 has a slope of 56.0 over the same range of inhibition, and a slope of 57.5 at the point of inflection. In other words, the deviation of the data from the dashed line is statistically significant.

The intermediate curves shown in Figs. 1–3 were calculated by postulating the presence of a mixture of normal and atypical esterase in the heterozygous serum. For this calculation, specific assumptions had to be made regarding the proportion of normal to atypical esterase activity in the uninhibited sample. Thus, in Figs. 1 and 3, the normal esterase was assumed to contribute 56 per cent to the total activity, while in Fig. 2, the contribution was taken as 66 per cent. Samples of heterozygous serum from different persons were used in the three experiments of Figs. 1–3. The apparent proportion of normal esterase differed between samples from different heterozygotes, not only if the inhibitors were changed, but also if only one inhibitor was used for the determination of concentration-inhibition curves. This is a confirmation of previously reported observations on the person-to-person variations in intermediate DN-values.²

The normal and the atypical curves have been determined for a number of other inhibitors; this appeared to be the primary task, since they set the limits for the intermediate curves. Particularly if there was little or no difference between the normal and atypical curves, measurements on heterozygous sera were omitted (Figs. 4 and 5).

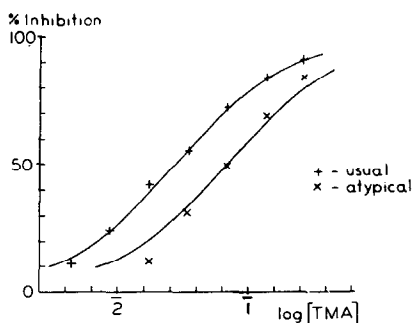


FIG. 4. Cholinesterase inhibition by tetramethylammonium bromide in sera from two different persons.

The abscissa indicates log molar concentration of inhibitor. Each point represents an average of duplicate determinations. The curves fitted to the data indicate the assumption of Zone A kinetics.

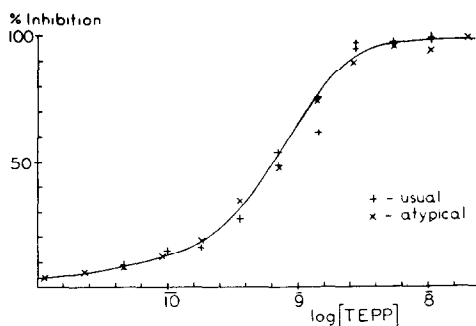


FIG. 5. Cholinesterase inhibition by tetraethylpyrophosphate, in sera from two different persons.

The abscissa indicates log molar concentration of inhibitor. Each point represents an average of duplicate determinations. An empirical curve is fitted to the data.

Other differences between the two enzymes became apparent when tests for competitive inhibition were made; an example is given in Fig. 6. The hydrolysis of benzoylcholine was followed in the presence of various concentrations of Win 4510, and the data were analyzed with the aid of double reciprocal plots. Each graph permits the evaluation of the apparent dissociation constants of the enzyme-substrate complex ES, the enzyme-inhibitor complex EI, and the complex ESI consisting of enzyme, substrate and inhibitor, the constants being K_{ES} , K_{EI} and K_{ESI} , respectively. The inhibition is termed competitive if the ESI complex cannot be formed, i.e., if K_{ESI} is infinitely large. Friedenwald and Maengwyn-Davies¹⁵ have defined $K_{ESI} = \alpha K_{ES}$, so that the degree of competition can be expressed in terms of the constant α which, for non-competitive inhibition, has a value of 1.0, and which becomes infinite for completely competitive inhibition. For the data obtained with the atypical esterase and Win 4510 (Fig. 6a), the constants are $K_{ES} = 2.5 \times 10^{-5}$, $K_{EI} = 6.5 \times 10^{-6}$ and

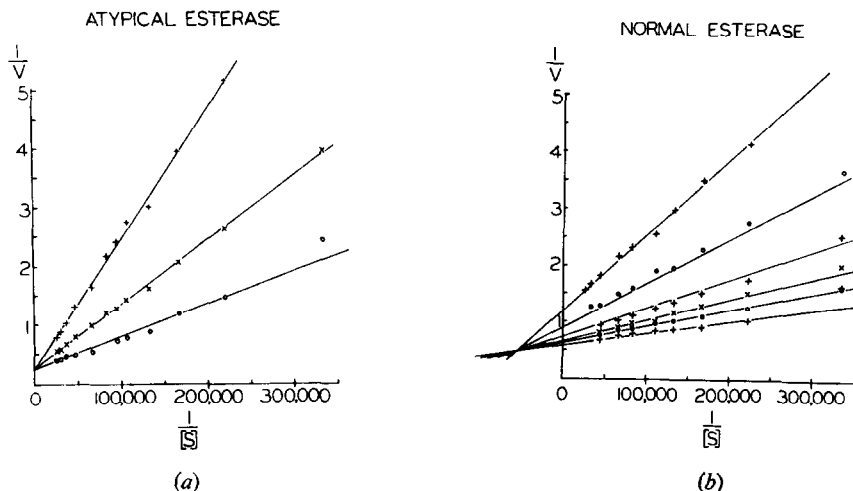


FIG. 6a and 6b. Tests on mode of inhibition of normal and atypical esterase by Win 4510.

The abscissa shows the reciprocal of the molar concentration of benzoylcholine; the ordinate indicates the reciprocal of the reaction velocity in arbitrary units. The data were gained as the tangents to continuous hydrolysis curves. The initial concentration of benzoylcholine was 5×10^{-5} M. The lines with the flattest slopes in each graph represent data obtained in the absence of inhibitor. The lines become steeper with rising concentrations of inhibitor. A common intercept at the ordinate indicates completely competitive inhibition, as in Fig. 6a, while the intercept to the left of the ordinate, as in Fig. 6b, indicates a partially competitive inhibition. See text for further explanations.

$\alpha = \infty$. For the data obtained with the usual esterase (Fig. 6b), the constants are $K_{ES} = 3.8 \times 10^{-6}$, $K_{EI} = 3.7 \times 10^{-6}$ and $\alpha = 6.2$. Similar investigations have been made for all those inhibitors which act rapidly and which were not hydrolyzed at an appreciable rate by the usual esterase; that is, for choline, TMA, meperidine, decamethonium, chlorpromazine, and dibucaine. All these compounds proved to be competitive inhibitors for atypical esterase, and choline, TMA, and meperidine also were completely competitive inhibitors for the usual enzyme. However, decamethonium ($\alpha = 10.7$), chlorpromazine ($\alpha = 9.3$), and dibucaine ($\alpha = 8.7$) were not completely competitive inhibitors for the usual cholinesterase.

Purified esterase (Cholase) behaved usually like normal homozygous serum. However, in some specific tests, differences between a normal sample of serum and cholase were established by statistical means (analysis of variance) with $p < 0.01$. Thus, the concentration of procaine causing 50 per cent inhibition (I_{50}) was 4.42×10^{-5} M for purified esterase and 3.94×10^{-5} for the tested sample of normal serum. No difference between the DN-values of cholase and this sample of serum could be established. A similar difference was proved statistically between the values for I_{50} of succinylcholine for cholase and for another sample of normal serum. Since previous observations² had already suggested the existence of differences between sera with a normal DN-value, cholase was chosen as a standard to represent normal esterase in some tests which have been described.

DISCUSSION

In support of the conclusions drawn from genetic studies, the data presented in this paper suggest strongly the existence of two different types of serum cholinesterase which may occur alone or in a mixture. One conceivable alternative to this conclusion can be dismissed. Let us assume that there exists only one type of serum cholinesterase, and that the observed differences are due to unrecognised materials in some sera, which modify the esterase activity as physiological or pathological inhibitors or activators. Such materials might be either competitive, non-competitive, uncompetitive, or "discontinuous" agents.^{15, 16} Non-competitive agents by definition do not change Michaelis constants, and thus could not alter the susceptibility of an enzyme to experimentally-added inhibitors. Competitive agents would depress the apparent affinities for both substrate and added inhibitors by a constant factor, and thus could not produce the changing ratios of " I_{50} -atypical/ I_{50} -usual", which are shown in Table 1. The behaviour of uncompetitive and "discontinuous" agents is more difficult to predict. However, in each case, the intermediate curves (cf., Figs. 1-3) should be essentially parallel to the limiting curves, which they are not.

In short, the observed differences cannot be explained as being due to the presence of any reversible modifier of esterase activity. The data presented in this paper leave the possibility open that an inherited specific modifier, such as another protein, might be firmly and irreversibly attached to the enzyme. The results of physico-chemical investigations, to be published, suggest that the two enzymes differ little, if at all, in molecular weight and overall charge, and they confirm that there is no reversible modifier.

A plot of pI_{50} -usual against pI_{50} -atypical for the data listed in Table 1, suggests that the inhibitors can be divided into three groups (Fig. 7). First, there is the majority of inhibitors with pI_{50} -values related by the equation pI_{50} -atypical = $0.0028 + 0.785 pI_{50}$ -usual. The number 0.0028, which is graphically presented as the ordinate intercept, is negligible. Hence, the equation can be presented in exponential form: I_{50} -atypical = $(I_{50}$ -usual)^{0.785}. In other words, the difference between the susceptibilities to inhibition of the two enzymes increased in an exponential fashion with increasing potency of the inhibitors. This rule became apparent only after measuring inhibitors with a 20,000,000-fold range of potencies.

The second group of inhibitors consists of the two bis-quaternary ammonium compounds, C_{50} and succinylcholine. For these compounds, the above equations would predict a roughly tenfold difference between I_{50} -atypical and I_{50} -usual, while

the observed difference is more nearly hundredfold. A conceivable explanation is that these bis-quaternary compounds have a two-point attachment to the usual esterase, but only a one-point attachment to the atypical enzyme. This hypothesis is supported by the observations of a difference in slope of the concentration-inhibition curves for the two enzymes (Fig. 3) and of a difference in competitiveness of inhibition. The inhibition of the usual enzyme appeared to be partially non-competitive. Hence, one

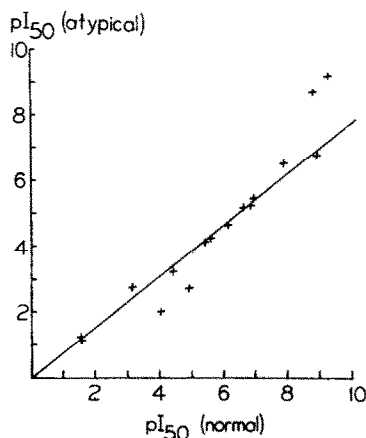


FIG. 7. Plot of pI_{50} -(normal = usual) against pI_{50} -(atypical) for the data listed in Table 1.

The two points above the regression line to the right represent DFP and TEPP; the two points below the line to the left represent decamethonium and succinylcholine. The indicated regression line was calculated excluding these four points. This regression is described by:

$$pI_{50}\text{-atypical} = 0.003 + 0.785 pI_{50}\text{-usual};$$

$$\text{correlation coefficient} = 0.995.$$

might visualize that both of the two quaternary nitrogens of, e.g. C_{10} , are attached to a negative charge on the surface of the usual enzyme, but that this negative charge is not always a part of the active centre of the enzyme; this also agrees with the slope in Fig. 3 which can be taken to indicate that every fourth molecule of C_{50} blocks simultaneously two active centres. By contrast, the completely competitive inhibition of the atypical enzyme by C_{10} , and the apparent 1:1 ratio of blocking molecule and active centre are both compatible with the assumption that only one quaternary nitrogen is attached to the enzyme; and that the point of attachment is always the anionic site¹⁷ of the active centre.

The third group of inhibitors consists of the phosphate inhibitors TEPP and DFP. The two enzymes were equally susceptible to these inhibitors. DFP and TEPP are postulated¹⁷ to react with the esteratic site of the active centre, without any direct attachment to the anionic site. By contrast, all other inhibitors which affected the two enzymes differently, probably react with either the anionic site exclusively, or both the anionic and esteratic sites.¹⁸ This suggests that the esteratic sites of the two choline esterases may be identical, but that the difference between the enzymes involves the anionic site. This difference could be due to a relatively weak effective charge in the anionic site of the atypical enzyme.

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BASIC TYPES OF CURARIFORM DRUGS

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Abstract—Curariform drugs may have a different mode of action in blocking neuromuscular transmission and thus in causing paralysis of skeletal muscle. This mode of action depends on the molecular properties of the drug and on the inherent properties of muscle and myoneural junction.

On basis of their chemical and pharmacological properties, curariform drugs have to be divided into at least three basic types.

Type I, the cholinomimetics or depolarizers, which have affinity to the ACh-receptors in the myoneural junction and which as well as ACh have a high intrinsic activity (e.g. suxamethonium).

Type II, the cholinolytics, curarimimetics or competitors, which have affinity towards the same receptors as Type I drugs but which have a very low intrinsic activity (e.g. D-tubocurarine).

Type III, the “non-competitors”, which have affinity towards other than ACh-receptors and which are non-competitive antagonists of ACh (e.g. Prestonal).

The features of these basic types were studied on the isolated frog’s rectus abdominis muscle and the nerve-muscle preparation of the cat and the chicken.

Gradual changes in the molecular configuration of curariform drugs cause gradual changes in the intrinsic activity and the affinity with consequently gradual changes from the one type to the other via intermediates or mixed types.

In his classical experiments Claude Bernard¹ showed that curare has its point of action where the motor nerve and striated muscle have their connections. Curare did not prevent conduction in the nerve, while the muscle as a result of the curare-action no longer responded to indirect stimulation, but continued to respond to direct stimulation.

Muscle relaxants, which obey these criteria and therefore interfere at the neuromuscular junction are called curariform drugs irrespective of whether or not their mechanism of action is identical with that of curare.

The mode of action of curariform drugs depends on the molecular properties of the drugs and on the inherent properties of muscle and myoneural junction.

Many vertebral skeletal muscles are composed of different types of muscle fibres: the “fast” or focally innervated twitch producing fibres and the “slow” or tonic contracture producing fibres.² Most mammalian muscles as e.g. the tibialis muscle of the cat contain only fast fibres, while especially avian and amphibian muscle may contain a considerable number of slow fibres. Experiments with the isolated frog’s rectus abdominis muscle give information about the slow fibres only, and the chicken gastrocnemius muscle-sciatic nerve preparation that of both fast and slow fibres.

Transmission through the myoneural junction is accomplished by the rapid release

		Curariform drugs
Type	Code	Chemical structure
I	SuCh	
	Imbretil	
I-II	C ₁₂ Me ₃	
	C ₁₀ Me ₂ Et	
	SuChMeEt ₂	
II	SuChEt ₃	
	Gallamine	
	D-tc	
II-III	Benzoquinonium	
	C ₁₀ Me ₂ Pr	
	C ₁₂ Me ₂ pNO ₂ Benz	
III	C ₁₂ Me ₂ Hept	
	Prestonal	
I-III	HeptNMe ₃	

of ACh at the nerve endings and the subsequent rapid enzymic breakdown by ACh-esterase. Drugs which interfere at the ACh-receptors or with the stimulus formed after occupation of the ACh-receptors with ACh, inhibit neuromuscular transmission and hence produce neuromuscular blockade. Owing to the inherent properties of fast and slow fibres, muscles containing only fast fibres are paralysed flaccidly, while the paralysis of muscles containing slow fibres as well, may be flaccid or spastic.

Curariform drugs usually are divided into two classes: Type I the depolarizers as e.g. suxamethonium and Type II the competitors as e.g. D-tubocurarine. Both types have affinity towards the specific ACh-receptors, but there is a difference in intrinsic activity.³ The depolarizers, including ACh, have a high and the competitors a very low intrinsic activity. When the enzymic hydrolysis of ACh is completely inhibited by high doses of ACh-esterase inhibitors a neuromuscular block of Type I is induced by neurogenous ACh.

In classifying curariform drugs one often encounters difficulties, for instance, the peculiar behaviour of dodecyltrimethylammonium has been revealed, when studying a series of alkyltrimethylammonium salts as muscle relaxants in the cat.⁴

Not being a depolarizer, this drug was hardly or not at all antagonized by neostigmine, while its action was synergistic to drugs of Type I. These findings were interpreted as a non-competitive inhibition of ACh by dodecyltrimethylammonium.⁵ A third type of curariform drug may therefore exist. Investigation of the series of alkyltrimethylammonium salts on the frog's rectus abdominis muscle showed that a non-competitive antagonistic action was introduced in the butyl homologue that became more pronounced with increasing length of the alkyl chain.⁶ Substitution of an alkyl group of increasing length for one methyl group on both quaternary N-atoms in decamethonium ($C_{10}Me_3$) led to a gradual change from drugs of Type I via drugs of Type II to a type of muscle relaxants which exert properties of Type III.⁷ The NN'-diheptyl homologue of decamethonium ($C_{10}Me_2Hept$) is a representative of this type.⁸ The features of the three basic types of curariform drugs may be summarized as follows:

Type I: the depolarizers, which also may be called cholinomimetics induce a flaccid paralysis of most mammalian muscles which is not reversible by neostigmine. In avian muscle they induce a spastic paralysis and in the isolated rectus abdominis muscle of the frog a contracture.

Type II: the curarimimetics, cholinolytics or competitors induce a flaccid paralysis of both mammalian and avian muscle which is easily reversed by neostigmine. They are mutual antagonists of drugs of Type I. The curarimimetics do not produce a contracture of slow muscles. On the rectus muscle of the frog they are competitive antagonists of cholinomimetics.

Type III: the non-competitive antagonists of ACh, which may be called "non-competitors" also produce a flaccid paralysis of both mammalian and avian muscle, which is however not reversed by neostigmine. They are synergists of drugs of Types I and II with respect to the paralysis of twitching muscle. The non-competitors also do not produce a contracture of slow muscles. On the frog rectus muscle they are non-competitive antagonists of the cholinomimetics. The non-competitive curariform drugs are supposed to block the transmission in the myoneural junction on receptors somewhere in the chain of events put in operation by the occupation of the ACh-receptors by ACh. Possibly the non-competitors have to be split up in different groups according to their point of action or their specific receptors.

Type IV: drugs, which block the synthesis of ACh in myoneural junction. The "hemicholiniums" possibly may serve as an example. The compound HC₃ produces a flaccid paralysis of both mammalian and chicken muscles which is reversed by choline¹⁷. On the frog rectus abdominis muscle HC₃ neither causes a contracture nor antagonizes the contracture as induced by ACh¹⁸.

The combination of the different types of curariform drugs result in various forms of drug interactions. Theories on drug actions and their mathematical development have been given earlier.^{3, 5, 6}

In searching for shortly-acting curarimimetics, Prestonal (3:14-dioxahexadecane-1:16-bis-(dimethyl, carbopropoxymethylammoniumbromide) was introduced. Preliminary clinical reports⁹ suggested that it was a muscle relaxant of a mixed Type (I-II). Further investigation¹⁰ revealed that edrophonium did not antagonize, but even potentiated the Prestonal induced neuromuscular block. On basis of these results Prestonal was supposed to be a relaxant of Type I. In, again, other clinical trials¹¹ Prestonal was mentioned to have a "dual blocking" mode of action (I-II). Other pharmacological studies¹² revealed a synergism of Prestonal to drugs of Type I (C₁₀Me₃) as well as to drugs of Type II (D-tc).

The structural resemblance of Prestonal and C₁₀Me₂Hept and the pharmacological properties hitherto known, led us to suppose that Prestonal is a curariform drug of Type III. The experiments confirmed this expectation.

On the isolated phrenic nerve-diaphragm muscle preparation of the rat, it could be demonstrated that Prestonal in a concentration of 2-5 μ mol/l, inhibits neuromuscular transmission without affecting the responses to direct stimulation of the muscle and without inhibiting the transmission along the nerve when applied directly to the phrenic nerve even in concentrations 100 times higher. Prestonal therefore is a curariform drug.

The different features of the three basic types of curariform drugs and their intermediates or mixed types were investigated upon the isolated frog rectus abdominis⁸ and the nerve-muscle preparation of the cat and the chicken.¹³ On the frog rectus cumulative log-dose-response curves were made for various cholinomimetics in the absence and in the presence of certain doses of drugs of Type II and Type III (see Fig. 1). The contracture induced by suxamethonium and Imbretil demonstrates a Type I, the competitive antagonistic properties of D-tc a Type II and the non-competitive antagonistic action of Prestonal a Type III action. On the chicken sciatic nerve-gastrocnemius muscle preparation suxamethonium (Fig. 2) induces a contracture (Type I), D-tc a flaccid paralysis, which is easily reversed by neostigmine (Type II), while Prestonal also induces a flaccid paralysis which, however, is not reversed but rather enhanced by neostigmine (Type III). The contracture induced by SuCh and other drugs of Type I is antagonized both by drugs of Types II and III as e.g. D-tc and Prestonal. The inhibition by D-tc can be overcome with higher doses of SuCh, which indicates a competition.

This is in contrast with the inhibition with Prestonal, which cannot be overcome by higher doses of SuCh, suggesting a non-competitive antagonism. On the peroneus nerve-tibialis muscle preparation of the cat SuCh, D-tc and Prestonal produced a flaccid paralysis. Contrary to D-tc, SuCh and Prestonal are not antagonized by neostigmine. It is therefore concluded that Prestonal is a curariform drug of the non-competitive type. The easily hydrolyzable ester bonds in Prestonal are mainly responsible for its short action.

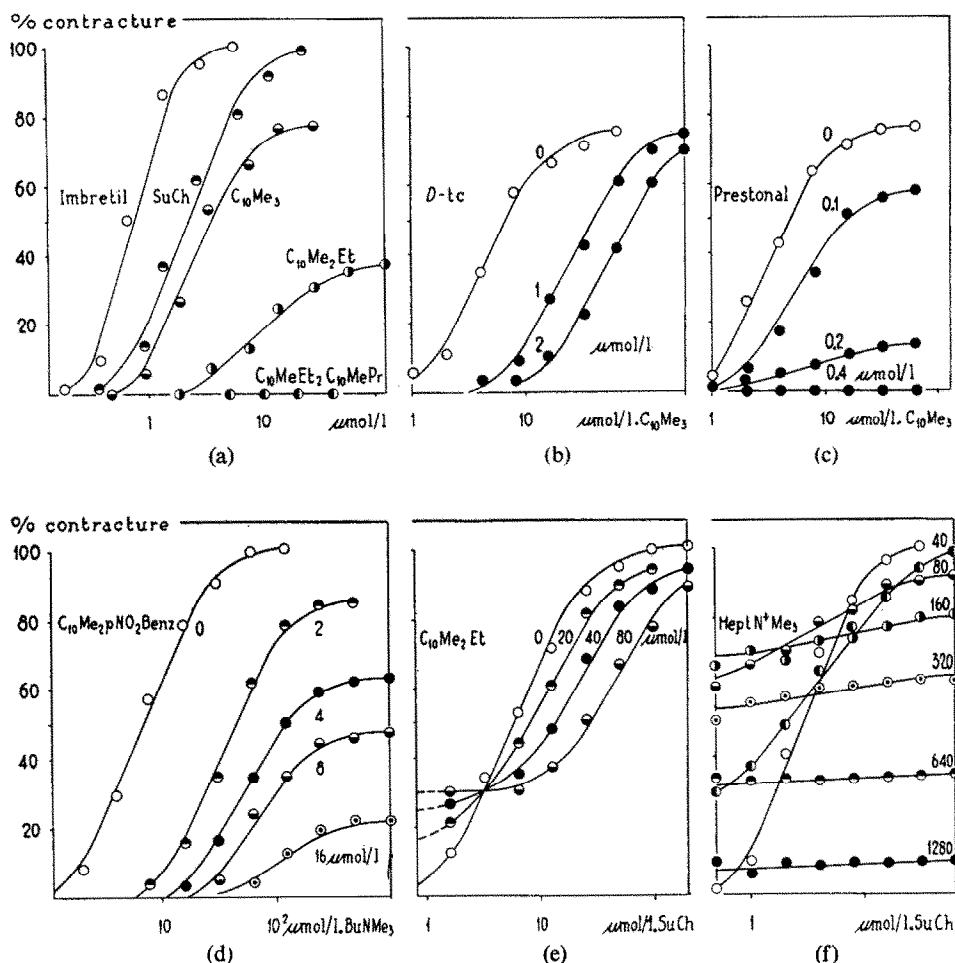


FIG. 1. Cumulative log dose-response curves for cholinomimetics performed on the isolated frog's rectus abdominis muscle in the absence and in the presence of constant doses of various curariform drugs. Note the agonistic properties of SuCh and Imbretil (Type I) as a contracture (Fig. 1a), the competitive antagonistic properties of D-tc (Type II) as a parallel shift of the curves (Fig. 1b), the non-competitive antagonistic properties of Prestonal (Type III), as a decline of the curves and a decrease of the maximal height (Fig. 1c), the competitive and non-competitive properties of $\text{C}_{10}\text{Me}_2\text{pNO}_2\text{Benz}$ (Type II-III) as a shift and a decline of the curves (Fig. 1d), the agonistic and competitive antagonistic properties of $\text{C}_{10}\text{Me}_2\text{Et}$ (Type I-II) as a contracture and a shift of the curves (Fig. 1e) and the agonistic and non-competitive antagonistic properties of Hept N^+Me_3 (Type I-III) as a contracture and a decline of the curves (Fig. 1f).

It is to be expected from the theories on drug actions and experimental data that with gradual changes in the molecular structure of curariform drugs, gradual changes in the parameter's intrinsic activity and affinity and consequently of one type into the other via intermediates will take place. So the introduction of heavier substituents on the quaternary nitrogen atoms results in a gradual change from Type I into Type II drugs (see the table). Introduction of substituents of increasing chain length as well as lengthening of the chain in between the onium groups results in an increase in

affinity to the non-competitive receptor system and thus in a gradual change from Type I or II into Type III drugs (see the table).

Depending upon the intrinsic activity, curariform drugs are mainly agonistic (I) when the i.a. is high, mainly competitive antagonistic (II) when the i.a. is very low and intermediate or mixed I-II when the i.a. is intermediate. Depending on the ratio, r , of the affinities to the specific acetylcholine receptors and the non-competitive receptors these drugs are mainly agonistic (I) or competitive (II) when r is large, both agonistic and non-competitive (I-III) or both competitive and non-competitive (II-III), when r is intermediate and mainly non-competitive (III) when r is small.

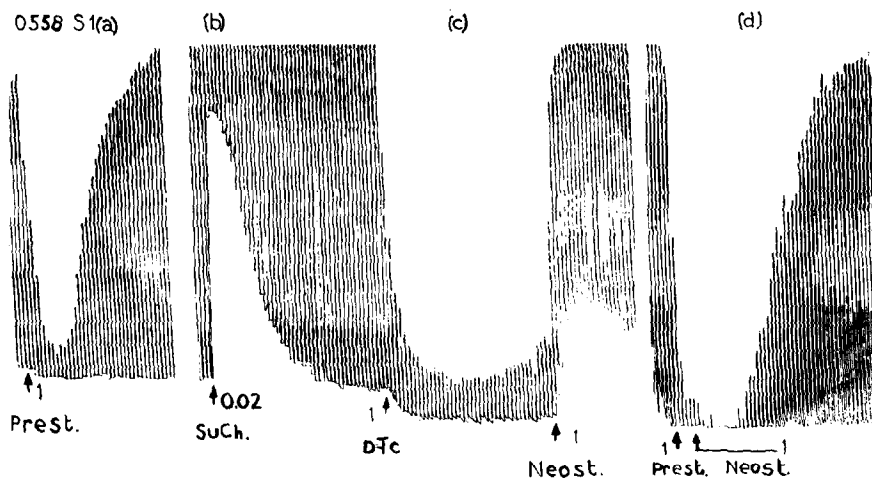


FIG. 2. The effects of three types of curariform drugs on the semi-isotonic responses of the chicken gastrocnemius muscle to faradic stimulation (300 H, 1 msec, 0.2 mA) of the sciatic nerve every 10 sec. Drugs were administered by rapid injection into the jugular vein. Prestonal (a) induces a flaccid paralysis of short duration. Suxamethonium (b) produces a contracture (Type I), D-tubocurarine (c) induces a flaccid paralysis which is completely reversed by neostigmine (Type II). The flaccid paralysis as induced by Prestonal (d) however is not reversed but rather enhanced by neostigmine (Type III). Doses were added in μmol to a chicken of 2 kg (1/3 of full size).

Examples of intermediates between I and II are the partially ethyl substituted derivatives of SuCh and C_{10}Me_3 . The dualistic behaviour (I-II) of SuChMeEt_2 was demonstrated by Ginzel *et al.*¹⁴ on the pigeon sciatic nerve-gastrocnemius muscle preparation. Also decamethonium exerts a dualism in action owing to the fact that its intrinsic activity is lower than that of SuCh (see Fig. 1a). $\text{C}_{10}\text{Me}_2\text{Et}$ has still a lower intrinsic activity than C_{10}Me_3 and more pronounced dualistic behaviour⁸ (see Fig. 1e).

Examples of intermediates between II and III are $\text{C}_{10}\text{Me}_2\text{Pr}$, $\text{C}_{10}\text{Me}_2\text{Bu}$ and $\text{C}_{10}\text{Me}_2\text{pNO}_2\text{Benz}$ (Fig. 1d). On the frog muscle they revealed a competitive and non-competitive antagonistic action.⁸ Also benzoquinonium, although mainly curarimimetic is of a mixed II-III type. It induces a flaccid paralysis on both cat and chicken muscle. The paralysis on the cat is not, and that on the chicken is to a variable extent antagonized by ACh-esterase inhibitors. On the frog rectus there is an indication of only a slight non-competitive component in its action. The ethyl homologue of benzoquinonium is more purely a curarimimetic.¹⁵ The compound $\text{C}_{10}\text{Me}_2\text{Pr}$ resembles $\text{C}_{10}\text{Me}_2\text{Et}$ and has an intrinsic activity not yet zero. So on the chicken muscle a slight

contracture is obtained. This implies that in fact $C_{10}Me_2Pr$ has characteristics of all three basic types.

Examples of intermediates between I and III are found in the trimethylalkyl-ammonium series for the pentyl, hexyl and heptyl derivatives. E.g. on the frog rectus they exhibit a non-competitive auto-inhibition (Fig. 1f). Since intrinsic activities and affinities are parameters of drug and biological object, especially the intermediate compounds may vary in their mode of action on different muscles of the same species and on those of different species. Of course also the possible ACh-esterase inhibiting properties of the curariform drugs may influence the picture obtained.

Investigations of ganglionic blocking agents on, for instance, the rectus abdominis muscle of the frog suggest that also these drugs may be divided into three classes.¹⁶

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REACTIVATION OF ALKYLPHOSPHATE INHIBITED ACETYLCHOLINESTERASE BY BIS QUATERNARY DERIVATIVES OF 2-PAM AND 4-PAM*

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Abstract—Symmetrical and unsymmetrical bis-quaternary derivatives of 2-PAM and 4-PAM were synthesized and studied as reactivators *in vitro* of acetylcholinesterase inhibited by TEPP and DFP. Certain of the 2-PAM-derivatives and all of the 4-PAM-derivatives were far superior to the parent compound in the sense of producing reactivations at lower concentrations. Despite the fact that 2-PAM is much more active than 4-PAM, the bis-quaternary derivatives of the latter were considerably more active in regard to DFP inhibition, but only slightly more active in regard to TEPP inhibition.

The best compound with regard to DFP inhibition was the unsymmetrical bis-quaternary salt of 4-PAM and pyridine with a pentamethylene linking chain.

In general, the addition of extra binding features in these series produced better reactivators.

ACETYLCHOLINESTERASE is inhibited irreversibly by a large number of organophosphates such as tetraethylpyrophosphate (TEPP) and di-isopropylfluorophosphate (DFP). The inhibited enzyme can be reactivated very readily by pyridine-2-aldoxime methiodide (2-PAM) and by the less active pyridine-4-aldoxime methiodide (4-PAM).^{1, 2} These compounds are especially active because they are bound by the inhibited enzyme in such a manner that the functional group the nucleophilic oximate oxygen atom, falls one bond length from and is directed towards the phosphorus atom (Fig. 1) of the phosphorylated (inhibited) enzyme.³

The mechanism and kinetics for reactivation are similar to that of an enzyme and its substrate: there is a binding constant for the reactivator and the phosphorylated enzyme and a maximum velocity.⁴ Therefore, it would appear that the addition of any binding features to the molecule would improve the activity in the sense of lowering the concentration at which reactivation occurs, provided of course that the maximum velocity was not changed. The maximum velocity will be unchanged if the orientation, as shown in Fig. 1, is not changed and if the nucleophilicity of the oxygen atom is maintained. In fact, considerations such as this led to the design of 2-PAM and 4-PAM.³ Since 2-PAM is extremely active, it might be assumed that the orientation is as good as can be achieved and that the lesser activity of 4-PAM is caused by a poorer orientation. Such a situation would imply a lower $V_{\max}$ for 4-PAM, which in fact is true.

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It is known that di-quaternary ammonium ions are better reversible, competitive inhibitors of acetylcholinesterase than are mono-quaternary ammonium ions. This is attributed to a second negatively charged region not too far removed from the active site of the enzyme. This second anionic region may be part of an adjacent enzymatic

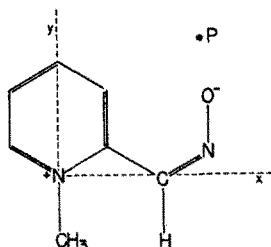
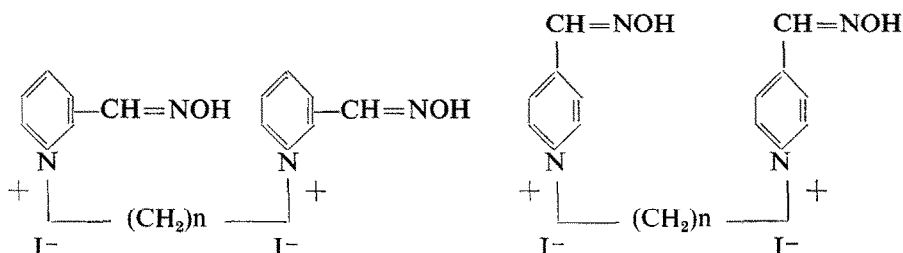


FIG. 1. The orientation of 2-PAM on the phosphorylated enzyme. The origin of co-ordinates is taken to coincide with the anionic site of the enzyme, the y axis with the preferred direction for the orientation of a ring and the x-y plane is defined by the plane of the ring. The phosphorus atom of the inhibited enzyme falls as shown at the point 3.4 Å, 3.3 Å, 0 Å.

site or may be accidental, i.e., it does not normally have a catalytic function. Therefore, introducing a second quaternary nitrogen function would appear to be an appropriate way to increase the activity of reactivators. This is most easily achieved by making bis-compounds such as:



Because of its greater activity, it appeared most reasonable, despite the greater synthetic difficulties, to devote greater attention to derivatives of 2-PAM, but the first compounds studied, $n = 3$ and $n = 10$, were disappointing. However, the derivatives of 4-PAM were much more rewarding. The synthesis of bis-4-PAM compounds ($n = 3, 4, 5, 10$) was reported in a talk by Poziomek, Hackley, and Steinberg of the Army Chemical Center;⁵ it was reported that at least one of these compounds is a more rapid antidote for ethoxymethyl fluorophosphonate (sarin) poisoning than is 2-PAM. Although they did not present enzyme reactivation data, it appeared that these compounds might well be more rapid as reactivators, *in vitro*, than is 2-PAM. These authors stated that they were unable to obtain bis-derivatives of 2-PAM. We have obtained these compounds, but only in very low yield.

In this paper we report on the synthesis of bis-compounds of the 2-PAM and 4-PAM series and on the reactivating capacities of these substances, with special emphasis on the theoretical considerations already outlined.

METHODS

Preparation of compounds

The bis-compounds were prepared from the tertiary oximes and straight chain dihalogen compounds.

*Bis-quaternary derivatives of the 4-PAM series.** General procedure:

aliphatic dihalide	5 mmoles
pyridine-4-aldoxime ⁷	20 mmoles
dimethyl formamide	10 ml

The solution was refluxed for 2-4 hr, and the diquaternary salt precipitated with absolute ether. Purification was effected by solution in 50 per cent methanol-water and extraction of unreacted dihalide and aldoxime with chloroform. The solution was dried under vacuum and the residue was either recrystallized from absolute methanol or washed with a small amount of cold ethanol.

TABLE 1

<i>n</i>	Halogen	Per cent yield	M.p. (°C)	Neutral calc.	equivalent found	Per cent halide calc.	halide found
1	I	3	188	256	256	49.6	50.3
2	Br	5	260	216	200	37.1	34.9
3	I	45	205	270	277	47.0	46.7
4	Br	35	244	230	236	34.8	35.0
5	I	23	126-7	284	290	44.7	50.1
6	Br	62	208	244	245	32.8	32.6
10	Br	28	216-7	272	279	29.4	29.0

Bis-quaternary derivatives of the 2-PAM series. The procedure was the same as above except that the reaction mixture was evaporated under vacuum and the dark oily residue dissolved in 25 per cent methanol and exhaustively extracted with chloroform. The residue was washed several times with either methanol, or absolute ethanol or isopropanol, until a satisfactory product was obtained. The yields were low.

TABLE 2

<i>n</i>	Halogen	Per cent yield	M.p. (°C)	Neutral calc.	equivalent found	Per cent halide calc.	halide found
3	Br	2	238	223	227	35.9	35.6
3	I	2	213	270	274	47.0	46.7
4	Br	3	262	230	238	34.8	35.1
5	I	7	208	284	279	44.7	44.5
6	Br	2	217-18	244	250	32.8	33.1
10	Br	7	97-8	272	276	29.4	29.0

5-Iodopentyl trimethyl ammonium iodide

1,5-di-iodopentane	7.5 g
benzene	10.0 ml
trimethylamine (25 per cent in methanol)	5 ml

Trimethylamine solution was added dropwise with stirring to the benzene solution of

* These compounds have been prepared by POZIOMEK, HACKLEY and STEINBERG. Their method of preparation is in press.⁶

di-iodopentane and the mixture was left overnight at room temperature. The methanol was evaporated until the salts began to separate. Precipitation was completed by addition of ether. The mixture of mono- and di-quaternary salts was extracted with boiling acetone, in which only the mono-quaternary is soluble. On cooling the product crystallized. Yield: 2.0 g, 35 per cent, m.p.: 164–165°C; per cent iodide: calc., 33.2; found, 32.8.

Unsymmetrical bis-quaternary salt of pyridine-4-aldoxime and trimethylamine in which the linking chain is pentamethylene

5-Iodopentyl trimethyl ammonium iodide	0.77 g
pyridine-4-aldoxime	0.30 g
dimethyl formamide	8 ml

Solution was heated on steam bath for 2 hr, cooled and an oil was thrown out with absolute ether. The oil was separated by decanting and was dissolved in warm acetone. On cooling, a small amount of oil separated and was discarded. The bis-quaternary salt was precipitated with ether and recrystallized from absolute ethanol. Yield: 0.38 g, 38 per cent; m.p.: 163–164°. Neutral equivalent: calc., 505; found, 500; per cent iodide: calc., 50.3; found, 50.0.

5-Iodopentylpyridinium iodide

1, 5-di-iodopentane	13 g
dimethyl formamide	20 ml
pyridine	2.5 ml

The pyridine was added dropwise with stirring to the solution of di-iodopentane in dimethyl formamide and left at room temperature overnight. The solution was evaporated under reduced pressure and the oily residue washed with ether, dissolved in boiling acetone, filtered hot and cooled. The product was precipitated with ether. Yield: 5.5 g, 46 per cent; m.p.: 73°; per cent iodide: calc., 31.5; found, 30.9.

Unsymmetrical bis-quaternary salt of pyridine-4-aldoxime and pyridine in which the linking chain is pentamethylene. This was prepared in the same manner as was the trimethyl ammonium derivative, and was recrystallized from methanol. Yield: 40 per cent; m.p.: 145–148°. Neutral equivalent: calc., 525; found, 548; per cent iodide: calc., 48.4; found, 47.9.

Unsymmetrical bis-quaternary salt of pyridine-2-aldoxime and trimethylamine in which the linking chain is pentamethylene. This was prepared in the same manner as was the 4-derivative; it was recrystallized first from acetone and then from methanol. Yield: 7 per cent; m.p.: 206–207°. Neutral equivalent: calc., 505; found, 520; per cent iodide: calc., 50.3; found, 51.1.

Alkylidides of 2-PAM and 4-PAM:

alkyl iodide	1 mmole
pyridine aldoxime	1.25 mmoles
dimethyl formamide	10 ml

The solution was kept at room temperature for two days (the two derivative was heated on a steam bath for 2 hr first), then it was evaporated under vacuum. The remaining oil was extracted with ether and dissolved in methanol. The product was precipitated with ether, redissolved in methanol, and again precipitated with ether.

TABLE 3

Compound	Per cent yield	M.p. (°C)	Neutral equivalent		Per cent halide	
			calc.	found	calc.	found
Pyridine-4-aldoxime pentiodide	40	119	320	315	39.7	39.5
Pyridine-2-aldoxime pentiodide	10	100	320	335	39.7	38.8
Pyridine-4-aldoxime dodecioidide	75	101	418	420	30.4	30.0

ANALYSIS

Iodides and bromides were assayed by the Volhard method. Neutral equivalents were determined by potentiometric titration with 0.1 N NaOH. This affords an additional check on the identity and purity of the compounds, because a "clean" curve should be obtained with proper pK_a values, namely, in the neighborhood of 8. For the dibasic acids only one break was obtained, so that the pH at one-half neutralization is the average of the two pK_a values. These compounds are yellow in alkaline solution.

Enzyme. A purified acetylcholinesterase from the electric organ of *Electrophorus electricus*⁸ which hydrolyzes 0.9 mmole of acetylcholine per min per mg of protein in a medium of 10^{-3} M substrate, 0.1 M NaCl and 10^{-4} M EDTA; the reaction was carried out at pH 7 and 25°C. To 0.2 ml of enzyme, 0.01 ml of TEPP solution containing 0.1 μ g (or 0.5 μ g of DFP) was added in the cold. After two hours, the solution was diluted 10-fold and used as a stock for inhibited enzyme. Usually this stock was diluted another 40-fold before reactivation. Reactivation was carried out in 0.03 M NaCl, 0.001 M sodium phosphate buffer of pH 7.8, 10^{-4} M EDTA, 25°C.

Of the reactivated enzyme solution, 0.1 ml is sufficient for measuring high reactivations, but for lesser reactivations a correspondingly larger aliquot was used.

Enzyme activities were measured under N_2 (to exclude CO_2) in 100 ml of solution by automatic continuous titration (Beckman). Alkali (0.01 N) was added in units of 0.7 λ from a screw-driven syringe. This screw was coupled to a rotary solenoid which was energized by the pulses from the titrator. The number of pulses was recorded by an electrical counter.

RESULTS AND DISCUSSION

Typical reactivations of diethylphosphoryl enzyme (TEPP inhibition) *in vitro* and of di-isopropyl phosphoryl enzyme (DFP inhibition) are given in Tables 1 and 2. In general, we note that all the 4-PAM derivatives, including two without a second quaternary group, are markedly better than the simple compound. It is evident (DFP data) that the maximum velocity has been increased suggesting that the extra binding contributions have also altered the binding orientation and, indeed, have improved it. In contrast, all derivatives of 2-PAM, except bis-quaternary compounds with $n = 5$ or 6, are poorer than the simple compound. It is suspected that the maximum velocity has been reduced. The very high activity of 2-PAM, and, in particular, its marked superiority to 4-PAM, would seem to demand that its binding orientation be very good and, therefore, any reorientation would necessarily be detrimental.

The bis-4-derivatives are very much better than 4-PAM, even for $n = 1$, and a plateau appears to be reached at $n = 3$, which is about 500 times better than the simple compound for TEPP inhibition. For DFP inhibition the comparison is even more startling, the bis-compound, $n = 5$, is 1000 times better than 4-PAM and 40 times better than 2-PAM in the sense of activity at very low concentration. However, we do not have data for the maximum velocities, with which the comparisons will surely be less extreme.

The bis-derivatives of 2-PAM are just about as active as 4-PAM derivatives with respect to TEPP inhibition, but the latter $n = 5$ is about 8 times better with DFP inhibition.

TABLE 4. REACTIVATION BY BIS-QUATERNARY DERIVATIVES OF 2-PAM AND 4-PAM

The reactivations are for 6 min at 25°C; pH = 7.8; 0.03 M NaCl

Compound	pH mid pt.	TEPP inhibition		DFP inhibition	
		concentration	per cent reactivation	concentration	per cent reactivation
2-PAM	8.0	10^{-6} 5×10^{-7}	40 23	10^{-4} 5×10^{-5}	50 28
$n = 3$	7.8	2×10^{-5} 10^{-5} 5×10^{-6}	45 32 17		
$n = 4$	7.8	10^{-6} 5×10^{-7}	27 15		
$n = 5$	7.9	2×10^{-7} 10^{-7}	22 13	10^{-5} 5×10^{-6}	26 14
$n = 6$	7.8	10^{-6} 5×10^{-7} 10^{-7}	17 13 5		
4-PAM	8.6	5×10^{-5} 10^{-5}	19 5	2×10^{-3} 10^{-3} 4×10^{-4} 10^{-4}	9.5 9.1 7.2 2.5
$n = 1$	7.6	2×10^{-6} 10^{-6}	15 8		
$n = 2$	7.8	10^{-6} 5×10^{-7}	26 16		
$n = 3$	8.2	10^{-7} 5×10^{-8}	18 9		
$n = 4$	8.2	10^{-7} 5×10^{-8}	18 11		
$n = 5$	8.3	10^{-7} 5×10^{-8}	18 11	2.5×10^{-6} 1.2×10^{-6}	46 26
$n = 6$	8.3	2×10^{-7} 10^{-7}	26 15		
$n = 10$	8.4	10^{-7} 5×10^{-8}	15 9		

Two functional groups are not necessary for the enhanced activity, as indicated by the unsymmetrical bis-4-PAM derivatives. Only the binding contribution is necessary. Interestingly enough, 4-PAM-pyridine, which is the best compound for DFP inhibition, is hardly more active against TEPP inhibition than it is against DFP inhibition. The unsymmetrical bis-compound of 2-PAM, although better than 2-PAM for TEPP inhibition, is poorer than 2-PAM against DFP inhibition.

The conclusion that the addition of extra binding features has improved the activity, in the sense of lowering the concentration at which activity occurs, is quite evidently justified.

TABLE 5. REACTIVATION BY UNSYMMETRICAL BIS-QUATERNARY AND OTHER DERIVATIVES OF 2-PAM AND 4-PAM

The reactivations are for 6 min at 25°; pH = 7.8; 0.03 M NaCl. Unsymmetrical bis-quaternary salts of pyridine aldoxime (2- or 4-) and trimethylamine or pyridine in which the linking chain is pentamethylene. Derivatives of pyridine aldoxime in which the N-methyl group is replaced by a longer aliphatic chain.

Compound	pH mid pt.	TEPP inhibition		DFP inhibition	
		concentration	per cent reactivation	concentration	per cent reactivation
2-PAM-trimethylamine	7.8	10^{-5}	62	10^{-4}	31
		2×10^{-6}	27	5×10^{-5}	18
		10^{-6}	13		
4-PAM-trimethylamine	8.3	2×10^{-7}	15	5×10^{-6}	23
		10^{-7}	9	12.5×10^{-6}	13
4-PAM-pyridine	8.2	10^{-6}	49	10^{-6}	39
		3×10^{-7}	26	5×10^{-7}	25
		1.5×10^{-7}	16		
Pyridine-2-aldoxime pentiodide	8.0	4×10^{-6}	34		
		2×10^{-6}	18		
Pyridine-4-aldoxime pentiodide	8.5	10^{-5}	33	10^{-4}	22
		5×10^{-6}	19	5×10^{-5}	13
Pyridine-4-aldoxime dodecioidide		10^{-6}	29		
		5×10^{-7}	16		

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THE REACTION OF MALIC DEHYDROGENASE WITH α -KETO β -FLUORO-SUCCINIC (β -FLUORO-OXALOACETIC) ACID*

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Abstract—The preparation of crystalline β -fluoro-oxaloacetic acid (FOAA) from its diethyl ester is described. FOAA is a competitive inhibitor of malic dehydrogenase.

MALIC dehydrogenase was obtained in pure form from pig heart by Wolfe and Neilands¹ and from mitochondria of ox heart by Davies and Kun.² A specific inhibitor of this enzyme has so far not been described. In the course of search for such an inhibitor β -fluoro-oxaloacetic acid (FOAA) was prepared in pure form and its effect on enzymatic reactions catalyzed by malic dehydrogenase² determined. The diethyl ester of FOAA and its Na-enolate have been previously tested for their inhibitory effects on multi-enzyme systems by Watland *et al.*³ and Gal,⁴ but these forms of FOAA are not suitable for more definitive enzyme inhibition studies. The free acid has hitherto not been prepared. In this paper the preparation and properties of FOAA will be described and it will be shown that it is a competitive inhibitor of malic dehydrogenase.

EXPERIMENTAL

Preparation and properties of FOAA

Diethyl fluoro-oxalacetate was prepared according to Rivett.⁵ It was converted to the free acid under conditions favoring transesterification.⁶ Hydrolysis of the ester by hydrochloric acid has been previously attempted without success.^{7, 8} The procedure used was as follows: 25 g of diethyl fluoro-oxaloacetate were dissolved in 240 ml of glacial acetic acid, then 120 ml of concentrated hydrochloric acid added. The mixture was kept at room temperature for three days, then evaporated to dryness at room temperature under reduced pressure by means of a Rinco rotating evaporator. The residue was 18.4 g of crude crystalline FOAA (85.5 per cent yield). This was purified by recrystallization from ether-petroleum ether (b.p. 30–60°C). After two recrystallizations which resulted in about 50 per cent recovery, the m.p. was 86–87° (decarboxylation) as determined with a Fisher-Johns block. Analysis: calculated for $C_4H_3O_5F$: 1.5 H_2O ; C, 27.13; H, 3.41; F, 10.73. Found: C, 27.59; H, 3.67; F, 10.7. Neutralization equivalent: calculated 88.5; found 89.0. 2,4-dinitrophenyl-hydrazone: m.p. = 210–214° (starts decomposing between 90° and 150°C depending on the rate

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† Established Investigator of the American Heart Association, Inc.

of heating). Calculated for $C_{10}H_7N_4O_8F$: C, 36.37; H, 2.14; N, 16.97. Found: C, 36.6; H, 2.3; N, 16.9. This derivative is very hygroscopic.

An aqueous solution of FOAA gives a deep purple-red color with aqueous $FeCl_3$. The color reaches its maximum intensity after about ten minutes.

The crystalline acid is stable for about a month at room temperature, then slowly decarboxylates. FOAA is extremely soluble in water.

Analysis and neutralization equivalent show that FOAA contains 1.5 moles of H_2O . Spectral evidence indicates that the keto group is in the hydrated form. This would be expected from the strongly electron attracting properties of fluorine which increase the positive character of the neighboring carbon atoms.

The UV spectra of aqueous solutions of OAA and FOAA were determined at pH 1.5 (OAA, $E^* = 612$; FOAA, $E = 96$) and at pH 11 (OAA, $E = 798$; FOAA, $E = 246$) at $\lambda_{max} = 265 m\mu$ for OAA and $\lambda_{max} = 270 m\mu$ for FOAA. The UV absorption spectra of both acids are shown in Fig. 1. It is apparent that in aqueous solutions FOAA is not appreciably enolized. This is further confirmed by infrared (IR) spectra, which are shown in Fig. 2.

Examination of the IR spectra shows that:

(1) FOAA does not exhibit the strong band at 6.1μ , which is given by OAA and has been attributed to the resonance-stabilized chelated enol.^{9†} The hydrated form could not give an enolic chelate. The changes in the OH region are consistent with this view.

(2) FOAA has only one carbonyl band in the 5μ region (carboxyl), whereas OAA has two bands in this region (keto group and carboxyl).

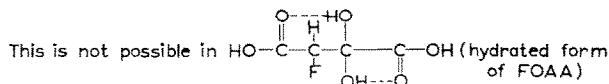
(3) FOAA has a band at 9.14μ , due to the C-F bond,⁸ which is absent in OAA.

Preparation of malic dehydrogenase

Malic dehydrogenase of rat liver mitochondria was prepared as described by Davies and Kun,² except the enzyme was not purified beyond the second stage. In calculating turnover numbers and expressing the amounts of enzyme, the molecular

$$* E = \frac{A}{\%} \times \frac{\text{Mol. wt.}}{10}$$

† This is due to a carbonyl in which the double bond character has been weakened by resonance as follows.



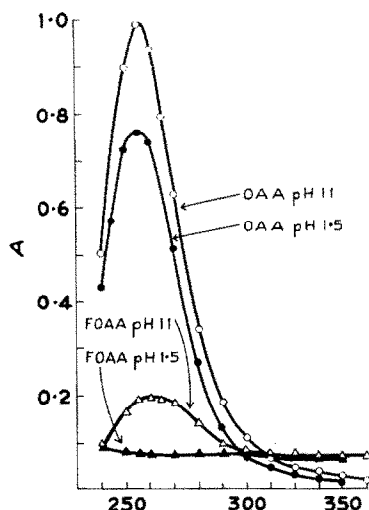


FIG. 1. UV. absorption spectra of OAA and FOAA at pH 1.5 and 11.0. OAA = 16.4 mg per cent, FOAA = 14.0 mg per cent. Readings were made with the Beckman DU spectrophotometer.

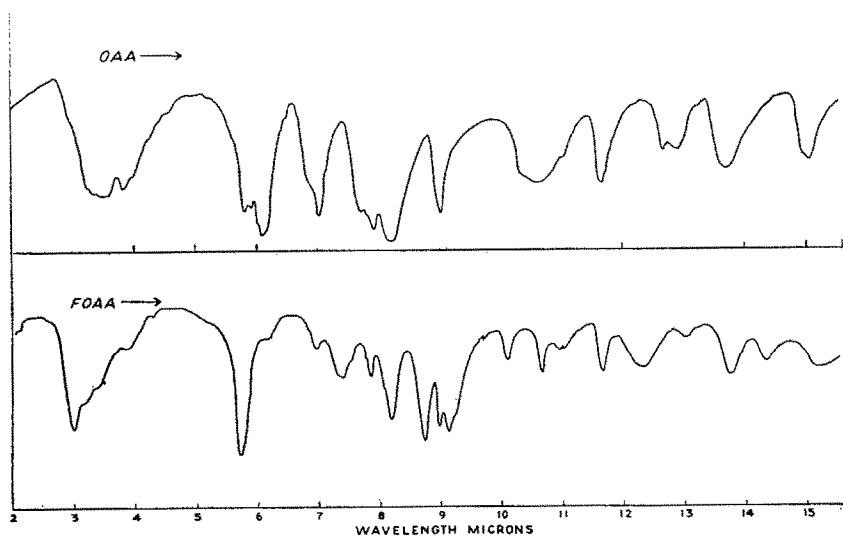


FIG. 2. I.R. spectra of FAA and FOAA were made with the PERKIN-ELMER 21 instrument in KBr disks.

weight of 15,000 was used for mitochondrial malic dehydrogenase.² Conditions of enzyme assay as well as specification of substrates were the same as previously described.² Determinations of K_1 were carried out by the method of Dixon.¹⁰

The effect of FOAA on reduction of OAA

The effect of FOAA on the reduction of OAA to malic acid by DPNH in the presence of malic dehydrogenase is shown in Fig. 3. In the presence of 20 μ moles of OAA per 3 ml reaction mixture, an approximately equal amount of FOAA (23.5

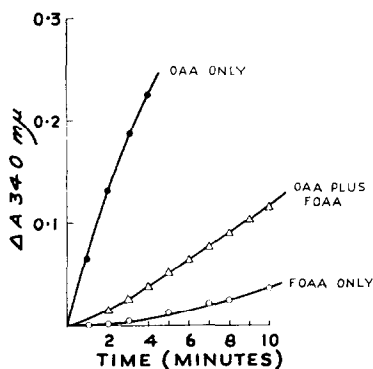


FIG. 3. The effect of FOAA on the reduction of OAA by DPNH in the presence of malic dehydrogenase. pH = 6.8; OAA = 20 μ moles; FOAA = 23.5 μ moles; enzyme = 0.3×10^{-3} μ moles; versene 69 μ moles; volume = 3.0 ml. All reductions of OAA and FOAA were made in 0.5 M PO_4 buffer.

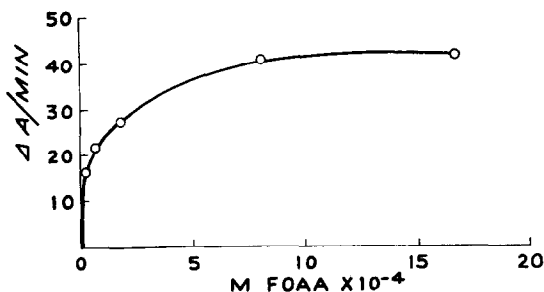


FIG. 4. The reduction of FOAA by DPNH in presence of dehydrogenase. DPNH = 0.28 μ moles. Enzyme = 0.72×10^{-3} μ moles; pH = 6.8; versene = 69 μ moles volume = 3.0 ml.

μ moles) caused marked inhibition. It was noticed that a slow but definite rate of DPNH oxidation occurred in the presence of FOAA alone. This reaction was studied in the presence of high enzyme concentration and it was clearly shown that FOAA is enzymatically reduced to fluoromalate. The relationship between FOAA concentration and reaction rate is shown in Fig. 4. K_m for FOAA was found to be 1.3×10^{-4} M. While the turnover number for OAA is 12,200,² for FOAA it is between 2 and 3. These observations suggest that FOAA is a competitive inhibitor of mitochondrial malic dehydrogenase. Kinetic measurements substantiated this prediction.

Competition between OAA and FOAA is shown in Fig. 5, which is a typical Dixon type plot.¹⁰ The K_i value of 2.8×10^{-5} was determined by this graphical method.

Since K_m for OAA was known,² $\frac{1}{V_{\max}}$ could be calculated. The horizontal line drawn through the $\frac{1}{v}$ axis at $\frac{1}{V_{\max}}$ meets the point of intersection of the lines constructed from rate values at two OAA concentrations.

The inhibition of malate oxidation by FOAA is shown in Fig. 6. The value of K_i of 7.4×10^{-6} was determined graphically from reaction velocity data obtained at various

INHIBITION OF
OAA $\rightarrow$ MALATE
REACTION BY FOAA

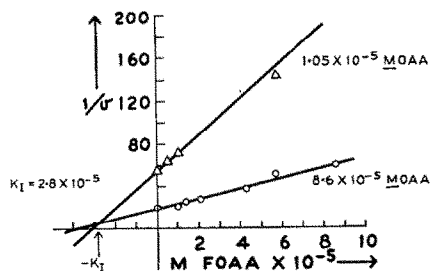


FIG. 5. Competition between OAA and FOAA. Enzyme = 0.35×10^{-3} μ moles; DPNH = 0.28 μ moles.

INHIBITION OF
MALATE $\rightarrow$ OAA
REACTION BY FOAA

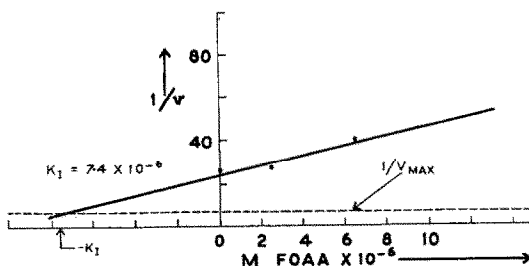


FIG. 6. Inhibition of malate oxidation by FOAA; malate = 50 μ moles; DPN = 1.5 μ moles; Tris buffer 0.1 M, pH 8.4; enzyme = 0.38×10^{-3} μ moles; volume = 3.0 ml.

FOAA concentrations and from known values of K_m for malate (between 5×10^{-4} to 10^{-2} M).²

Similar competitive type of inhibition was obtained with other substrates of mitochondrial malic dehydrogenase such as meso-tartrate (Fig. 7) and dioxosuccinate. The extreme instability of dioxosuccinate made kinetic measurements somewhat uncertain and the K_i value of 1.8×10^{-5} is a close approximation for this substrate.

INHIBITION OF
MESOTARTRATE $\rightarrow$ DHF
REACTION BY FOAA

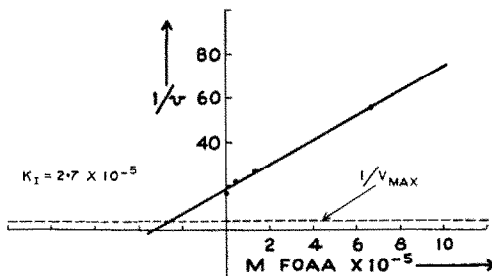


FIG. 7. Inhibition of oxidation of meso-tartrate by FOAA. Mesotartrate = 150 μ moles; DPN = 1.5 μ moles; Tris buffer 0.1 M, pH 8.4; enzyme = 1.4×10^{-3} μ moles.

DISCUSSION

The simplest interpretation of the kinetic data is that FOAA inhibits malic dehydrogenase by combining with the enzyme at the site of OAA. The introduction of the fluorine atom into OAA increases its acid strength* and markedly reduces enolization, as shown by UV and IR spectra. These two effects may well contribute to the inhibitory action of FOAA. Since an increase in FOAA concentration results in progressive increase in inhibition at fixed levels of substrate, this inhibition appears to be of the fully competitive type (Dixon and Webb).¹¹

It is interesting that K_i values for OAA, mesotartrate and dioxosuccinate are of the same order of magnitude (between 2 to 3×10^{-6}). This fact supports the view proposed by Davies and Kun² that malic dehydrogenase reacts with all these substrates. It is predictable from the lower K_i value for L(+) malate (7.4×10^{-6}) that the reaction from malate to OAA is inhibited by FOAA to a greater extent than the reverse. It is to be expected that in a multienzyme system in which malate is formed, FOAA would cause the channeling of malate metabolism in directions other than oxidation to OAA. FOAA is a slowly reacting substrate for malic dehydrogenase. The product, fluoromalate, was shown by Krebs and Davies¹² and by Gal⁴ to inhibit the malic enzyme.

From the inhibitory properties of FOAA it appears likely that pharmacological effects of this compound would not show up in the form of acute toxicity since an alteration rather than inhibition of the tricarboxylic acid cycle is the result. Investigations dealing with the metabolic effects of chronic FOAA feeding as well as reactions of FOAA with other enzymes are in progress.

$$\begin{array}{l} * \text{OAA } \text{pk}_1' = 2.7 \\ \text{pk}_2' = 4.1 \end{array}$$

$$\begin{array}{l} \text{FOAA } \text{pk}_1' = 2.2 \\ \text{pk}_2' = 3.3 \end{array}$$

Acknowledgement—It is a pleasure to acknowledge the contribution of Mr. M. K. Hrenoff from the Spectrographic Laboratory of the University of California Medical Center for the preparation of infra-red spectra.

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THE ACTION OF NITROGEN MUSTARD ON AMINO ACID INCORPORATION INTO RAT LIVER PROTEIN

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Abstract—The action of the nitrogen mustard di-(2-chloroethyl) methylamine hydrochloride (HN2) on the *in vitro* incorporation of labelled amino acids into the proteins of rat liver slices has been compared with its action on other biosynthetic processes in the liver. Uptake of ^{14}C -glycine was found to be less sensitive to HN2 than was incorporation of ^{32}P into ribonucleic acid. Liver slices were treated with different concentrations of HN2 and nuclear and cytoplasmic fractions isolated separately after incubation. Uptake of ^{14}C -glycine by the proteins of these two fractions was similarly affected by HN2. The action of HN2 on ^{14}C -phenylalanine incorporation and on *p*-aminohippuric acid synthesis by liver slices were then compared. At all concentrations of the inhibitor, essentially the same response was obtained with both systems. It has been concluded that the inhibition of amino acid incorporation produced by HN2 acting on liver slices is due to general inhibition of synthetic reactions rather than to a specific action on the mechanism of protein synthesis.

INTRODUCTION

CRATHORN AND HUNTER^{1, 2} have described the action of a number of nitrogen mustards on amino acid incorporation into the proteins of *Staphylococcus aureus*. They found that, in general, the compounds that caused the greatest reduction in amino acid incorporation were also those most effective in inhibiting growth of the Walker carcinoma in rats. They suggest accordingly that, if the processes of protein synthesis follow similar pathways in all living cells, then the primary inhibitory action of nitrogen mustards is to produce a block at some stage in protein synthesis.

The extensive use of nitrogen mustards in the experimental chemotherapy of cancer makes it a matter of some importance to know whether, in the case of mammalian cells, inhibition of protein synthesis is the most important effect of mustards on cellular metabolism. We have therefore carried out some experiments to study the action of the nitrogen mustard di(2-chloroethyl)methylamine hydrochloride (HN2) on the *in vitro* incorporation of labelled amino acids into the proteins of rat liver slices and have compared these results with the action of HN2 under the same conditions of incubation on the metabolism of RNA. Although amino acid uptake by the tissue proteins and ^{32}P uptake by the RNA of the slice were both inhibited, it was concluded that the action of HN2 was not primarily on protein synthesis but was more a general inhibition of biosynthetic reactions. This conclusion was supported by the observation that synthesis of *p*-amino hippuric acid by liver slices was inhibited by HN2 at concentrations similar to those affecting amino acid incorporation.

Some of our findings have been briefly reported in a preliminary communication.³

EXPERIMENTAL

The action of nitrogen mustard on ^{14}C -glycine uptake by protein and ^{32}P uptake by RNA

Tissue slices were prepared with a tissue chopper⁴ from the pooled livers of several adult male albino rats and 400 mg samples were suspended in 4 ml Krebs-Ringer bicarbonate solution⁵ in 25 ml conical flasks under aseptic conditions. Amino acid incorporation into the proteins of the slices was studied by adding $1\ \mu\text{C}$ ^{14}C -2-glycine to some of the flasks and RNA synthesis was investigated by addition of $25\ \mu\text{C}$ ^{32}P -orthophosphate to other flasks. Different concentrations of the nitrogen mustard di-(2-chloroethyl)-methylamine hydrochloride (HN2) (Boots Pure Drug Co. Ltd.) were prepared in Krebs-Ringer bicarbonate buffer and allowed to stand for 15 min at room temperature in order to allow cyclization of the mustard to its biologically active form before being added to the incubation flasks.⁶ After gassing with 95 per cent O_2 -5 per cent CO_2 , the flasks were shaken at 120 oscillations/min in a water-bath at 37° for periods of 1 to 3 hr.

Incubation was terminated by adding 0.5 vol. 30 per cent (w/v) trichloroacetic acid (TCA) to each flask. After separating the precipitate centrifugally, it was washed twice with ice-cold 10 per cent TCA followed by lipid extraction⁷ and allowed to dry in air. In the case of ^{14}C -glycine uptake, the dry powder was suspended in 5 per cent (w/v) TCA and heated for 15 min at 90° in order to extract nucleic acids.⁸ The protein precipitate was recovered by centrifuging, followed by dehydration in absolute ethanol and then ether, and was allowed to dry off in air. The dry powder was counted on $0.5\ \text{cm}^2$ polythene planchets at infinite thickness using an end-window Geiger-Müller tube. The data are expressed in counts/min per planchet.

In the case of ^{32}P uptake by RNA, the dry, lipid-extracted powder was digested with 0.3 N KOH for 18 hr at 37° , the protein and deoxyribonucleic acid precipitated by acidification of the digest, and the ribonucleotides separated individually by ionophoresis (procedure 1 of Davidson and Smellie⁹). The specific activity of each of the ribonucleotides so isolated was obtained by measuring the radioactivity in a liquid counter¹⁰ and relating it to the amount of phosphorus present.¹¹ The data are expressed in counts/min per 100 μg phosphorus, and are the average figures obtained for the four ribonucleotides.

Control flasks in which the tissue was inactivated with TCA at the start of incubation showed slight uptake of both isotopes, and these zero time controls were subtracted from the activities found at the end of incubation.

Comparison of the inhibitory action of nitrogen mustard on uptake of ^{14}C -glycine by nuclear and cytoplasmic proteins of rat liver

Liver slices were prepared as before and 800 mg portions were incubated in 8 ml Krebs-Ringer bicarbonate in the presence of $1\ \mu\text{C}$ ^{14}C -2-glycine and various concentrations of HN2 under the conditions described above. At the end of incubation, the flasks were chilled and the tissue was separated into nuclear and cytoplasmic fractions by the citric acid procedure of Smellie, Humphrey, Kay and Davidson.¹² The nuclear fraction was washed with 0.01 M citric acid as described by these authors, the washing being controlled by microscopic examination. The nuclear fraction was treated with 10 per cent (w/v) TCA and 0.5 vol. 30 per cent (w/v) TCA was added to the cytoplasmic fraction. The nuclear and cytoplasmic precipitates were then washed with 10

per cent (w/v) TCA, lipid-extracted,⁷ and dried in air. Radioactivity was measured at infinite thickness on an 0.3 cm² polythene planchet, using an end-window counter.

Comparison of the action of nitrogen mustard on ¹⁴C-phenylalanine uptake and p-aminohippuric acid synthesis

In these experiments, liver slices were used in the proportion of 100 mg wet weight to 4 ml Krebs-Ringer bicarbonate medium containing 0.01 M glycine and 0.001 M p-aminobenzoic acid.¹³ Owing to the presence of non-radioactive glycine in the medium, ¹⁴C-glycine could not be used to measure amino acid incorporation, and in its place 1 μ C ¹⁴C-L-phenylalanine (generally labelled) was added to each flask. Different concentrations of HN₂ were prepared and added as before. The reaction was terminated by addition of TCA to give a final concentration of 0.16 M.¹³ The precipitate was spun down and the amount of p-aminohippuric acid formed was estimated on the supernatant fluid by the method of Cohen and McGilvery.¹³ The precipitate was twice washed with 5 per cent (w/v) TCA and was then subjected to lipid extraction.⁷ After drying in air, the radioactivity of the powder was assayed at infinite thickness on an 0.3 cm² polythene planchet using an end-window counter.

RESULTS

The action of nitrogen mustard on ¹⁴C-glycine uptake by protein and ³²P uptake by ribonucleic acid

The action of high concentrations of the nitrogen mustard on the characteristics of incorporation of ¹⁴C-glycine into the protein and ³²P into the RNA of liver slices is shown in Fig. 1. Although 10⁻³M concentration markedly inhibited uptake of both

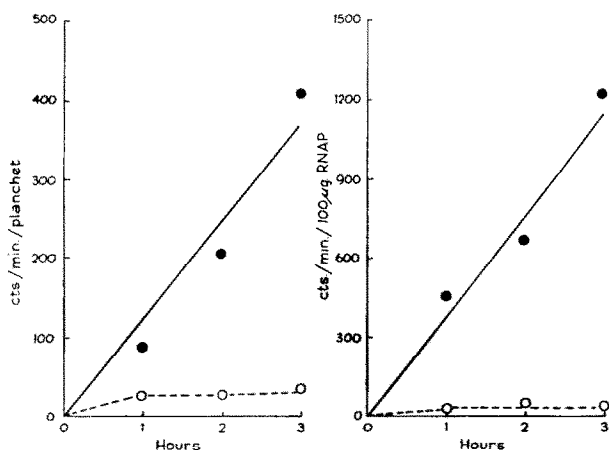


FIG. 1. Effect of 10⁻³ M HN₂ on incorporation of ¹⁴C-glycine into protein (left-hand graph) and ³²P into RNA (right-hand graph) by rat liver slices. Data are the mean results obtained in two experiments. Control specimens ●; treated specimens ○.

isotopes, in neither case was incorporation completely eliminated. After 1 hr of incubation in the presence of mustard, an appreciable uptake of ¹⁴C-glycine and a small uptake of ³²P were observed. On the other hand, more prolonged incubation did not produce any further increase in incorporation. The most likely explanation

of the initial incorporation is that it occurs before the nitrogen mustard has fully penetrated the liver cells. Consequently, the full inhibitory action of 10^{-3} M HN2 is represented by the subsequent period without incorporation. In order to compare the inhibitory actions of different concentrations of HN2, it is thus necessary to measure isotope uptake after enough time has elapsed for the action of the mustard to become fully developed. This has been done in the case of the data shown in Fig. 2. Incorporation of ^{14}C -glycine and of ^{32}P into RNA were measured after 1 hr and after 3 hr

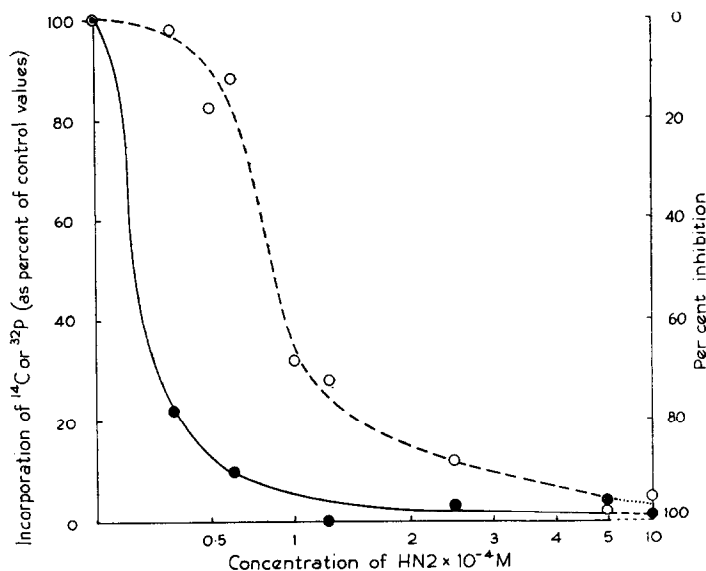


FIG. 2. Comparison of the effect of increasing concentrations of HN2 on the uptake of ^{32}P by the RNA (●) and of ^{14}C -glycine by the protein (○) of rat liver slices. Data are the mean results obtained in three experiments.

of incubation, the uptake during the intervening 2-hr period being taken as a true measure of the inhibitory action of HN2. In each repetition of the experiment, isotope uptake by the mustard-treated specimens during this 2-hr period has been expressed as a percentage of the uptake obtained with untreated control slices of liver incubated under the same conditions. Inhibition of glycine incorporation was apparent at concentrations exceeding 3×10^{-5} M and was essentially complete at 5×10^{-4} M, 50 per cent inhibition occurring at a concentration of 9×10^{-5} M. The incorporation of ^{32}P into RNA was appreciably more sensitive, complete inhibition occurring at 1×10^{-4} M concentration and 50 per cent inhibition at about 2×10^{-5} M concentration. Thus 50 per cent inhibition of RNA synthesis occurs at a concentration at which amino acid uptake into proteins is not affected.

Comparison of the action of nitrogen mustard on uptake of ^{14}C -glycine by nuclear and cytoplasmic proteins

The preceding experiments show that there is nothing unique about the action of HN2 on amino acid incorporation by the mixed proteins of the liver cell. In fact, the system is somewhat less sensitive to HN2 than is the mechanism by which ^{32}P is

incorporated into RNA. This does not exclude the possibility that the synthesis of one particular protein fraction may be critically affected by low concentrations of HN2. The most likely site of such an action is the nucleus, since there is considerable evidence in the literature to suggest that the process of nuclear division is specifically affected by mustards.¹⁴ We therefore compared the uptake of ^{14}C -glycine by the proteins of the nuclei and of the cytoplasm of liver cells following exposure to different concentrations of HN2. Slices of liver were incubated with 6.25×10^{-5} M HN2 and 5×10^{-4} M HN2, these concentrations being chosen in the light of our previous experiments (Fig. 2) to provide respectively slight and nearly complete inhibition of amino acid uptake by the mixed proteins of the cell. At the end of incubation, nuclear and cytoplasmic fractions were isolated from the slices and their proteins assayed for radioactivity. Table 1 shows that amino acid uptake by the nuclear proteins was not more sensitive to HN2 than was uptake by the cytoplasmic proteins.

TABLE 1. EFFECT OF NITROGEN MUSTARD ON UPTAKE OF ^{14}C -GLYCINE BY NUCLEAR AND CYTOPLASMIC PROTEINS* OF RAT LIVER SLICES

Conc. of mustard	Uptake of ^{14}C -glycine†		Per cent inhibition	
	Nuclei	Cytoplasm	Nuclei	Cytoplasm
6.25×10^{-5} M	46	214	—	—
	39	158	16	26
5×10^{-4} M	4	1	92	99

* The data are the mean results obtained in two experiments.

† Expressed as the difference between uptake at 3 hr and at 1 hr of incubation, in cts/min per planchet, measured at infinite thickness.

*Comparison of the action of nitrogen mustard on ^{14}C -phenylalanine uptake and on *p*-amino-hippuric acid synthesis*

In order to test whether the action of HN2 on amino acid incorporation by liver slice proteins is part of a general inhibitory action on synthetic processes, we compared it with the effect of this mustard on *p*-aminohippuric acid (PAH) synthesis by the liver. Fig. 3 shows that the action of 5×10^{-4} M HN2 on ^{14}C -phenylalanine uptake and on

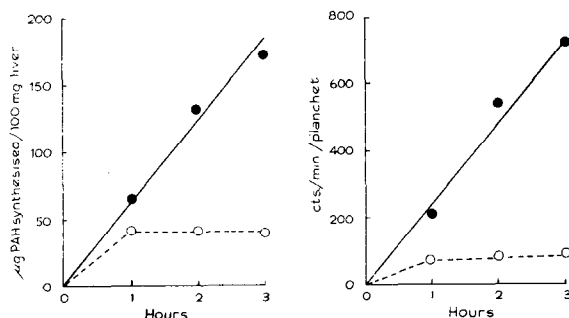


FIG. 3. Effect of 5×10^{-4} M HN2 on synthesis of *p*-aminohippuric acid (left-hand graph) and ^{14}C -phenylalanine incorporation (right-hand graph) by rat liver slices. Data are the mean results obtained in three experiments. Control specimens ●; treated specimens ○.

PAH synthesis in the same liver slice preparations. In agreement with McKinney¹⁵, HN2 causes inhibition of PAH formation, the pattern of response of this system and of ¹⁴C-phenylalanine incorporation being similar to that noted in our earlier studies on ¹⁴C-glycine uptake (Fig. 1), namely an initial period of synthesis followed by complete cessation with more prolonged incubation. We have therefore assessed the action of different concentrations of HN2 on these biosynthetic reactions in the same way as before, that is, by measuring the increase in synthesis after 1 hr of incubation has elapsed. In Fig. 4 the increments in PAH synthesis and ¹⁴C-phenylalanine incor-

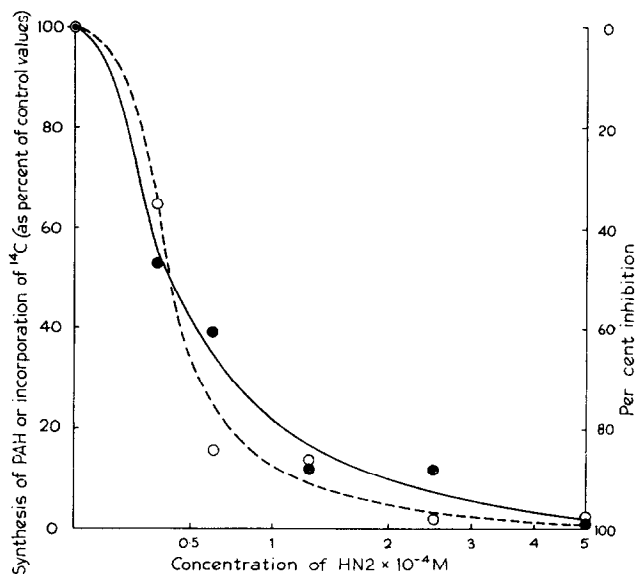


FIG. 4. Comparison of the effects of increasing concentrations of HN2 on *p*-aminohippuric acid synthesis (●) and on ¹⁴C-phenylalanine incorporation (○) by rat liver slices. Data are the mean results obtained in three experiments.

poration obtained in the presence of different concentrations of HN2 between the first and third hours of incubation are expressed as a percentage of the values observed in untreated control flasks. Although inhibition of incorporation of phenylalanine occurs at lower concentrations than those found to affect glycine uptake (Fig. 2) direct comparison is not justified since the conditions of incubation differ. The data recorded in Fig. 4 show essentially the same response of protein and PAH synthesis to the inhibitory action of HN2 at all concentrations. There is accordingly nothing specific about the sensitivity of amino acid incorporation towards HN2.

DISCUSSION

Crathorn and Hunter^{1, 2} have observed that the ability of various nitrogen mustards to inhibit incorporation of labelled amino acids into the protein of *Staphylococcus aureus* can be correlated with their effectiveness in reducing tumour growth in rats. These authors have accordingly postulated that nitrogen mustards act primarily by producing a block at some stage in protein synthesis. If this general view of the action of nitrogen

mustards is correct, it might be anticipated that amino acid incorporation by mammalian tissues would be particularly sensitive to these inhibitors. However, our studies of the action of a nitrogen mustard, HN2, on liver cells reveal that amino acid incorporation is unaffected by concentrations of the inhibitor which greatly reduce ^{32}P uptake by the RNA of the same tissue (Fig. 2). The greater sensitivity of nucleic acids towards HN2 has also been noted by other workers. LePage and Greenlees¹⁶ incubated ascites tumour cells with HN2 and observed inhibition of ^{14}C -2-glycine uptake by the purine bases of the nucleic acids without appreciable diminution in its incorporation into protein. Using rats pretreated with HN2, Heidelberger and Keller¹⁷ studied uptake of ^{14}C -formate into the purine bases and ^{14}C -alanine into the proteins of the Flexner-Jobling carcinoma and of the spleen. Although their data show a greater effect of HN2 on ^{14}C -incorporation into the purine bases, parallel studies with ^{32}P uptake into nucleic acids failed to show any inhibitory effect. Their experimental conditions differ appreciably from those of LePage and Greenlees and also from our own.

The published literature contains further evidence that systems other than protein synthesis are the first to be affected by mustards. Application of threshold concentrations of HN2 to corneal epithelium causes cessation of mitosis without appreciable diminution in growth of cell volume.¹⁸ A similar observation has been made with salamander embryos exposed to mustard.¹⁹ This suggests that protein synthesis can continue in the presence of concentrations of mustard sufficient to inhibit nuclear division. Similarly, in a study of the action of mustards on bacterial growth, the formation of protein was shown to be unaffected by concentrations of the inhibitor which interfered with DNA synthesis.²⁰

Other evidence points to a specific action of mustards on nuclear metabolic processes. Concentrations of sulphur or nitrogen mustards sufficient to cause cessation of DNA synthesis have been shown to produce little or no action on the synthesis of RNA by bacteria,^{20, 21} salamander embryos,¹⁹ the small intestine of the rat,²² on several of the tissues of rabbits treated *in vivo* with mustard,^{23, 24} and on cells grown in tissue culture.²⁴ On the other hand, a number of workers^{16, 17, 24} have failed to demonstrate a specific effect of mustards on DNA synthesis by tumour cells. Our own examination of the sensitivity of the nuclear proteins of liver cells in comparison with the cytoplasmic proteins shows that they do not differ in their response to HN2 (Table 1). Although this does not exclude the possibility that some subfraction of the mixed nuclear proteins is more sensitive to HN2, it does suggest that no major fraction is primarily affected by the mustard.

All the evidence discussed above conflicts with the view that the primary action of mustards is on the mechanism of protein synthesis. The inhibition of amino acid uptake by HN2 would therefore seem to represent one aspect of a more general inhibitory action. Comparison of the reaction of amino acid incorporation and of *p*-aminohippuric acid (PAH) synthesis in liver slices towards HN2 has demonstrated that both biosynthetic systems are affected to the same extent (Fig. 4). There is good reason to believe that the peptide bonds of proteins²⁵ and of PAH²⁶ are formed at different sites in the cell, and it has been demonstrated that thyroxine affects the two systems differently.²⁷ Thus our observation that both processes respond similarly towards HN2 implies that its action at these concentrations involves general inhibition of synthetic processes, rather than a specific action on protein synthesis.

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ÜBER EINE NEUE ADRENALINCARBONSÄURE UND EINIGE IHRER DERIVATE

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Abstract—Synthesis and pharmacological properties of a new carboxylic acid of epinephrine and of some derivatives are reported.

The carboxylic acid produced qualitatively the same effects on blood pressure and denervated nictitating membrane of the cat as epinephrine, the relative potency being 0.0003. The effects on the blood pressure could be reversed by previous administration of adrenalytics. We believe that the effects on the blood pressure and the nictitating membrane are caused by admixture of epinephrine contained in the original substance. An enzymatic decarboxylation *in vitro* could not be observed.

On the other hand, the effects of the esters derived from the new epinephrine carboxylic acid were similar to those of the *isopropyl*norepinephrine. The most active ester derived from the new acid equalled *isopropyl*norepinephrine in its potency on all test subjects. In all experiments, the increasing effects on heart frequency, the bronchodilating and the depressing effect on the blood pressure of the esters run parallel. The corresponding derivatives of the *p*-oxyephedrin were practically completely ineffective. The connections between chemical constitution and pharmacological activity is discussed.

SEIT der Entdeckung der "Dioxyphenylalanin-Decarboxylase" durch P. Holtz *et al.*¹ wurde jenen Aminosäuren, welche durch Abtrennung der Carboxylgruppe zu Adrenalin und Noradrenalin hätten führen können, erhebliches Interesse entgegengebracht. Vor allem die Threo-Form des 3 : 4-Dioxyphenylserins (DOPS) kann durch Extrakte aus den Nieren und Nebennieren mancher Tiere wie auch aus sympathischen Nerven zu 1-Noradrenalin umgewandelt werden.^{2, 3} Die enzymatische Decarboxylierung der von Bolhofer⁴ hergestellten Serine (D,L-erythro und threo DOPS; D,L-erythro und threo *m*-Oxyphenylserin; D,L-threo-*p*-Oxyphenylserin) ist im Vergleich mit DOPA von einer Gruppe amerikanischer Forscher unter der Leitung von Drell ebenfalls untersucht worden.⁵ Die Decarboxylierung besonders der Threo-Form geht jedoch weit langsamer vor sich als die der als Vorstufe für 1-Noradrenalin nicht in Betracht kommenden Erythro-Form und die von DOPA. Auch ist die Frage erörtert worden, ob die beiden Decarboxylasen für DOPA und DOPS identisch sind, was Werle *et al.*⁶ ablehnen, während Holtz *et al.*^{7, 8} es für wahrscheinlich halten.

Frühzeitig hatte Blaschko⁹ gefunden, daß Methyl-DOPA nicht decarboxyliert wird. Dies gilt auch für die von Dalgiesh und Mann¹⁰ synthetisierte, am Stickstoff methylierte Aminosäure, aus der Adrenalin entstehen müßte (Methyl-DOPS).¹¹ Es scheint demnach, daß am Stickstoff substituierte Adrenalincarbonsäuren nicht durch die Aminosäure-Decarboxylase angegriffen werden.

Dennoch schien es uns interessant, die Eigenschaften einer neuen Adrenalincarbonsäure zu untersuchen, deren Synthese der eine von uns (Brücke) angeregt hatte. Es handelt sich um ein substituiertes Glycin, nämlich N-[2-(3:4-Dihydroxyphenyl)-2-hydroxyäthyl]glycin (DOPOG). Der chemische Teil über die Synthese des Stoffes und einiger Analoges, welche im Zentrallaboratorium der Philips-Roxane in Weesp (Holland) durchgeführt wurde, ist in diese Arbeit eingeschlossen.

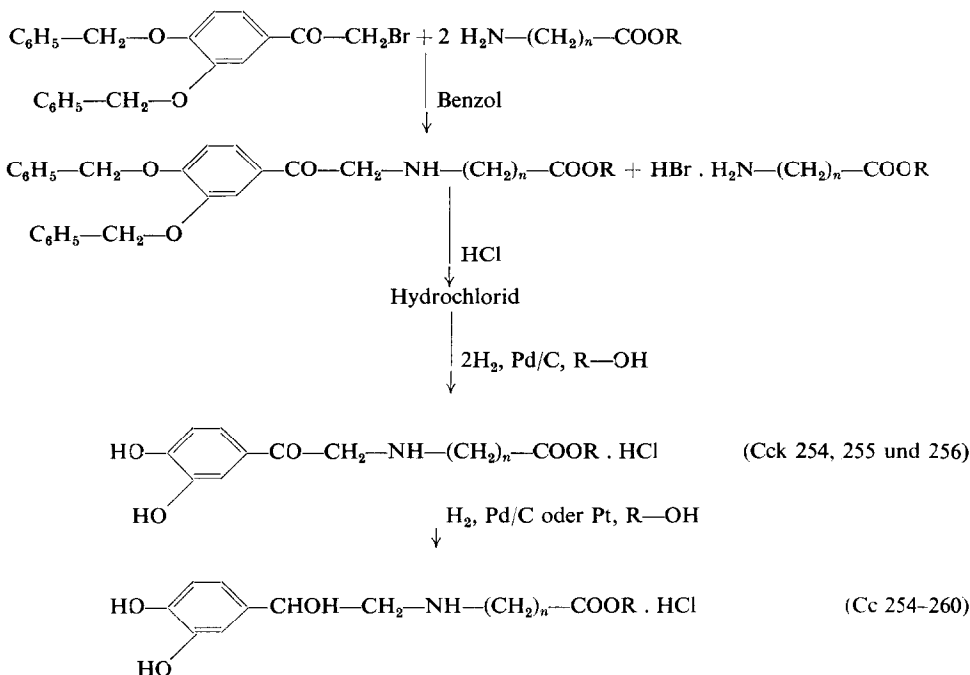
Besonders bemerkenswert erschien es uns, daß offenbar Ester der hier in Frage kommenden Aminosäuren bisher nicht untersucht worden sind.

METHODIK

Beschreibung der Synthesen

N-[2-(3:4-Dihydroxyphenyl)-2-hydroxyäthyl]glycin (DOPOG). Man kocht eine Lösung (10%) von Cck 254* 2 Stunden lang in 2 N Schwefelsäure und neutralisiert bis zu pH 3,5. Die abgeschiedenen Kristalle der Ketosäure werden abgesaugt und getrocknet. Ausbeute 60% der Theorie. Schmelzpunkt 270° (u.Z.). Hierauf suspendiert man 1,37 g der Ketosäure und 50 ml Wasser und hydriert mit Palladium-Tierkohle Katalysator. Man filtriert den Katalysator ab, engt die Lösung ein in N₂ Atmosphäre und isoliert 0,9 g DOPOG, einer weißen Substanz, welche bei Zimmertemperatur bis zu 0,5% in Wasser löslich ist. Schmelzpunkt 220–225° (u.Z.). U.V.-Spektrum siehe Abb. 1.

Die Ester des DOPOG. Die Darstellung der Ester Cc 254, 255, 256, 259, 260, Cck 254, 255 und 256 wird durch die unterstehenden Gleichungen ausgedrückt:



$n = 1$ ($\text{R} = \text{—CH}_3, \text{—C}_2\text{H}_5$ und $\text{—CH(CH}_3)_2$); $n = 2$ und 3 ($\text{R} = \text{—C}_2\text{H}_5$).

Die Ester schmelzen unter Zersetzung oder zersetzen sich bevor sie schmelzen. Für Prüfung auf Reinheit sind die U.V. Spektren sehr geeignet. Die Spektren der Diphenole zeigen das charakteristische Bild der Adrenalinderivate.¹⁸ In wässriger Lösung werden die Ester ziemlich rasch hydrolysiert.

Als Beispiel beschreiben wir die Darstellung von Cc 254 und Cck 254: Eine Lösung von 16,8 g (0,041 Mol) 2-Brom-3':4'-dibenzyl-oxy-acetophenon in 100 cm³ Benzol wird

gemischt mit 7,6 g (0,083 Mol) Glycinmethylester. Man lässt die Lösung 30 min bei Zimmertemperatur stehen. Hierauf filtriert man die abgeschiedenen Kristalle Glycinmethylester Hydrobromid ab, wäscht sie mit 50 cm³ Benzol nach und versetzt das Filtrat mit 30 cm³ 1,45 N alkoholischer Salzsäure. Das abgeschiedene Hydrochlorid von N-[2-(3:4-Dibenzoyloxyphenyl)-2-oxoäthyl]glycinmethylester wird abgesaugt und eine weitere Menge des rohen Salzes nach dem Abdampfen des Alkohols aus der Mutterlauge isoliert. Die Mischung beider Kristallisate wird aus 130 cm³ absolutem Methylalkohol umkristallisiert. Nach mehrstündigem Stehen in der Kälte

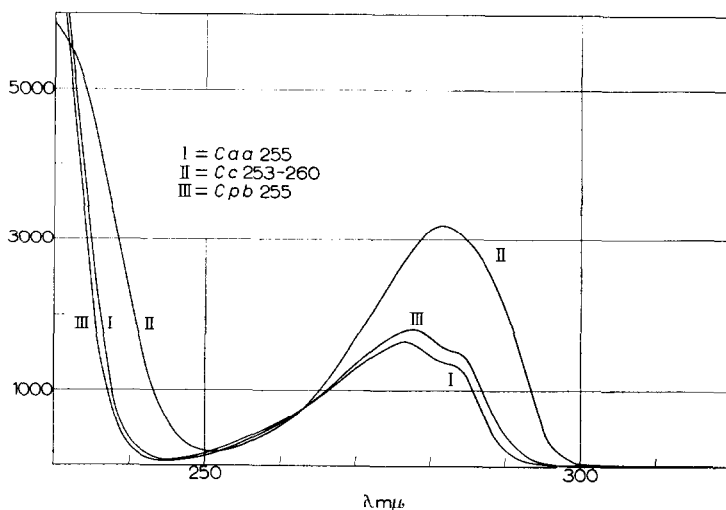


ABB. 1. U.V. Spektra,

filtriert man die Kristalle ab, engt die Mutterlauge noch weiter ein, filtriert nach längerem Stehen auch die zweite Kristallisation ab und trocknet die reine Substanz zuerst an der Luft und dann im Exsiccator über konz. Schwefelsäure. Schmelzpunkt 154–157° (u.Z.). Ausbeute etwa 7,3 g oder 39% der Theorie.

Man löst diese Substanz (7,0 g, 0,0154 Mol) in 200 cm³ Methylalkohol, suspendiert 0,7 g 10% Palladium-Tierkohlein die Lösung und schüttelt die Mischung unter geringem Überdruck und bei einer Temperatur von etwa 40° mit Wasserstoff. Nachdem die notwendige Menge Wasserstoff aufgenommen ist, wird der Katalysator abfiltriert und der Methylalkohol verdampft. Man rührt den Rückstand mit 20 cm³ Azeton, versetzt die Suspension mit 20 cm³ Äther und filtriert nach 24 Stunden Stehen in der Kälte die abgeschiedenen Kristalle ab. Die Ausbeute beträgt 3,9 g (92%) Hydrochlorid des N-[2-(3:4-Dihydroxyphenyl)-2-oxoäthyl]glycinmethylesters (Cck 254). Die Substanz schmilzt unter Zersetzung bei etwa 210–211°C.

Man löst 2,0 g (0,0073 Mol) dieses Hydrochlorids in 80 cm³ Methanol und hydriert mit 1,5 g Palladium-Tierkohle. Nachdem die berechnete Menge Wasserstoff aufgenommen ist, wird der Katalysator abgesaugt, der grösste Teil des Lösungsmittels

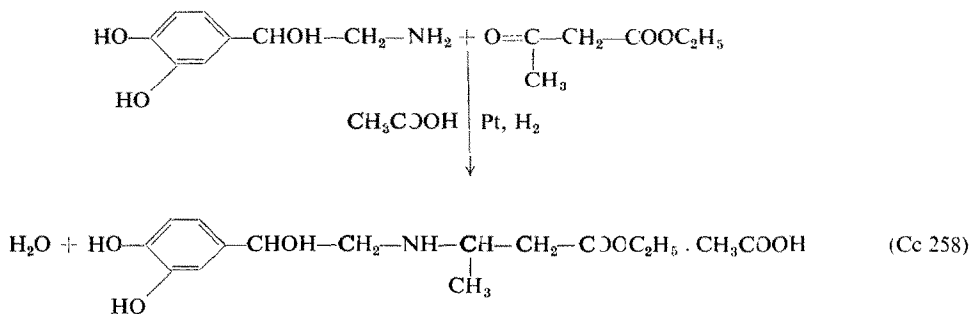
verdampft und der hinterbleibende Rest mit 4 cm³ Azeton gemischt. Man impft und wartet während 24 Stunden. Hierauf wird das abgeschiedene Hydrochlorid des N-[2-Hydroxy-2-(3:4-dihydroxyphenyl)äthyl]glycinmethylesters (Cc 254) scharf abgesaugt und im Vakuumexsiccator getrocknet. Die Substanz schmilzt unter Zersetzung bei 126–130°. Die Ausbeute beträgt 1,32 g (65%). Die Substanz kann gereinigt werden durch Umkristallisieren aus einer Mischung von absolutem Methanol und Äther (U.V. Spektrum siehe Abb. 1).

Die Cc Substanzen können auch ohne Isolierung der Cck Substanzen dargestellt werden, nämlich durch katalytische Hydrierung der "Dibenzoyloxyverbindungen" unter Aufnahme von 3 Mol Wasserstoff.

Die Substanz Caa 255 wurde auf analoge Weise dargestellt.

Man suspendiert 11,2 g (0,080 Mol) Hydrochlorid des Glycinäthylesters und 25,6 g (0,080 Mol) 4'-Benzyloxy-2-brompropiofenon in eine Lösung von 16,4 g Triäthylamin in 120 cm³ absolutem Alkohol und erwärmt unter Rühren bis zum Sieden. Nachdem sich der feste Stoff gelöst hat, hält man noch 3 Stunden lang im Kochen, lässt abkühlen, verdünnt mit 500 cm³ Äther und schüttelt fünfmal mit insgesamt 600 cm³ Wasser aus. Der Äther wird mit wasserfreiem Natriumsulfat getrocknet, filtriert und unter Rühren mit 37,5 cm³ 3,27 N alkoholischer Salzsäure versetzt. Man saugt die abgeschiedene Menge ab, reinigt sie durch Umkristallisieren aus 100 cm³ absolutem Äthylalkohol und 2 cm³ konzentrierter Salzsäure und waschen mit Azeton und trocknet im Vakuumexsiccator. Ausbeute 13,6 g oder 45% der Theorie. Schmelzpunkt 188–190° (u.Z.). Eine Lösung von 10,0 g (0,026 Mol) dieses Hydrochlorids in 350 cm³ absolutem Alkohol wird katalytisch hydriert mit 0,5 g 10% Palladium-Tierkohle. Nach Aufnahme von 2 Mol Wasserstoff engt man die filtrierte Lösung ein, verdünnt den Rückstand (20 g) mit 60 cm³ Äther, impft und saugt nach einiger Zeit das Hydrochlorid des N-[2-Hydroxy-2-(4-hydroxyphenyl)-1-methyläthyl]glycinäthylesters ab. Man wäscht mit Äther und trocknet. Ausbeute 6,2 g (80%). Schmelzpunkt 141–142° (u.Z.) Spektrum siehe Abb. 1.

Die Darstellung des Aminosäureesters Cc 258 geschah auf folgende Weise:

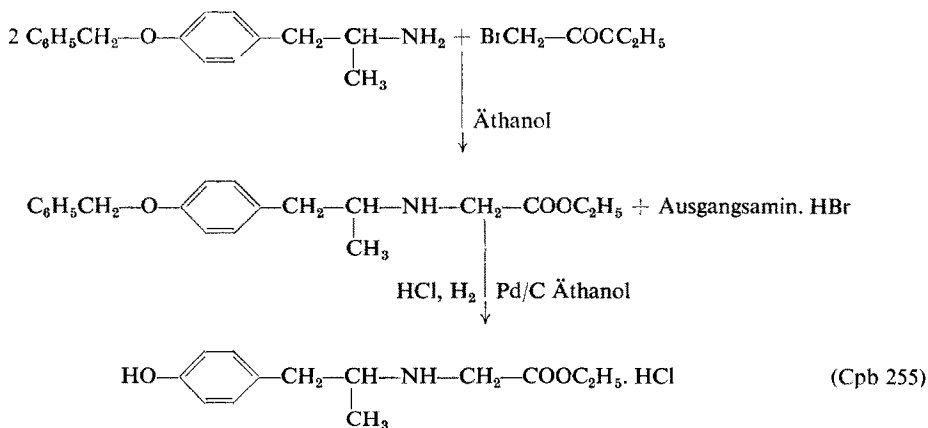


Die intermediär gebildete sog. Schiff'sche Base wird hier zum *sec.*-Amin reduziert.

Man mischt 6,9 g (0,053 Mol) Acetessigester und 2,8 cm³ Essigsäure mit 160 cm³ Äthanol und versetzt die Lösung mit 6,8 g (0,040 Mol) α-(Aminomethyl)-3:4-dihydroxybenzylalkohol (Nor-Adrenalin Base) und 0,4 g Platinoxidkatalysator nach Adams. Unter geringem Überdruck und bei Zimmertemperatur schüttelt man die Mischung mit Wasserstoff. Nach und nach löst sich der Aminoalkohol. Nachdem

die berechnete Menge Wasserstoff aufgenommen ist, saugt man den Katalysator ab, engt die Lösung ein bis das Gewicht des hinterbleibenden Restes 24 g beträgt und löst den Rückstand in einer Mischung von 20 cm³ Azeton und 2 cm³ Essigsäure. Hierauf versetzt man die Lösung nach und nach mit 70 cm³ trockenem Äther, impft, und nachdem die Kristallisation begonnen hat, versetzt man aufs Neue mit 10 cm³ dieses Lösungsmittels. Nach 6 Stunden Stehen in der Kälte (0°) isoliert man die Kristalle, wäscht sie mit einer Mischung von 10 cm³ Azeton und 10 cm³ Äther nach und trocknet im Vakuumexsiccator. Die Ausbeute beträgt 7,56 g (55%) des Azetats des 3-[2-(3:4-Dihydroxyphenyl)-2-hydroxyäthylamino]buttersäure äthylesters. Schmelzpunkt 114–117°, U.V. Spektrum siehe Abb. 1. Durch Einengen der Mutterlauge kann die Ausbeute mit etwa 2 g (15%) gesteigert werden. Die Substanz ist wahrscheinlich ein Gemisch zweier Razemate. Durch zweimal Umkristallisieren aus einer Mischung von Azeton, Essigsäure und Äther (20:1:20) erhält man eine Substanz mit scharfem Schmelzpunkt: 126,5–127°. Dieses Azetat ist möglicherweise identisch mit einem der zwei Razemate.

Die Substanz Cpb 255 wurde nach einer anderen Methode dargestellt.



Nach einer Methode von Heinzelman²³ wurde 1-(4-Benzoyloxyphenyl)-2-nitropropen (Schmp. 147–148°) dargestellt und mit LiAlH₄ in Tetrahydrofuran reduziert. Man erhält dann 1-Methyl-2-(4-benzoyloxyphenyl)äthylamin. Ausbeute 68%; Schmelzpunkt 103–104°. Eine Lösung von 5,30 g (0,022 Mol) dieses Amins und 1,67 g (0,010 Mol) Bromessigesters in 10 cm³ Äthanol hält man 10 Minuten lang auf etwa 50°. saugt das abgeschiedene Hydrobromid des Ausgangsamins ab, versetzt das Filtrat mit 5 cm³ 4 N Salzsäure und kühlt auf 0°. Nach einer Stunde filtriert man das Hydrochlorid des N-[2-(4-Benzoyloxyphenyl)-1-methyläthyl]glycinäthylesters ab, gewinnt eine zweite Portion durch Einengen der Mutterlauge und trocknet. Ausbeute 2,74 g (75%); Schmelzpunkt 145–146°.

Man unterwirft eine Lösung von 1,1 g (0,003 Mol) dieses Salzes in 50 cm³ Äthanol einer katalytischen Hydrierung, filtriert nach Abspaltung der Benzylgruppe, verdampft das Lösungsmittel und verdünnt den Rückstand mit 5 cm³ Äthanol und 30 cm³ Äther. Das Hydrochlorid des Cpb 255 wird abgesaugt, mit Äther gewaschen und getrocknet. Ausbeute 0,4 g (50%). Schmelzpunkt 157–158°. U.V. Spektrum siehe Abb. 1.

TABELLE 1. SCHMELZPUNKTE UND ANALYSEN DER DOPOG UND ESTER

Kode No.	Schmp* (°C)	Formel† (Mol.-gew.)	Analysen‡							
			%C		%H		%N		%Cl	
			gef.	ber.	gef.	ber.	gef.	ber.	gef.	ber.
Caa 255	141–142	C ₁₃ H ₁₉ NO ₄ .HCl (289,76)	53,8	53,88	7,0	6,96	4,8	4,83	12,2	12,24
Cc 253 (DOPOG)	220–225	C ₁₀ H ₁₃ NO ₅ (227,22)	52,9	52,86	5,8	5,77	6,2	6,16		
Cc 254	126–130	C ₁₁ H ₁₅ NO ₅ .HCl (277,71)	47,5	47,57	5,8	5,81			12,8	12,76
Cc 255§	127–129	C ₁₂ H ₁₇ NO ₅ (255,27)	53,8	56,45	6,6	6,71	5,5	5,48		
Cc 256§	154–155,5	C ₁₃ H ₁₉ NO ₅ (269,30)	57,1	57,98	7,5	7,11	5,0	5,20		
Cc 258	126,5–127	C ₁₄ H ₂₁ NO ₅ .C ₂ H ₄ C ₂ (343,39)	56,4	55,96	7,5	7,34	4,1	4,08		
Cc 259	163,5–164,5	C ₁₃ H ₁₉ NO ₅ .HCl (305,77)	51,2	51,05	6,9	6,59	4,4	4,58	11,5	11,60
Cc 260	114–115	C ₁₄ H ₂₁ NO ₅ .HCl (319,80)	52,3	52,57	7,1	6,93	4,2	4,38		
Cck 254	210–211	C ₁₁ H ₁₃ NO ₅ .HCl (275,70)	47,9	47,91	5,1	5,12			13,1	12,86
Cck 255	174–175	C ₁₂ H ₁₅ NO ₅ .HCl (289,73)							12,3	12,24
Cck 256	201–202	C ₁₃ H ₁₇ NO ₅ .HCl (303,75)							11,7	11,67
Cpb 255	157–158	C ₁₃ H ₁₉ NO ₃ .HCl (273,76)	56,7	57,02	7,4	7,36	5,0	5,11		

* Nicht korrigiert.

† Strukturformel siehe Tabelle 2 (Relative Wirksamkeit . . .)

‡ Die C, H und N Mikroanalysen wurden ausgeführt unter Leitung des Herrn W. Hazenberg im Org. Chem. Lab. der Universität-Groningen.

§ Dieser Ester wurde als Base isoliert durch die alkoholische Lösung des Hydrochlorids mit einer äquivalenten Menge C₂H₅ONa zu versetzen, zu filtrieren und im Vakuum einzuziehen.

Biologische Versuche

Zunächst wurden an Spinalkatzen nach Dale¹² Blutdruckversuche mit DOPOG durchgeführt, wobei eine Nickhaut chronisch denerviert war. Die Kontraktionen beider Nickhäute wurden registriert. Substanzen mit blutdrucksenkender Wirkung wurden an vagotomierten Katzen in Chloralosenarkose (0,08 g/kg) untersucht.

Broncholytische Wirkungen wurden am durch Acetylcholin hervorgerufenen Bronchospasmus bei 700 bis 800 g schweren männlichen Meerschweinchen in einer Modifikation der Methode von Konzett und Rössler¹³ ausgewertet. Als Vergleichssubstanz diente *iso*Propylnoradrenalinsulfat.

Herzfrequenzsteigerungen wurden an zwei 10 kg schweren, wachen Hunden dadurch verfolgt, daß die R-Zacke des EKG's verstärkt und mit einem Ordinatenschreiber (Totalisator) über die ganze Wirkungsdauer registriert wurde. Die Steigerungen der Herzfrequenz durch verschiedene Dosen wurden in Prozenten der Ausgangswerte berechnet, kurvenmäßig aufgetragen, durch Auswägen planimetriert und die relative Wirksamkeit gegenüber *iso*Propylnoradrenalinsulfat berechnet.

Um uns darüber zu orientieren, ob die biologische Aktivität von DOPOG auf Verunreinigung mit Adrenalin aus dem Synthesevorgang oder aus der Instabilität der Substanzen in wässriger Lösung beruhen könne, haben wir einige papierchromatographische Auftrennungen versucht. Zur Trennung des DOPOG von Adrenalin wurde Eisessig, Pyridin und Wasser (35%–50%–15%) verwendet; die Trennung gelang

jedoch nur unvollständig. Das Filterpapier (Whatman I) wurde mit 0,01 N HCl gewaschen, nach Trocknung mit einer Lösung von 50 mg Ascorbinsäure/100 ml Wasser besprüht und wieder getrocknet. Die Wanderung erfolgte in CO₂ Atmosphäre. Mit DOPOG ließen wir gleichzeitig 1-Adrenalin mitwandern, entwickelten diesen Streifen mit 0,44 %igem Kaliumferricyanid (pH 7,8) und schnitten in der entsprechenden Höhe den Streifen bei der Versuchssubstanz aus. Nach Entfernung der Lösungsmittel wurden die Streifen erschöpfend eluiert (Elutionsflüssigkeit 0,4% NaH₂PO₄ + 1 mg% Ascorbinsäure), im Vakuum eingengt und sowohl biologisch an sensibilisierten Ratten als auch fluorometrisch nach Euler¹⁴ getestet.

Die hierzu verwendeten Ratten wurden mit 1,2 g/kg Äthylurethan intraperitoneal narkotisiert (Druckregistrierung aus der Carotis mit entsprechend dimensioniertem Hg-Manometer; Injektionen in die Vena femoralis). Nach dem Vorgehen von Vogt¹⁵ wurden einige Tiere mit 2 mg/kg Atropinsulfat und 16 mg/kg Hexamethoniumchlorid intravenös vorbehandelt, wodurch ihre Empfindlichkeit gegen Adrenalin auf etwa das Zehnfache steigt. Ein Nachteil dieser Methode besteht nur darin, daß die Empfindlichkeit mit dem Wiederanstieg des Blutdrucks nach etwa einer halben Stunde wieder abnimmt. Besser war es den Tieren 5 Stunden vor Versuchsbeginn 5 mg/kg Reserpin i.p. verabreichen, wodurch eine ähnliche Sensibilisierung über einen längeren Zeitraum erreicht wird und das Blutdruckniveau konstant bleibt. Das Injektionsvolumen betrug stets 0,5 ml.

Zur Bestimmung der Decarboxylierung im Warburgapparat wurde als Fermentansatz ein Extrakt aus Meerschweinchennierenhomogenat in 0,067 N Soerensenphosphatpuffer 1:3 bei pH 7,4 verwendet. Es wurde bei 37° in N₂ Atmosphäre gearbeitet. Am Ende des Versuches wurde 0,2 ml 10%ige H₂SO₄ zur Freisetzung des gelösten CO₂ eingekippt. Die Inkubationsdauer betrug 4 Stunden.

ERGEBNISSE

Am Blutdruck der Spinalkatze wirkte DOPOG rein pressorisch, wobei etwa 5 mg der Wirkung von 1 µg Noradrenalin bzw. 1,5 µg Adrenalin entsprach. Da wir wenig Substanz zur Verfügung hatten konnten wir nur solche Dosen geben, welche ausschließlich die denervierte Nickhaut beeinflussten. Die Dauer der Wirkung war in allen Fällen sowohl am Blutdruck wie auch an der Nickhaut bei gleicher Blutdruckwirkung gleich lang wie die von Adrenalin.

Wurden Katzen mit Hydergin (4 mg) bzw. Dibenamin (15 mg/kg) vorbehandelt, dann kam es bei DOPOG und Adrenalin zu einer Wirkungsumkehr. Auf Grund dieser Versuche nehmen wir an, daß die biologische Wirksamkeit der Aminosäure im wesentlichen von Verunreinigungen mit Adrenalin herrührt.

Papierchromatographisch gelang die Trennung des Adrenalins von DOPOG nur unvollständig. Die Substanz selbst zeigt eine sehr starke Eigenfluoreszenz. Bei der biologischen Austestung steigerten sowohl die Eluate aus den Streifen der Adrenalinhöhe als auch der Aminosäurebasis den Blutdruck.

Bei den Warburgversuchen gelang es nicht während einer Inkubationsdauer von 4 Stunden eine Decarboxylierung von DOPOG nachzuweisen.

Während wie oben ausgeführt wurde, DOPOG am Katzenblutdruck eine sehr geringe steigernde Wirkung hatte, die durch Adrenolytica umgekehrt werden konnte, waren die untersuchten Ester hochwirksame blutdrucksenkende Stoffe.

Es wurde nun entweder die esterbildende Gruppe verändert, oder die Länge der Seitenkette zwischen dem N-Atom und der Carboxylgruppe, wobei auch eine Verbindung mit verzweigter Seitenkette untersucht wurde (Cc-Derivate). In anderen Fällen wurden einige Keto-Verbindungen dieser Adrenalinderivate untersucht (CcK-Derivate). Schließlich wurde auch der aromatische Ring variiert, indem nur eine OH-Gruppe in *p*-Stellung zur Seitenkette eingeführt wurde.

Die Resultate der biologischen Auswertung von blutdrucksenkender, herzfrequenzsteigernder und bronchodilatatorischer Wirkung sind aus der Tabelle zu entnehmen. Die Wirkungen wurden jeweils mit der Wirkung von *iso*Propylnoradrenalinsulfat verglichen und diese gleich 1 gesetzt. Es zeigte sich, daß sie stets parallel liegen. Die wirksamste Substanz erreicht an allen Testobjekten die Wirksamkeit von *iso*Propylnoradrenalinsulfat. Es handelt sich um die Verbindung Cc 258, welche in der Seitenkette vor der Carboxylgruppe einen *iso*Propylrest enthält und als Äthylester vorliegt.

TABELLE 2. RELATIVE WIRKSAMKEIT GEGENÜBER *ISO*PROPYLNORADRENALIN-SULFAT = 1,0

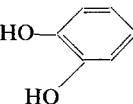
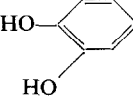
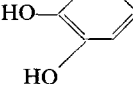
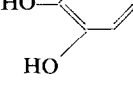
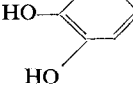
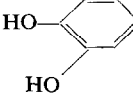
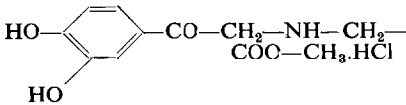
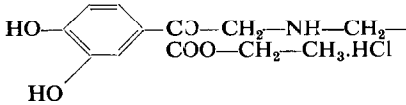
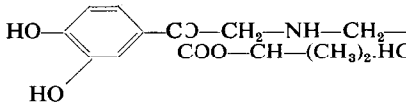
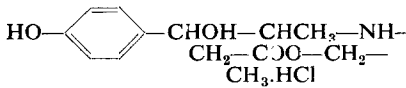
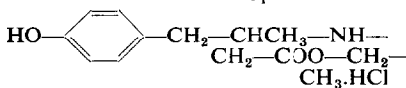
Substanz	Broncholytische Aktivität	Blutdrucksenkende Wirkung	Steigerung der Herzfrequenz
Cc 254 	0,007	0,009	0,007
Cc 255 	0,05	0,1	0,15-0,05
Cc 256 	0,01-0,02	0,04	0,01
Cc 258 	1,0	1,0	1,1-0,9
Cc 259 	0,33-0,2	0,33	0,2-0,3
Cc 260 	0,13	0,1	0,06-0,13

TABELLE 2.—*Continued*

Substanz	Broncholytische Aktivität	Blutdrucksenkende Wirkung	Steigerung der Herzfrequenz
CcK 254 	0,0001	0,0001–0,00005	—
CcK 225 	0,0001	0,0002–0,0001	—
CcK 256 	0,0005–0,0003	0,0005–0,001	—
Caa 255 			
Cpb 255 			

Die Verbindung, welche vor der Carboxylgruppe eine Äthylgruppe enthält (Cc 259) ist etwa ein Drittel so wirksam als Cc 258. Am unwirksamsten sind sowohl am Blutdruck als auch am Bronchospasmus die Ketoderivate, wobei stets der Methylester am schwächsten, der Äthylester am stärksten wirkt.

Der Äthylester der *p*-Oxyephedrin-carbonsäure (Caa 255) hatte am Bronchus bis 200 μ g keine Wirkung und 5 mg/kg der Substanz senkten den Blutdruck kurzdauernd, wie etwa 1 μ g/kg *iso*Propylnoradrenalin-sulfat. Noch unwirksamer war die Substanz Cpb 255 an beiden Testobjekten.

DISKUSSION

Die Tatsache, daß die zu Adrenalin gehörige Carbonsäure pharmakologisch unwirksam ist scheint an sich nicht bemerkenswert, da dies ja für alle Aminosäuren zutrifft, während die dazugehörigen Amine höchst wirksam sind. Dies hängt offenbar mit dem sauren Charakter jener Gruppe des Rezeptors zusammen, welche mit der Aminogruppe in Verbindung tritt. DOPOG hat allerdings insofern einen verschiedenen Aufbau, als hier die aliphatische Kette, welche die COOH-Gruppe trägt, direkt am Stickstoff sitzt. Es handelt sich demnach um ein substituiertes Glycin bzw. Sarkosin. Tatsächlich finden wir, daß diese Verbindung nicht enzymatisch zum primären Amin oder zu Adrenalin decarboxyliert wird, was übrigens auch für Methyl-DOPS gilt.¹¹

Unser auffallendster Befund ist jedoch, daß aliphatische Ester von DOPOG starke und zwar dem *iso*Propylnoradrenalin ähnliche Wirkungen haben. Es ist merkwürdig, daß offenbar ähnliche Ester von DOPS und Methyl-DOPS, die allerdings die Substitution mit der Dioxyphenyl-Oxyäthylgruppe nicht am Stickstoff, sondern am C-Atom tragen würden, bisher nicht bekannt worden sind; es wäre vielleicht lohnend auch diese zu untersuchen.

Berücksichtigt man bei den uns vorliegenden Estern die Substitution am Stickstoffatom und beachtet zunächst nur die Anzahl und Anordnung der C-Atome vor der COOH-Gruppe, dann ergeben sich gewisse Parallelen zu den Befunden von Konzett¹⁶ an entsprechend substituiertem Noradrenalin. Dieser Autor fand nämlich, daß die stärkste broncholytische Wirkung dem *iso*Propylnoradrenalin zukommt und auch unsere wirksamste Substanz (Cc 258) trägt vor der COOH-Gruppe einen *iso*Propylrest am Stickstoff. Unsere Substanzen Cc 259 und Cc 260 lassen sich ebenfalls in dieses Schema einfügen. Liegt jedoch nur eine Methylgruppe zwischen dem Stickstoff und der Carboxylgruppe wie bei unseren Substanzen Cc 254, Cc 255 und Cc 256, dann ist die broncholytische Wirkung erheblich schwächer. Einen gewissen Einfluss scheint jedoch auch die Estergruppe zu haben, nachdem der Äthylester (Cc 255) wirksamer ist als der *iso*Propylester (Cc 256) und erheblich wirksamer als der Methylester Cc 254. Die Einführung einer Ketogruppe statt der Hydroxylgruppe in der Seitenkette schwächt die broncholytische Wirkung sehr erheblich ab, der Einfluß der Estergruppe ist hier weniger deutlich (CcK-Reihe).

Entsprechende Derivate des *p*-Oxyephedrins waren praktisch unwirksam (Caa und Cpb 255).

Abgesehen von den eben geschilderten strukturellen Einflüssen der Seitenkette haben alle untersuchten Ester im wesentlichen die charakteristischen Wirkungen von *iso*Propylnoradrenalin, d.h. hemmende Wirkungen auf glatte Muskelfasern der Gefäße und der Bronchien und als erregende Wirkung Tachycardie. Auch in dieser Reihe, wie in früher untersuchten¹⁷⁻²⁰ ging die tachycarde Wirkung stets parallel mit der broncholytischen und blutdrucksenkenden.

Man hat gelegentlich den Unterschied in den Wirkungen von Noradrenalin und *iso*Propylnoradrenalin (als typische Vertreter zweier Gruppen von Sympathicomimeticis) auf das Vorhandensein von zwei verschiedenen Rezeptoren zurückgeführt, die Ahlquist²⁷ mit "Alpha" für den Noradrenalinotyp und "Beta" für den *iso*Propylnoradrenalinotyp bezeichnet hat.

Nach unseren Versuchen müssen wir annehmen, daß gleichgültig wie das übrige Molekül gebaut ist, die Hauptbindung und zwar wohl eine heteropolare Bindung mit einer sauren Gruppe des Rezeptors an der basischen Aminogruppe stattfindet. Barger und Dale²² und auch Blaschko¹¹ haben daneben auf die Wichtigkeit der Bindung der Brenzkatechylgruppe hingewiesen, jedoch genügt diese allein nicht dazu, um eine wirksame Verbindung mit dem Rezeptor herzustellen, wenn in der Nähe der Aminogruppe eine freie Carboxylgruppe entweder am C-Atom, oder am Stickstoff eingeführt wird. Die OH-Gruppe in der Seitenkette ist nach Blaschko¹¹ ein dritter Verankerungspunkt, welcher nach seiner Auffassung vor allem die Intensität der Wirkung beeinflusst. Dabei darf nicht vergessen werden, daß diese Gruppe auch die basische Eigenschaft der Aminogruppe beeinflusst: Wird sie durch eine CO-Gruppe ersetzt, dann wird die Wirkung erheblich schwächer. Wenn nun jenseits der Aminogruppe noch aliphatische—oder Aralkylgruppen eingeführt werden, dann bedeutet

das eine zusätzliche Besetzung der Rezeptoroberfläche und schon die Einführung einer Methylgruppe, wie im Adrenalin, anstelle der primären Aminogruppe, führt besonders an der Bronchialmuskulatur zu einer Wirkungsveränderung, welche sich dem *iso*Propylnoradrenalin nähert. Man sollte jedoch als Prototyp dieser Wirkung nicht das *iso*Propylnoradrenalin ansehen, weil wir in früheren Untersuchungen¹⁷⁻²⁰ gezeigt haben, daß es noch stärker broncholytische Stoffe gibt. Optimal scheint die Substitution mit einer *iso*Propylphenolgruppe am Stickstoff zu sein.

Wird jedoch die primäre Bindung durch eine am Stickstoff selbst befindliche oder in unmittelbarer Nachbarschaft stehende COOH-Gruppe verhindert, dann werden die Substanzen wirkungslos. Die Wirkung kann jedoch wieder eintreten, wenn durch Esterbildung die COOH-Gruppe neutralisiert ist.

Anerkennungen—Wir danken Herrn Dr. J. Brug für wertvolle Ratschläge, Herrn H. Niewind und Herrn A. Vrijbergen für die Durchführung einiger Synthesen

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SHORT COMMUNICATIONS

Reserpine depletion of adenosine triphosphate from the rat suprarenal medulla

(Received 13 August 1958)

RESERPINE interferes with oxidative phosphorylation in rat brain and liver *in vivo* but has little effect on oxidative phosphorylation in skeletal muscle or heart.¹ Studies *in vitro* have shown that reserpine reduces the P/O ratio in rat brain mitochondria by about 40 per cent.²

A number of reports^{3, 4} have shown that reserpine depletes the brain and adrenal medulla of catechol amines. Since the adrenal medulla contains not only catechol amines but also a high concentration of adenosine triphosphate (ATP),^{5, 6} it seemed of interest to investigate whether reserpine had an effect upon the adenosine nucleotides of the rat adrenal medulla. If a relationship exists between adrenal adenosine nucleotides and synthesis or storage of catechol amines,^{7, 8} then reserpine which depletes the medulla of catechol amines should also affect the nucleotides.

Experiments were carried out using groups of 9 female rats, each rat weighing about 200 g. The control groups were injected with an appropriate volume of the vehicle, while the treated groups were given 1 mg of reserpine by intraperitoneal injection. Food was withheld and the animals were killed 16 hr after the injection.

The adrenal medullae were carefully dissected out and crushed in a centrifuge tube containing 1 ml of 1.5 per cent perchloric acid. A sandpaper-roughened glass rod and a little silver sand facilitated crushing. The adrenal medullae from each group of rats were pooled. All operations were carried out at 0°. The crushed samples were centrifuged at 2000 r.p.m. for 10 min, the supernatant transferred and the residue extracted twice with 1 ml of 1.5% perchloric acid. An equivalent amount of succinate buffer (pH 6.1) using a pH meter. The final volume was adjusted to 10 ml with succinate buffer.

Total catechol amines were estimated on the blood pressure of the spinal cat and calculated in *noradrenaline* equivalents. Adenosine nucleotides were estimated by the specific enzymic method of Kalckar.⁹

The results are presented in Table 1.

TABLE 1. EFFECTS OF RESERPINE ON THE CONTENT OF CATECHOL AMINES AND ATP OF THE ADRENAL MEDULLA

Group No.*	Treat-ment	Wt. of pooled medullae (mg wet wt)	Total catechol amines in <i>noradrenaline</i> (equiv.mg/g wet wt)	Total nucleotides (expressed in μ moles/g wet wt)	ATP	ADP	AMP
1	Control	29.5	11.7	10.10	10.0	nil	0.10
2	Control	29.7	12.15	9.25	9.0	nil	0.25
3	Reserpine (1 mg)	39.1	2.90	3.32	3.0	nil	0.32
4	Reserpine (1 mg)	39.9	2.10	3.30	2.95	nil	0.35

* Each group contains 18 medullae taken from 9 rats.

It is clear that reserpine depletes the medulla both of catechol amines and ATP. No adenosine diphosphate (ADP) and little adenosine monophosphate (AMP) are found in the adrenal medullae of the drug-treated animals, i.e. there is no increase in the concentration of these compounds over the

control level as might have been expected. The fact that the catechol amines and ATP disappear in roughly the same proportions during reserpine treatment may point to a specific role for ATP in the storage or release of catechol amines. In a recent publication Schümann¹⁰ has obtained similar results in the hen.

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La 8-azaguanine provoque-t-elle la formation de protéines anormales?

(Received 17 September 1958)

DANS des travaux récents,^{1, 2, 3} nous avons montré que la 8-azaguanine dissocie la synthèse des acides nucléiques de celle des protéines chez *Bacillus cereus*, d'une façon qui rappelle à plusieurs points de vue les effets de la chloromycétine sur *Escherichia coli*. Par exemple, l'azaguanine, comme la chloromycétine, inhibe fortement la synthèse des protéines sans entraver celle des parois cellulaires.^{1, 3}

Mandel⁴ a observé de son côté que l'incorporation des acides aminés contenant du soufre est inhibée tandis que celle de l'acide glutamique ne le serait pas. Ceci indiquerait que la composition des protéines formées en présence d'azaguanine est anormale.

Nous croyons que le choix de l'acide glutamique comme précurseur marqué dans de telles expériences est regrettable, car cet acide aminé s'incorpore non seulement dans les protéines mais encore dans les parois cellulaires de *B. cereus*, dont la synthèse n'est pas inhibée par l'azaguanine.^{1, 3} Il est certain que l'incorporation d'acide glutamique observée par Mandel correspond pour une large part à la croissance des parois cellulaires.

Toutefois, des résultats d'une tout autre nature indiquent que la synthèse résiduelle observée en présence d'azaguanine pourrait en effet correspondre à la formation de substances protéiques anormales. Nous avons signalé² que la synthèse de pénicilline, enzyme constitutif dans la souche utilisée, est plus fortement inhibée que l'incorporation de méthionine-³⁵S dans les protéines. En outre, si la synthèse des protéines est inhibée par l'azaguanine (40 µg/ml) chez *B. cereus* en croissance exponentielle et que de la guanosine soit ajoutée au système 60 min après l'analogue, l'incorporation de phénylalanine-¹⁴C dans les protéines et la synthèse de pénicilline sont toutes deux rétablies, mais pas simultanément. Il s'écoule 2 heures entre le moment où l'incorporation de phénylalanine reprend et celui où la synthèse de pénicilline se rétablit (Fig. 1).

A moins que la pénicilline constitutive ne se comporte autrement que la plupart des protéines de la bactérie, tout permet de penser que l'incorporation de phénylalanine qui est rétablie par la guanosine ne représente pas dès le début la synthèse de protéines parfaites.

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Nous croyons que l'action de l'azaguanine s'exerce à deux niveaux différents: l'inhibition générale qui se produit quelques minutes après l'addition de l'analogue serait due à des modifications de dérivés guanyliques à métabolisme rapide^{2, 4, 5} tels que l'acide guanosinetriphosphorique, des oligonucléotides ou une fraction particulière de l'acide ribonucléique, tandis que l'effet plus profond sur

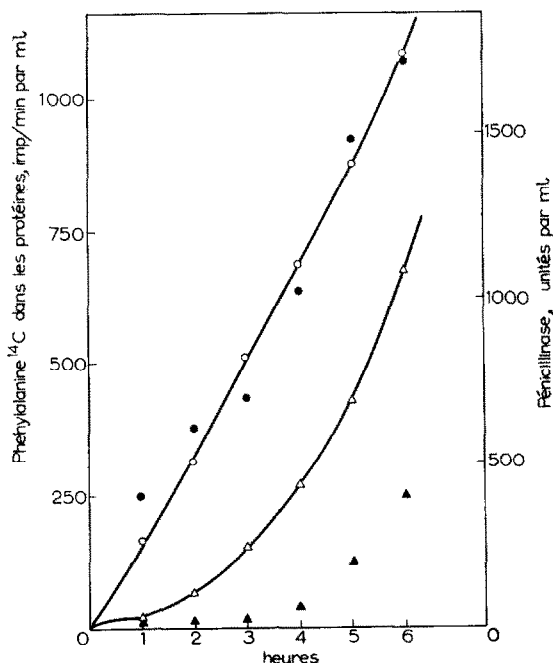


FIG. 1. Restauration par la guanosine de la synthèse de pénicilline et de l'incorporation de phénylalanine après inhibition par l'azaguanine. *B. cereus*, mutant pénicilline constitutive, dans un hydrolysate de caséine contenant de la phénylalanine radioactive, et Tween 80.

Témoin: 40 μ g azaguanine et 100 μ g guanosine au temps zéro.

○: ¹⁴C dans le précipité trichloroacétique.

●: pénicilline.

Essai: 40 μ g azaguanine au temps zéro, et 100 μ g guanosine au temps 60 min.

△: ¹⁴C dans le précipité trichloroacétique.

▲: pénicilline.

les propriétés des protéines formées résulterait de modifications des organisateurs polynucléotidiques ("templates"). Ce second effet pourrait être rapproché de celui que les analogues de purines⁶ et de pyrimidines⁷ exercent sur les virus.

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Presence of biogenic amines in the bovine pineal body

(Received 8 September 1958)

THE sub-microscopic anatomy of the pineal body of the rat reveals a structure well suited to a secretory function¹ which is reminiscent of the morphology of the neurohypophysis described by Palay.² Recently, Lerner and his co-workers³ have reported the presence of a new hormone, melatonin, in the pineal body. This substance which is believed to be an indole derivative, inhibits the melanocyte-stimulating hormone (MSH) of the neurohypophysis. These findings have prompted us to seek other neurohormonal substances in the bovine pineal body. We have found this structure relatively rich in three biogenic amines: 5-hydroxytryptamine (serotonin); catechol amines (determined as noradrenaline); and histamine. Table 1 summarizes these results and presents data on enzymes concerned with the metabolism of these amines.

TABLE 1.

Amine or enzyme studied	Found
Serotonin	0.4 µg/g
5-Hydroxytryptophan decarboxylase	Present
Tryptophan hydroxylase	Absent
Monoamine oxidase	Present
Catechol amines (as noradrenaline equivalent)	0.4 µg/g
Histamine	10 µg/g
Histidine decarboxylase	Absent
Histaminase	Absent

Serotonin was determined by bioassay on the heart of *Venus mercenaria*. Histamine was extracted by Code's method⁴ and assayed on the isolated ileum of the guinea pig. Catechol amines were extracted and separated by means of alumina, following a modification of the method of Von Euler and Floding,⁵ and the fluorescence of their oxidation products served as the basis for their determination in an Aminco-Bowman spectrophotofluorometer.

It appears from this preliminary work that the pineal body may serve as a convenient and fruitful model for neuro-chemical study.

Acknowledgement—We are indebted to Dr. A. B. Lerner for supplying us with bovine pineal bodies, both in fresh and in lyophilized forms.

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Some biochemical effects of hypoglycin

(Received 28 August 1958)

THE structure and synthesis of hypoglycin, α -amino-methylene-cyclopropane-propionic acid, have recently been reported.^{1, 2, 3, 4} We wish to report some biochemical studies with this powerful hypoglycemic agent, as well as a comparison of its effects with those induced by insulin and tolbutamide.* A possible metabolic site of action of hypoglycin is proposed as a working hypothesis.

Eighteen-hour-fasted rats weighing about 100 g were used in all experiments.

Groups of 4 litter-mates were given the following intramuscular injections: (a) saline, (b) 15 mg of hypoglycin,[†] (c) 0.3 unit of insulin, (d) 50 mg of tolbutamide. Five minutes later 100 mg of glucose and 3 μ curies of uniformly C^{14} -labeled glucose were injected subcutaneously. The rats were placed in a small respiratory apparatus for 2 or 4 hr and expired CO_2 was collected in KOH or NaOH and counted as $BaCO_3$. Liver and muscle (rectus femoris) glycogen⁵ and liver total lipid⁶ were determined after sacrificing the animals and the extent of C^{14} -incorporation was determined. The data obtained from one such experiment are shown in Table 1. In comparison to the control, treatment with insulin

TABLE 1. THE DISTRIBUTION OF C^{14} IN RAT TISSUES TWO HOURS AFTER ADMINISTRATION OF HYPOGLYCEMIC AGENTS FOLLOWED BY UNIFORMLY C^{14} -LABELLED GLUCOSE

	Total Number Counts per Min/Rat			
	Control	Hypoglycin	Insulin	Tolbutamide
Liver lipid	8,181	5,788	922	721
Liver glycogen	4,540	0	0	0
Muscle glycogen*	10,788	19,632	38,400	8,376

* Calculated from the gastrocnemius muscle on the basis that total muscle mass is 40 per cent of body weight.

or tolbutamide resulted in marked inhibition of C^{14} -incorporation into liver lipid, whereas hypoglycin did not exert this effect. Similar results were obtained in two other experiments. All three treatments resulted in minor incorporation (zero in the experiment shown) of glucose into liver glycogen. Furthermore, no major differences between treatments were observed in the excretion of $C^{14}O_2$ (data not shown). About 40–50 per cent of the administered C^{14} was excreted as CO_2 .

In other studies, hypoglycin, insulin, tolbutamide or saline was administered and the livers were excised 4 hr later. Liver total lipid⁶ levels were significantly higher (25–30 per cent) than those of control animals only in the case of treatment with hypoglycin (Table 2). Similarly, only liver mitochondria prepared from the hypoglycin-treated rats showed significant impairment in the ability to form high-energy phosphate bonds associated with oxidation of pyruvate and malate. When added *in vitro* at a final concentration of 10^{-3} M to liver mitochondria prepared from normal rats, hypoglycin produced no effect on oxidative phosphorylation.

It was of further interest to determine serum non-esterified fatty acid (NEFA) levels after hypoglycin treatment. Four hours after hypoglycin treatment, NEFA levels were found to be 4.5 fold higher (about 2000 μ equivalents/liter) than those of control animals (Table 2). Both insulin and tolbutamide induced a pronounced fall in NEFA as well as in glucose levels.^{7, 8}

In other experiments 100 mg of glucose were administered 5 min after the hypoglycemic agents or saline, and blood glucose⁹ levels were determined at 0, 15, 30, 60 and 120 min. Both insulin and tolbutamide lowered the peak rise and caused a rather rapid fall in the blood glucose level. In contrast, the curves obtained with hypoglycin-treated rats were similar to those of the saline-treated rats for the first 2 hr, but at a time when the glucose levels of rats treated with tolbutamide or insulin were returning to normal the glucose levels of the hypoglycin-treated rats continued to fall.

Other experiments were performed with diagram and liver slices from hypoglycin-treated rats. When removed 2 hr after hypoglycin treatment no significant differences in glucose uptake or glycogen formation, as compared to diagram from untreated rats, were observed. Similarly no effect on insulin stimulation of glucose uptake was observed. The respiratory quotient (R.Q.) of liver slices obtained

from hypoglycin-treated rats was not significantly different from that of controls. However, when hypoglycin was added *in vitro* to respiring liver slices from untreated rats at a concentration of 4×10^{-3} M a significant increase in R.Q. was observed.

TABLE 2. LIVER TOTAL LIPID LEVELS AND SERUM NON-ESTERIFIED FATTY ACID LEVELS (NEFA) FOUR HOURS AFTER ADMINISTRATION OF HYPOGLYCEMIC AGENTS

Treatment	Liver total lipid level*	Serum NEFA†
	(mg/g)	(microequivalents/liter)
Saline	57.5 ± 2.3	457‡
Hypoglycin	78.2 ± 2.5	1655‡
Insulin	57.0 ± 4.0	—
Tolbutamide	58.8 ± 4.0	—

* Determined in 100 g rats starved for 18 hr. Values given are the means $\pm$ standard error. Six rats per treatment.

† Rats were given 100 mg glucose intramuscularly at the time of injection of saline or hypoglycin.

‡ Values shown are the mean of 7 determinations.

The experiments reported herein suggest that hypoglycin may impair utilization of fat. The observed increases in liver total lipid and serum NEFA levels especially point to this conclusion. Fat formation from glucose is apparently uninfluenced by the administration of hypoglycin while treatment with insulin or tolbutamide significantly lower the net incorporation of glucose into liver lipid.

Of further interest is the fact that α -ketomethylene cyclopropane-propionic acid* and methylene cyclopropane acetic acid* have also been found to have about the same order of hypoglycemic activity as hypoglycin. A recent report¹⁰ indicated that Δ -4-pentenoic acid was also hypoglycemic. These latter findings, along with the observed effects of hypoglycin on lipid metabolism described above, offer the further suggestion that the amino acid is metabolized to an acid metabolite, the true hypoglycemic agent.

* These compounds were prepared by Mr. J. C. Van Meter and Mr. E. H. Snedcker of these laboratories.

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Different effects exerted by isoniazid upon the coenzyme A and pyridoxine contents of the host's tumour and tissues

(Received 15 September 1958)

THE inhibiting effect exerted by isoniazid upon the development of certain experimentally-induced tumours¹ and the fact that such an effect is enhanced by desoxyypyridoxine² may well be regarded as a still unclarified facet of the relationship between the metabolism of vitamin B6 and the mechanism through which isoniazid expresses its antitlastic action. It has been furthermore reported that the lowering of the activity of coenzyme A in the liver of rats when anti-vitamin B6 is administered can be offset by a simultaneous administration of isoniazid,³ a finding which seems to be inconsistent with the reported reduction² of the pyridoxine contents in the tissues of such animals as are treated with this drug.

In view of the foregoing, a research programme was undertaken to assess the effects of isoniazid (a) upon coenzyme A contents and (b) upon vitamin B6 contents in several tissues of tumour-bearing animals.

We used male albino rats averaging in weight from 15 to 18 g, each bearing an adenocarcinoma-755 grafted 5 days before in the axillary area by the usual trocar technique. Starting from the fifth day, the animals were divided into two groups of twenty subjects each, one group receiving a daily intramuscular injection of 20 mg isoniazid (INH) per kilogram of body weight, while injections of physiological solution were administered to the other group. After 20 days of the above treatment the subjects were bled to death and their organs and tumours removed under aseptic conditions, frozen, weighed and individually divided into two sections which were also weighed, one part being used to determine the coenzyme A contents by the Kaplan and Lipmann method,⁴ while the other was used to assess the vitamin B6 contents by the method developed by Parrish *et al.*⁵ Our results are summarized in Table 1.

TABLE 1. CHANGES IN COENZYME A AND PYRIDOXINE CONTENTS OF SEVERAL TISSUES AFTER ISONIAZID TREATMENT (AVERAGE VALUES $\pm$ S.E.)

Tissue	Control		Treated	
	CoA (U/g)	Pyridoxine (μ g/g)	CoA (U/g)	Pyridoxine (μ g/g)
Liver	110.8 $\pm$ 7.3	5.19 $\pm$ 0.46	126.1 $\pm$ 11.0	4.56 $\pm$ 0.38
Kidney	91.5 $\pm$ 6.6	4.02 $\pm$ 0.31	99.8 $\pm$ 8.2	3.41 $\pm$ 0.31
Heart	64.1 $\pm$ 5.2	4.41 $\pm$ 0.38	70.6 $\pm$ 6.1	3.68 $\pm$ 0.33
Lung	14.6 $\pm$ 0.9	0.73 $\pm$ 0.06	15.9 $\pm$ 1.2	0.55 $\pm$ 0.04
Brain	23.2 $\pm$ 1.6	2.65 $\pm$ 0.19	25.2 $\pm$ 2.8	2.34 $\pm$ 0.19
Muscle	7.9 $\pm$ 0.4	2.58 $\pm$ 0.22	9.0 $\pm$ 0.6	2.01 $\pm$ 0.18
Tumour	13.3 $\pm$ 0.7	0.66 $\pm$ 0.04	10.3 $\pm$ 0.9	0.49 $\pm$ 0.03

The fact can be observed that a decrease in vitamin B6 contents and an increase in the coenzyme contents are simultaneously induced by INH in all tissues excepting the neoplastic one, where both contents are lowered, this effect being accompanied by a slight, non-significant reduction in tumour weight (1.22 g. average in treated subjects against 1.36 g in the controls).

Obviously, neoplastic and normal tissues appear to react to INH in different ways, and it would seem only reasonable to assume that in normal tissues the drug can interfere with only some of the reactions in which the vitamin is involved, while the other would not be affected or, possibly, would benefit from it, while suitable conditions for such an action to be profitably exerted by the INH molecule would seem to lack in the neoplastic tissue.

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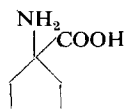
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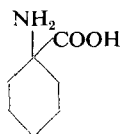
The tumour growth inhibitory activity of 1-amino-cyclopentane-1-carboxylic acid* and related peptides
(Received 20 December 1958)

IN THE course of the examination of potential amino-acid antagonists as inhibitors of tumour growth we have been particularly interested in α -substituted derivatives¹ since these are believed to be strongly concentrated intracellularly.² As a logical extension of this work we have examined some cyclic compounds which can be regarded as derived from such α -alkylated amino-acids.³

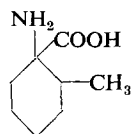
Amongst the compounds now tested are 1-amino-cyclopentane-1-carboxylic acid (I), 1-amino-cyclohexane-1-carboxylic acid (II), 2-methyl- (III), and 3-methyl- (IV) -1-amino-cyclohexane-1-carboxylic acid, 1-amino-tetrahydronaphthalene-1-carboxylic acid (V), and 2-amino-tetrahydronaphthalene-2-carboxylic acid (VI).



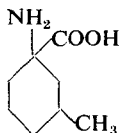
(I)



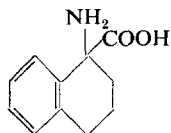
(II)



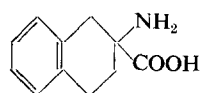
(III)



(IV)



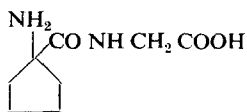
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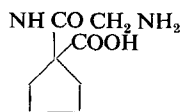
(VI)

The cyclohexane derivatives (II, III, and IV) and the two tetrahydronaphthalene amino-acids (V and VI) had no effect on the growth of the transplanted Walker rat carcinoma when administered by intraperitoneal injection of an aqueous solution in ten daily doses of 100 mg per 200 g. rat. The cyclopentane derivative (I) is rather more toxic and could only be given at a dose level of 25 mg per 200 g rat per day. The average tumour weight in a control series of animals eight days after implant was 55 g whereas in a parallel series of animals which had 25 mg of (I) on each of six days after implant the average tumour weight on the eighth day was 1.8 g (Control/treated ratio $55/1.8 = 30.5$).

At this dose level the compound was still producing toxic symptoms and in an attempt to reduce this toxicity we have examined some simple dipeptides, such as (VII) and (VIII).



(VII)



(VIII)

In an experiment where the treated rats received six daily injections of 50 mg of the hydrochloride of (VIII) the C/T ratio was 9.0 but this dose was not well tolerated. Eight daily doses of 25 mg of the hydrochlorides of (VII) and (VIII) gave C/T ratios of 7.5 and 5.3 respectively and these amounts were well tolerated by the animals.

In preliminary haematological investigations a single intraperitoneal injection of an aqueous solution of (I) at a dose of 750 mg/kg caused death after severe weight loss in three out of four rats in

* While this work was in progress it was learned from the Cancer Chemotherapy National Service Center, Bethesda, U.S.A., that this amino-acid is also being studied under their auspices. It is of interest to note that a related compound—1-hydroxylamino-cyclopentane-1-carboxylic acid has been reported to prolong the life of mice afflicted with an ascites tumour (Wilson and Irwin, quoted by Neelakantan and Hartung, Abstracts of papers, 134th meeting of the American Chemical Society, p. 24/0).

eight to ten days. In all cases death was preceded by a very marked depression of circulating leukocytes particularly of neutrophils which disappeared completely from the circulation 6 days after administration of the compound. A delayed, rather less severe fall in blood platelets also occurred shortly before death. The surviving animal showed complete suppression of blood neutrophils 6 days after treatment with the amino-acid (I) and some fall in lymphocyte count together with a slight fall in platelet count also occurred. Neutrophils were still absent at 10 days by which time the lymphocyte count had returned to normal.

Following the demonstration of the tumour growth inhibitory action of 1-amino-cyclopentane-1-carboxylic acid (I) and the two glycine peptides we are now preparing for test certain analogues of (I) and further simple peptides for eventual conversion into cyclic peptides.

Acknowledgements—This investigation was supported by grants to this Institute from the British Empire Cancer Campaign, the Jane Coffin Childs Memorial Fund for Medical Research, the Anna Fuller Fund, and the National Cancer Institute of the National Institutes of Health, U.S. Public Health Service. The authors thank Professor A. Haddow, F.R.S. for permission to quote the results of tumour growth inhibition studies.

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ERRATA

P. EMMELOT and C. J. BOS: *Biochemical Pharmacology* **1**, 105-110.

Page 106, line 11. For "cytochromec" read "cytochrome-c."

Page 107, footnote, line 11. For "aerobic" read "anaerobic."

P. EMMELOT, C. J. BOS, B. J. VISSER and C. T. BAHNER: *Biochemical Pharmacology* **1**, 111-114.

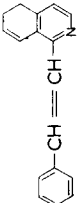
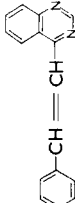
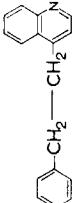
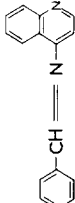
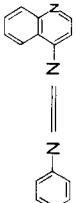
Page 112, Table 1. This table is now printed in its correct form on pages 242 and 243 overleaf.

TABLE 1. CONDENSED SUMMARY OF THE METABOLIC AND TUMOUR-INHIBITORY EFFECTS OF STYRYLQUINOLINES AND ANALOGUES

For details of methods compare the previous paper² and the present text. The solubility of the various compounds was measured colorimetrically (after incubation at 37°C in the absence of biological material the sediment and supernatant, obtained by centrifugation, were acidified after addition of extra ethanol. Aliquots were measured at the wave length of maximum absorption of each compound).

ATPase activity was calculated from the phosphate release from ATP after 20 min of incubation at 27°C. The compounds were tested at 1.5×10^{-4} M in the Warburg assays, at 1.3×10^{-4} M in the ATPase assay and at 1.3×10^{-5} M in the spectrophotometric determination of the glutamic dehydrogenase activity. The data on tumour growth were collected from reference 1 and unpublished experiments.

Compound	Solubility	Crystall. glutamic dehydrogenase (DPN-reduction)	Liver Mitochondria		Ascites Tumour Cells			Relative activity against Lymphoma 8 and Walker 256 tumours
			Glutamate oxidation (O ₂ -consumption)	ATPase (phosphate release)	Respiration endogenous (O ₂ -consumption)	Glycolysis		
						Anaerob.	Aerob.	
per cent of added dose			per cent of controls					(75 mg/kg)
I:	80	100; —	0-5	400-500	30-50	150-200	150-200	moderately active
II:	22	45; 36*	0-15	700-900	30-50	200-280	200	(very) active
III:	23	69; —	10-20	700-900	20-50	200-250	200	very active
IV:	15	—; 80	80-100	500	80-100	100-120	100-120	very slightly active

V: 	?	90; 80	0-10	500-700	20-30	200	(very) active
VI: 	30	90; 75	90-100	200-250	100	100-110	slightly active†
VII: 	36	100; —	90-100	110-150	100	100-110	very slightly active
VIII: 	25	—; 87	70	400	100	150	inactive
IX: 	?	—; 84	0-10	400	20-30	150	slightly active‡
X: 	?	—; 60	20	500-600	30-50	150-175	inactive‡
XI: 	33	31; 26*	50-60**	700-800	60	150-19 ^v	inactive
<i>o</i> -aminoazotoluene	60	100; 90	50**	700-800	50-80	150-180	

* No inhibition in the presence of 0.1 per cent albumine.

† Active at three times the dose required for 4-DSQ (II).

‡ Not tested at higher dose levels.

Papers to be published in future issues

- R. L. NOBLE, C. T. BEER and J. H. CUTTS: Further biological activities of Vincalukoblastine—an alkaloid isolated from *Vinca rosea* Linn.
- JEAN M. HAGEN: Inhibition of an action of adrenaline by D-tartrate.
- A. J. THERKELSON: Combined treatment of transplantable mouse tumour by cysteamine (*p*-mercaptoethylamine) and nitrogen mustard.
- A. J. THERKELSON: Studies on the protective action of sulphadryl compounds and amines against nitrogen mustard (HN2) and Roentgen irradiation in mice.
- N. J. GIARMAN and S. SCHANBERG: The intracellular distribution of 5-hydroxytryptamine (HT; serotonin) in the rat's brain.
- G. H. DELTOUR, J. M. GHUYSEN and A. KLAUS: Effet de la mescaline, du LSD et de dérivés de l'adrenochrome sur la decarboxylase glutamique de cerveau.
- T. FREDERICKSON and G. TIBBLING: Reversal of effects on the rat nerve-diaphragm preparation produced by methyl fluoro-phosphorylcholines.
- W. L. NASTUK and BARBARA O. ALVING: Further study of 3-hydrophenyldimethylethylammonium (edrophonium) and its closely related analogues with respect to activity at the neuromuscular junction.
- A. S. FAIRHURST, R. E. SMITH and E. M. GAL: The effects of fluorocompounds on oxidative phosphorylation—I. The effects on P/O ratios with fumarate and with reduced diphosphopyridine nucleotide.
- A. S. FAIRHURST, R. E. SMITH and E. M. GAL: The effects of fluorocompounds on oxidative phosphorylation—II. Effects on the breakdown of nucleotides by liver mitochondria.
- N. K. CHAUDURI, K. L. MUKHAJEE and CHARLES HEIDELBERGER: Studies of fluorinated pyrimidines—VII. The degradative pathway.
- O. A. TROWELL: The cytotoxic action of barbiturate *in vitro*.
- RICHARD SCHINDLER: The conversion of C¹⁴-labelled tryptophan to 5-hydroxytryptophan by neoplastic mast cells.
- MONIQUE REGINSTER: A propos l'effet Delaporte.
- V. P. WHITTAKER: Acrylylcholine: a new, naturally occurring, pharmacologically active choline ester from *Buccinum undatum*.
- TH. J. DE MAN and J. R. ROBORGH: The hypercalcemic activity of dihydrotachysterol₂ and dihydrotachysterol₃, and of the vitamins D₃ and D₂. Comparative experiments in rats.
- ADAMO VERGINE: Une méthode pour l'étude des lipides hématiques.

COMBINED TREATMENT OF A TRANSPLANTABLE MOUSE TUMOUR WITH CYSTEAMINE (β -MERCAPTO-ETHYLAMINE) AND NITROGEN MUSTARD (HN2)

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(Received 4 September 1958)

Abstract—The toxic effect of cysteamine has been studied after repeated injections into AKA mice.

In a series of experiments in which a lymphocytic neoplasm was transplanted into AKA mice it was shown:

(1) That cysteamine and nitrogen mustard (HN2) exert an additive effect on the size of the subcutaneously transplanted tumour, but that this effect is probably due to the weight loss which occurs during the treatment of the animals, since the growth of the tumour occurred at a slower rate when the animals were subjected to moderate starvation. It is mentioned that the results obtained by Peczenik¹⁰ may possibly be explained in the same way.

(2) That cysteamine has no therapeutic effect on the tumour used.

(3) That cysteamine given 4-6 hr before HN2 does not affect the increased survival time produced by HN2.

(4) That cysteamine given 15-20 min before HN2 does not reduce the increased survival time produced by HN2, but that a considerably greater increase in survival time can be obtained by a combination of the two drugs than when HN2 is given alone, since cysteamine seems to afford a certain protection against the lethal effect of HN2 without a simultaneous reduction in the tumour-inhibitory effect of the latter.

It is therefore concluded that the protective effect of cysteamine against HN2 can scarcely be explained by an inactivation of the latter by its linkage to the SH group of cysteamine, as has been claimed by Salerno and Friedell.¹³

Other possible explanations of the effect are discussed.

THE PROTECTION afforded by cysteamine against the lethal effect of x-rays and nitrogen mustard is well known from animal experiments.^{2, 13}

It has been claimed by Peczenik¹⁰ that cysteamine does not reduce the inhibitory action of nitrogen mustard (HN2) on the Walker carcinoma transplanted into rats, but that it, on the contrary, increases the tumour-inhibitory effect of HN2.

It was previously shown¹⁷ that cysteamine gives good protection of mice against a 98.5 per cent lethal dose of HN2 when injected within $\frac{1}{2}$ hr before HN2 injection, and that it especially gives a good primary protection of the bone marrow.

If this protection of the bone marrow could be achieved without a simultaneous reduction in the tumour-inhibitory effect of HN2, a combination of cysteamine and HN2 might prove to be of significance in the clinical treatment of malignant disorders.

MATERIALS AND METHODS

Apart from one of the first experiments (Fig. 2), mice weighing from 21 to 27 g were used; the animals (inbred AKA mice) were divided into groups of eight with an average weight of 24 g and with a uniform sex and age distribution in the individual groups.

* Lady Tata Memorial Scholar. Aided by grants from the Research Council of the Danish Civil Defence Board, the Anders Hasselbalch Anti-Leukaemia Foundation and C. P. Schepler and wife's bequest, the Irma Foundation.

The tumour used was what Dunn⁵ terms a "lymphocytic neoplasm", which had arisen spontaneously in one of our AKA mice in December 1954 and since maintained by continuous subcutaneous transplantation in the same strain.

In the initial experiments, subcutaneous transplantation of 0.1 ml of finely minced tumour was used, but in the later experiments in which intraperitoneal transplantation was employed, a suspension with a known number of tumour cells/ml in physiological saline was prepared; amounts of 0.1 ml of this suspension, containing $1.0-1.5 \times 10^6$ cells, were used for the injections.

All the animals were weighed daily or every other day. Injections of HN2 and cysteamine were usually commenced 2 days after the transplantation and were given daily (except on Sundays) as long as at least one group of animals in the experiment concerned was complete in number.

Autopsy was performed on all the animals, and histological section of the kidneys, liver, radix mesenterii, spleen, thymus and lungs were studied after staining with haematoxylin and eosin. Specifically, sections were prepared from all animals in which the gross appearance did not unquestionably show whether death was due to the tumour growth or to intoxication by the injected HN2.

The nitrogen mustard used was HN2 (N-methyl-bis- β -chloro-ethylamine-hydrochloride) manufactured by A/S Ferrosan under the proprietary name of Erasol and supplied in ampoules containing 10 mg of HN2 in 1 ml of absolute alcohol.

The desired dilution of HN2 was obtained by addition of physiological saline, and the HN2 solution was at once injected intraperitoneally in amounts of 0.1 ml.

The cysteamine used in the first experiments was synthesized by the Chemical Institute of the University of Copenhagen (Professor K. A. Jensen). As this institute could not continue the synthesis, the cysteamine used in later experiments was supplied by the chemical factory Fluka; the latter preparation proved to have the same toxicity and the same protective effect against HN2 in mouse experiments as the former. The iodometric titration value does not change when the drug is stored in ampoules with an N_2 atmosphere.

The cysteamine preparation was dissolved in physiological saline and neutralized by hydrochloric acid immediately before the injection.

RESULTS

1. Toxicity of cysteamine after repeated daily injections

The toxicity of cysteamine after a single injection has previously been determined in "street" mice; in animals weighing from 21 to 27 g (average weight 24 g) the LD_{50} was found to be 6.5 mg/mouse.

With a view to the planned tumour experiments it was necessary to study the toxic action of the drug after repeated daily injections into AKA mice.

After intraperitoneal injection of 3 mg of cysteamine each day for 14 consecutive days, 11 out of 11 AKA mice survived. The animals did not seem to be affected by the injections, but a weight loss occurred, averaging 3 g as compared with the animals of a control group injected with physiological saline (Fig. 1).

Following injections of 3 mg of cysteamine twice daily at intervals of 4-6 hr for 15 consecutive days, 7 out of 8 mice survived; after having received a total of 6×3 mg the eighth mouse died with clonic convulsions persisting for 24 hr. The weight loss in the surviving animals did not exceed that observed in the aforementioned mice, which

were given only one injection daily (Fig. 1). The toxic effect of a still higher dosage was not studied, but an amount of 3 mg twice daily for 15 days is likely to be very close to the lethal dose for AKA mice of an average weight of 24 g, since 4 mice which in another experiment received that dose all developed clonic convulsions before they were killed and autopsied on the day following the last injections (Table 1).

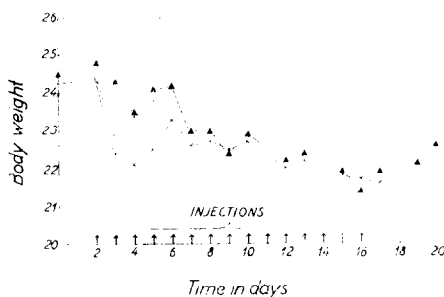


FIG. 1. Average body weight (g) of 3 groups of 8 AKA mice which were given injections on 15 consecutive days as follows: ●—●, physiological saline, 0.2 ml daily; ×—×, cysteamine, 3 mg daily; ▲—▲, cysteamine, 3 mg twice daily.

As Peczenik¹⁰ has claimed that cysteamine produces shrinkage of the spleen in rats, groups of 4 mice were subjected to autopsy at different times within the experimental period in the toxicity studies in order to ascertain the weight of the thymus and spleen.

The result appears from Table 1. It is seen that in spite of the fact that the doses injected were considerably higher than those used by Peczenik, no definite change in the weight of the spleen was found as compared with a control group of the same age and weight after injections of physiological saline. On the other hand, a considerable decrease in the weight of the thymus occurred in all 4 groups of animals.

TABLE 1. TOTAL WEIGHT OF THE THYMUS GLANDS AND SPLEENS OF FOUR EXPERIMENTAL ANIMALS AND FOUR CONTROLS AFTER THE INJECTION OF VARIOUS DOSES OF CYSTEAMINE

Dose of cysteamine (mg)		Weight of thymus (mg)		Weight of spleen (mg)	
Daily	Total	Exp.	Controls	Exp.	Controls
3	3 × 14	318	525	214	237
3 × 2	3 × 9	290	516	221	223
3 × 2	3 × 17	264	427	260	205
3 × 2	3 × 30	288	561	281	331

Histological sections of the kidneys, spleen, liver, lungs and thymus, stained with haematoxylin and eosin, showed no definite abnormalities. Specifically, the structure of the thymus showed no changes; the cellular density seemed to be normal, and the cortex and medulla could readily be distinguished. Thus, the weight loss was due to a concentric atrophy of the organ.

2. Effect of cysteamine and HN2 on subcutaneously transplanted tumours

In these initial experiments, attempts were made to assess the effect of the treatment on the basis of the tumour size and the survival time of the animals.

Both in these and in later experiments with intraperitoneal injections the following experimental set-up with 5 groups each of 8 animals was used:

Group 1: 0.2 ml physiol. saline i.p. 15–20 min before 0.1 ml HN2 solution i.p.

Group 2: 0.2 ml cysteamine sol. i.p. 15–20 min before 0.1 ml HN2 solution i.p.

Group 3: 0.2 ml cysteamine sol. i.p. 4–6 hr before 0.1 ml HN2 solution i.p.

Group 4: 0.2 ml cysteamine sol. i.p. 15–20 min before 0.1 ml physiol. saline i.p.

Group 5: 0.2 ml physiol. saline i.p. 15–20 min before 0.1 ml physiol. saline i.p.

In this way, the studies showed the effect of

(1) HN2 alone.

(2) Cysteamine given so shortly before HN2 that a protective effect could be expected.

(3) Cysteamine given such a length of time before HN2 that a protective effect could not be expected, and of

(4) cysteamine alone.

The last group was used as a control group which received injections of physiological saline only.

The results of a preliminary experiment are seen in Fig. 2, showing the survival time, tumour size, body weight and the number of injections, which were commenced on the day when the transplantation was performed and continued for a total of 14 days. The daily doses of cysteamine and HN2 were 3 mg and 0.0075 mg, respectively.

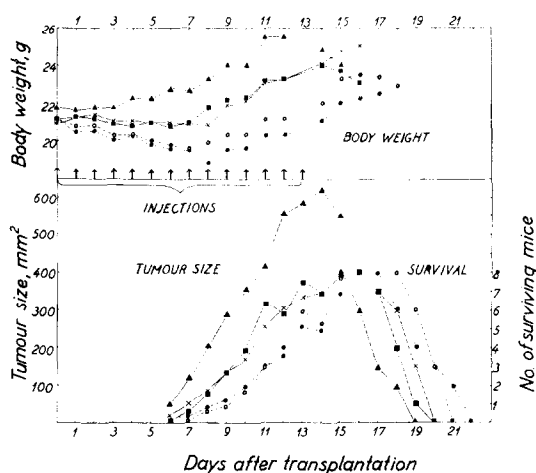


FIG. 2. Average body weight (g), average tumour size (mm^2) and survival curves for 5 groups of 8 AKA mice which from the day of subcutaneous transplantation of 0.1 ml of tumour mass were given injections as follows: $\blacktriangle$ — $\blacktriangle$, no injections (controls); $\blacksquare$ — $\blacksquare$, 0.2 ml physiol. saline 30 min before 0.0075 mg HN2; $\times$ — $\times$, 3 mg cysteamine 30 min before 0.1 ml physiol. saline; $\circ$ — $\circ$, 3 mg cysteamine 30 min before 0.0075 mg HN2; $\bullet$ — $\bullet$, 3 mg cysteamine 6 hr before 0.0075 mg HN2. All injections ($\uparrow$) were given intraperitoneally.

It is seen that the experiment points in the same direction as the results obtained by Peczenik,¹⁰ since the two groups which received both cysteamine and HN2 had the smallest tumours and also the longest survival times, but the extension of the survival time as compared with the group which was given cysteamine alone and that which was given HN2 alone was slight. In addition, it is seen that cysteamine alone also has an effect on the tumour size, and that the body weight and tumour size are correlated in such a manner that the animals with the highest body weights have the largest tumours.

As it is known that starvation is capable of reducing the size of a subcutaneous tumour,¹⁵ it was necessary to study if the reduction in the tumour size should simply be due to the weight loss which accompanies the treatment with cysteamine and HN2, i.e. if it should merely be a non-specific effect.

This was investigated in an experiment with a group of mice which were given restricted amounts of food (Fig. 3). It is seen that the smallest tumours were found in this group, while the starvation did not influence the length of the survival time. In this experiment, too, there was a correlation between the body weight and tumour size; there was no definite additive effect of cysteamine and HN2 as measured by the length of the survival time.

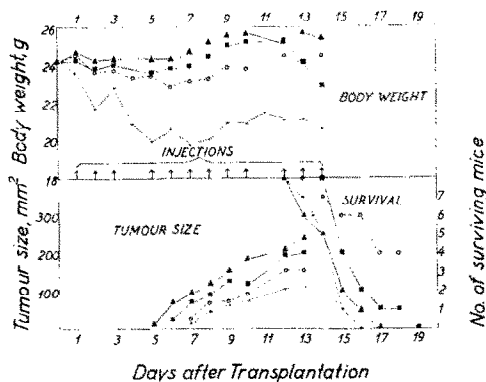


FIG. 3. Average body weight (g), average tumour size (mm^2) and survival curves for 4 groups of 8 AKA mice which from the day following subcutaneous transplantation of 0.1 ml tumour mass were given injections as follows: ▲—▲, 0.2 ml physiol. saline 20 min before 0.1 ml physiol. saline (controls); ■—■, 0.2 ml physiol. saline 20 min before 0.009 mg HN2; ○---○ 3 mg cysteamine 20 min before 0.009 mg HN2; ●---●, one group that was given restricted amounts of food, but no injections. All injections (↑) were given intraperitoneally.

The experiment thus suggests that the effect on the tumour size observed in these two initial experiments is, either wholly or at least in part, of a non-specific nature. It is uncertain whether the additive effect of cysteamine and HN2 on the size of the Walker carcinoma in rats observed by Peczenik¹⁰ can be explained in the same way, but in this connexion it must be pointed out that Peczenik assessed the effect only on the basis of the tumour size and, apparently, did not weigh the experimental animals. As already mentioned, the present experiments did not reveal any definite additive effect of the two drugs as measured by the length of the survival time. As, thus, measurements of the tumour size did not seem to be of value in studies on the type of tumour used, intraperitoneal transplantation was used in the subsequent experiments. Another reason for this change in technique was that an experiment in which the

effect of the same dose of HN2 (0.015 mg daily) was compared in a group given intraperitoneal injections and one given the same number of cells subcutaneously showed that HN2 had a better effect on the survival time in intraperitoneally transplanted mice.

3. *Effect of cysteamine and HN2 on intraperitoneally transplanted tumours*

As already mentioned, a known amount of cells ($1.0\text{--}1.5 \times 10^6$) was injected in these experiments. The injections of cysteamine and HN2 were commenced 2 days (in one experiment, 3 days) after the transplantation. The injections were given daily (except on Sundays) as long as at least one of the groups of animals in the experiment was complete in number. The number of injections and the doses of cysteamine and HN2 given in the individual experiments are shown in Fig. 4.

The experimental set-up was usually as stated above, 5 groups each of 8 mice being used in each experiment. Fig. 4 shows the survival curves for the experimental groups 1, 2 and 5, whereas those for group 3 (cysteamine 4–6 hr before HN2) and group 4 (cysteamine alone) have been left out in order to facilitate a survey.

(a) *Effect of cysteamine alone.* The effect of cysteamine alone was studied only in four of the experiments.

In experiments 1 and 7 a group of mice was given 3 mg of cysteamine 15–20 min before 0.1 ml of physiological saline intraperitoneally (total ten injections). The average survival time in these two groups were 13.0 and 13.5 days as compared with 13.4 and 12.4 days in the control groups.

In experiments 3 and 6 the daily dose of cysteamine was only 2 mg. The average survival times in these experiments were 12.9 and 11.6 days as compared with 12.0 and 11.6 days in the control groups.

Thus, cysteamine does not seem to have any effect on the survival time of the animals. Nor did autopsy or histological examination of the organs reveal any difference between the cysteamine-treated and untreated animals.

A therapeutic effect of cysteamine alone might have been expected, since it has been claimed by Bacq *et al.*¹ that the drug is of benefit in patients with leukaemia.

(b) *Effect of cysteamine given 4–6 hr before HN2.* In the aforementioned experiments performed by Peczenik,¹⁰ cysteamine was given either 3 or 6 hr before HN2. It was therefore thought to be of interest to study the effect of the two drugs given at similar time intervals. Incidentally, when the interval between the administration of the two drugs is so long, a protective effect of cysteamine against HN2 cannot be expected.¹⁷

This was studied in five of the experiments, viz. experiment 1, in which the daily cysteamine dose was 3 mg, and in experiments 3, 4, 6 and 8, in which the daily cysteamine dose was 2 mg.

The average survival times in these five experiments were 15.3, 15.6, 18.0, 18.0 and 20.6 days, respectively, while those for group 1 (HN2 alone) in the same experiments were 14.9, 15.0, 18.0, 18.5 and 19.3 days.

Cysteamine given such a length of time before HN2 injection does not have any influence at all on the survival time of the animals. In particular, it should be noted that in experiment 8, cysteamine did not afford any protection against HN2, which was here given in such a high dosage that 6 of the 8 animals died with signs of HN2 intoxication and without microscopically demonstrable tumour infiltration in the organs.

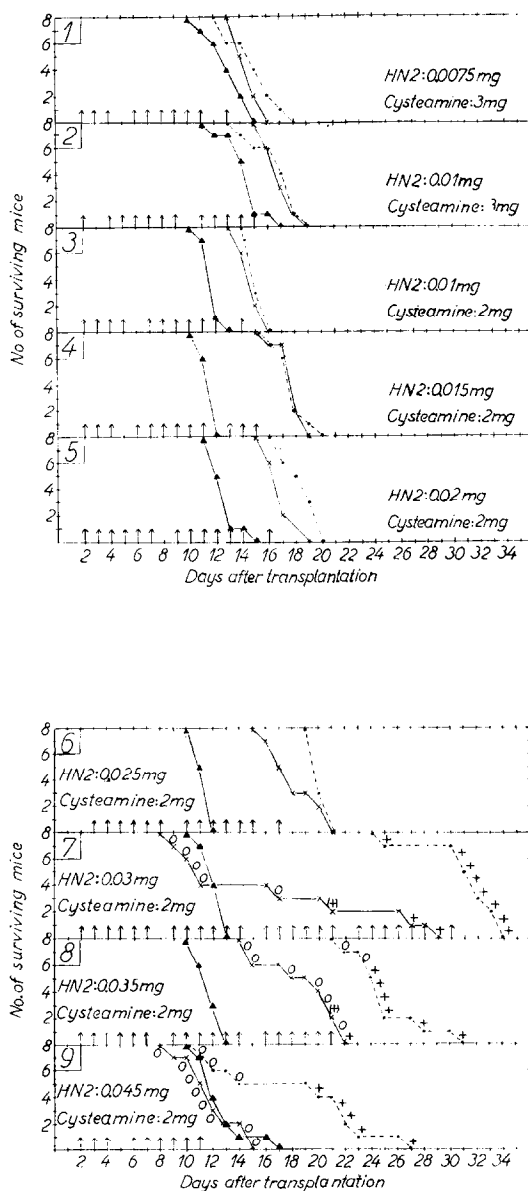


FIG. 4. Survival curves for groups of 8 AKA mice. A total of 9 experiments are illustrated, each showing survival curves for 3 groups of 8 mice. Two days after intraperitoneal transplantation of $1.0-1.5 \times 10^6$ cells a series of cysteamine and HN2 injections were commenced. The injections ($\uparrow$) and doses of cysteamine and HN2 are shown at the curves. The injections were given as follows: $\blacktriangle$ — $\blacktriangle$, 0.2 ml physiol. saline 15–20 min before 0.1 ml physiol. saline (controls); $\times$ — $\times$, 0.2 ml physiol. saline 15–20 min before 0.1 ml HN2 solution; $\bullet$ — $\bullet$, 0.2 ml cysteamine sol. 15–20 min before 0.1 ml HN2 solution.

For experiments 7, 8 and 9, the principal results of the histological examinations are indicated for each animal as follows: +, tumour infiltration in all the organs examined; (+), tumour infiltration in some of the organs examined, and $\circ$ no tumour infiltration in any of the organs examined.

(c) *Effect of cysteamine given 15–20 min before HN2.* This form of administration was of particular interest, since it has previously been shown¹⁷ that cysteamine gives good protection against HN2 when given within half an hour before HN2. The mechanism of the protective action is unknown, but it has been claimed by Salerno and Friedell¹³ that HN2 is inactivated simply by being bound to the SH group of cysteamine. If this be the case, it should be expected that cysteamine given within $\frac{1}{2}$ hr before HN2 would reduce the effect of the latter on the tumour. The results of the experiments appear in Fig. 4.

In experiments 1, 2, 3 and 4, cysteamine apparently did not exert any influence at all on the action of HN2, since the survival curves for the groups which were given HN2 alone ($\times$ — $\times$) coincide with those for the groups which received cysteamine 15–20 min before HN2 ($\bullet$ — $\bullet$).

In experiments 1 and 2, the daily cysteamine dose was 3 mg, which is probably very close to the lethal dose in mice transplanted with the tumour, since it was seen in both experiments that clonic convulsions developed in the two animals which died first in the cysteamine-treated group. As mentioned above, such convulsions are seen in cysteamine intoxication, whereas they are not observed in the animals treated with HN2 alone or in the untreated animals.

However, histological examinations of sections of the organs of these four animals showed marked infiltration with tumour cells in all the organs examined, just as was seen in the remaining mice used in the experiment.

In experiments 5 and 6, the cysteamine-treated group survived a little longer than the unprotected group which was given HN2 alone. This effect became very pronounced when the HN2 dose was increased to 0.003 mg daily as in experiment 7, in which the cysteamine-treated mice showed an average survival time of 31.5 days, while the unprotected, HN2-treated animals survived for 16.9 days and the controls for 12.4 days. The difference of 14.6 days between the average survival time of the protected and the unprotected group is significant as the standard deviation of the difference calculated according to Student's *t*-test for small samples is 3.0 ($t = 4.9$, $P < 0.001$). The difference between the average survival time of the protected group and the average survival times of the two treated groups in experiment 6 is highly significant, too ($t = 10.6$ and $t = 10.7$).

In experiment 7, the protection afforded by a daily cysteamine dose of 3 mg was also studied. However, three of the animals in this group died with clonic convulsions 7, 10 and 10 days after the transplantation, which, like the results of experiments 1 and 2, suggests that a daily dose of 3 mg is very close to the lethal dose in mice previously transplanted with the tumour. Thus, it does not seem to be possible to increase the cysteamine dose appreciably beyond 2 mg daily. The remaining 5 animals attained an average survival time of 31.0 days.

If the daily HN2 is further increased to 0.035 mg, as was done in experiment 8, the cysteamine-treated animals still have a significantly longer survival time than the unprotected mice ($t = 4.4$, $P < 0.001$), but the average survival time is not so long as in the protected group in experiment 7. In experiment 8, the survival times for the control group, the group treated with HN2, and that treated with cysteamine and HN2 were 11.1, 19.3 and 25.5 days, respectively.

In experiment 9, the daily HN2 dose was increased to 0.045 mg. It is seen that the HN2-treated animals now died just as quickly as the controls, while the cysteamine-

treated group survived somewhat longer, although the first two animals of the group succumbed after 11 and 12 days, respectively.

The protective effect of 3 mg. of cysteamine daily was also studied in this experiment (cf. experiments 1, 2 and 7), but the survival curve for this group almost completely coincided with that shown in Fig. 4 (2 mg of cysteamine daily), indicating that an increase of the cysteamine dose would not afford better protection at this dosage level of HN2.

(d) *Histological studies.* As previously pointed out, in all cases where it was not quite clear whether the animals had died from the tumour infiltration into the organs or of HN2 intoxication, sections of the organs were studied histologically after staining with haematoxylin and eosin.

The following organs were usually studied: the liver, spleen, kidneys, thymus, lungs and a portion of the radix mesenterii in which it was endeavoured to include not only the retroperitoneal lymph nodes, but also the pancreatic head and part of the duodenum.

The controls invariably showed extensive tumour infiltration in all the organs. The sections of the radix mesenterii showed pronounced infiltration with invasion into the pancreatic head and usually into the duodenum. The liver revealed infiltration especially in the periportal regions, but usually also around the central vein, and scattered tumour cells were observed in the sinusoids.

The kidneys showed marked infiltration in and around the renal pelvis and small infiltrates scattered in the cortex.

In the spleen and thymus, the normal structure had always completely disappeared, and tumour cells were seen everywhere.

The lungs invariably revealed tumour cells in all the septa; small infarcts were often seen, and tumour-cell thrombi were occasionally observed in the arterioles.

As the principal purpose of the histological examination of the treated animals was to decide whether the animals had died from the tumour infiltrations in the organs or of HN2 intoxication, the histological studies are of the greatest interest in the animals which had received the highest doses of HN2, especially in experiment 5 to 9 inclusive, in which the unprotected animals died before those protected by cysteamine, probably because the HN2 dose had reached a toxic level.

The histological findings in experiments 5 to 9 are therefore considered in detail below.

Experiment 5. Macroscopically, the radix mesenterii showed considerably less infiltration in the HN2-treated animals than in the controls. Similarly, microscopic studies showed that the infiltration and destruction of the pancreas were considerably less in the two HN2-treated groups; only in 1 mouse was the duodenum invaded; in 1 mouse from each group no tumour cells at all could be demonstrated in the sections of the radix mesenterii.

No difference between the cysteamine-treated and the unprotected animals could be demonstrated.

In addition, autopsy studies showed that the liver was greatly enlarged, of a yellowish pale colour and had rounded margins in the control group, while it was only slightly enlarged and of normal colour in the two groups treated with HN2. Similarly, micro-

scopic studies showed that the cytoplasm of the liver cells was markedly vacuolated in the controls, while this change was inconspicuous in the other two groups. Macroscopically, it could not be decided whether the animals of the control group were more anaemic than those in the groups treated with HN2.

Otherwise, macroscopic and microscopic studies did not reveal differences between the animals of the various groups, in all groups, the organs studied showed tumour infiltration to such an extent that it must be assumed to have been the cause of death in all the animals. This assumption is also supported by the fact that none of the animals showed clinical signs of HN2 intoxication, such as emaciation, diarrhoea or dehydration.

Experiment 6. This showed the same findings as in experiment 5, i.e. the same macroscopic and microscopic differences between the various groups. Only 4 mice in each group were studied histologically, but the animals examined showed pronounced tumour infiltration in all the organs.

Experiment 7. The result of the histological examination in this and the two following experiments is illustrated in Fig. 4, the presence or absence of microscopic tumour infiltration being indicated at the survival curves as follows: +, tumour infiltration in all the organs examined; (+), tumour infiltration in some of the organs examined; and 0, no tumour infiltration in any of the organs examined.

It appears that in experiment 7, 5 of the 8 mice in the unprotected HN2-treated group died without signs of tumour infiltration in any organs, which is in harmony with the fact that these animals died with signs of typical HN2 intoxication, such as diarrhoea, emaciation, dehydration and greatly diminished thymus and spleen.

One of the animals is graded (+) because definite tumour infiltration could be demonstrated only in the spleen and thymus. The last 2 animals in the group showed tumour infiltration in all the organs examined.

In the cysteamine-treated group, microscopic tumour-cell infiltration was revealed in all the organs examined, which suggests that, in all probability, these animals died of their tumours. This is in agreement with the fact that none of the animals showed clinical signs of HN2 intoxication.

Experiment 8. As appears from Fig. 4, 6 of the unprotected, HN2-treated mice died without demonstrable tumour infiltration. One animal in the group is graded (+) because tumour infiltration was found in the thymus and in one section of the radix mesenterii, but not in the liver or lungs. By a mistake, sections of the kidneys and spleen of this mouse were unfortunately not prepared. The last animal in the group showed pronounced tumour infiltration in all the organs. It is seen that of the protected mice in the experiment, 2 died without signs of tumour infiltration, while the remaining 6 showed such infiltration in all the organs.

Experiment 9. All the unprotected mice died without demonstrable tumour infiltration in the organs, but with typical signs of HN2 intoxication. Among the cysteamine-treated animals, tumour infiltration could not be demonstrated in 3, while there was pronounced infiltration of all the organs examined in the remaining 5 of the group.

DISCUSSION

The mechanism of the toxic action of cysteamine given in repeated injections is unknown, and the toxicity of the drug by this mode of administration does not seem to have been considered in the literature. On the other hand, the toxic effect of single injections of cysteamine has been studied,^{9, 16} and it has been shown that a toxic dose of the drug is capable of producing clonic convulsions.

Atrophy of the thymus gland may be explained by the weight loss alone, as it has been shown by Selye¹⁴ that weight loss following starvation is accompanied by atrophy of the thymus. Incidentally, atrophy of the thymus may be produced by a large number of drugs of a widely different nature¹⁴ and is often associated with hypertrophy of the adrenals and decrease in liver size. Whether this was the case in my experiments is unknown, as the problem was not studied.

From the experiments reported here it must be concluded (a) that cysteamine *per se* does not exert any therapeutic effect on the tumour studied, (b) that cysteamine given 4–6 hr before HN2 does not influence the action of the latter on the tumour, and (c) that by administration of cysteamine 15–20 min before HN2 it is possible to obtain an extension of the survival time of the animals which is considerably longer than that obtained after HN2 alone, probably because cysteamine affords a certain protection against the lethal effect of HN2 without a simultaneous reduction in the tumour-inhibitory effect of the latter drug, so that a larger dose of HN2 can be given without producing a lethal intoxication.

It is therefore difficult to harmonize the results of the present investigation with the above-mentioned theory advanced by Salerno and Friedell¹³ in explanation of the protective effect of cysteamine against HN2. From their investigations, these authors concluded: "It appears that the protective action against nitrogen mustards is a result of the direct interaction of the free sulfhydryl groups of cysteine or Becaptan with the highly reactive imonium ion of HN2. . . ."

However, on the assumption that this theory be correct, one would expect that cysteamine would have reduced the tumour-inhibitory effect of HN2, and that it would have been impossible to improve this effect by a combination of the two drugs, such as was seen in the present studies.

Nevertheless, the explanation might be that because of the uneven distribution of cysteamine in the organism demonstrated by Eldjarn and Nygaard,⁶ some organ—for example, the bone marrow—might be protected to a greater extent than the tumour.

Eldjarn and Nygaard⁶ found high concentrations of cysteamine in the bone marrow of rats, and if the theory be correct, one would expect a good protection of the bone marrow, but neither Weisberger *et al.*,¹⁸ nor Preziosi *et al.*¹² were able to demonstrate any protective effect of cysteamine against the leukopenia produced by injection of HN2 into rabbits. The writer has investigated the protective effect of cysteamine against HN2-induced leukopenia in mice; in two preliminary experiments, no such effect was demonstrable.

In previous experiments,¹⁷ cysteamine was found to afford protection of the bone marrow, as assessed by counts of nucleated cells in sections from the marrow of animals killed 24 hr after the injection of cysteamine and a LD_{93.5} of HN2.

These seemingly contradictory results might be explained, since it is known that cysteamine is toxic to isolated cells in tissue culture,¹⁶ and that the compound can

arrest mitosis at metaphase. If cysteamine prolongs the process of mitosis, we might find a higher number of cells in the marrow without a larger number of cells in the peripheral blood, but in that case the larger number of cells in the marrow would not be an expression of an inactivation of HN2. It might also be difficult to differentiate cells in incipient pyknosis from normal ones in the sections.

So far, it is therefore difficult to explain the present results on the basis of the theory of Salerno and Friedell,¹³ even if the uneven distribution of cysteamine in the organism is taken into account.

Another theory in explanation of the protection afforded by cysteamine against HN2 has been advanced by Eldjarn and Pihl,⁷ who suggested that the effect might be explained by assuming that the drug brings about a transitory blockage of vital SH groups and thus protects these against HN2.

If the uneven distribution of cysteamine in the organism is without significance, this theory is also unable to explain the present results, unless the normal cells are protected to a higher degree than the malignant ones.

Further, it must be mentioned that it is questionable if the principal point of attack of HN2 in the cells is the SH groups; much evidence is available suggesting that the point of attack should be sought in the nucleic acids.^{3, 11}

In the two theories mentioned it is presupposed that the action of cysteamine is exerted at a cellular level.

It has not been unquestionably proved that cysteamine protects isolated cells against the lethal effect of HN2. It is true that Deysson and Truhaut⁴ have shown that cysteamine protects *Pisum sativum* against the toxic effect of nitromin (the N-oxide of HN2), but in these experiments both substances were dissolved in Knoop's solution before this solution was given to the plants, so that the effect may simply be due to an inactivation of HN2 by its linkage to the SH group of cysteamine.

That such a linkage does not occur in the animal organism is probably due to the fact that cysteamine, as shown by Eldjarn and Pihl,⁷ is very rapidly bound to the serum proteins when it is injected into mice. Thus, proof that the protection afforded by cysteamine against HN2 occurs at a cellular level still seems to be missing, and the possibility that it is due to a systemic effect must therefore also be considered.

Thus, further investigations are necessary to clarify these problems, and so far it must be concluded that the present results speak against the assumption that cysteamine brings about an inactivation of HN2 in the mouse organism.

An interesting parallel to the present experiments is found in the investigations of Kluyskens,⁸ who showed that cysteamine protects mice against the toxic effect of dihydrostreptomycin on the vestibular apparatus. At the same time, he demonstrated that cysteamine did not reduce the bacteriostatic effect of dihydrostreptomycin *in vitro*.

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STUDIES OF THE MECHANISM OF THE PROTECTIVE ACTION OF SULPHYDRYL COMPOUNDS AND AMINES AGAINST NITROGEN MUSTARD (HN₂) AND ROENTGEN IRRADIATION IN MICE

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Abstract—It is shown that glutathione (GSH) lowers the lethal effect of cysteamine in mice, just as previous investigations¹⁹ suggested that GSH lowers the cytotoxic effect of cysteamine in tissue-culture experiments.

It is also shown that GSH does not change the radioprotective action of cysteamine, and that it increases the ability of cysteamine to protect the animals against nitrogen mustard (HN₂). Accordingly, the present investigation weighs against the assumption that the mechanism of the toxic effect of cysteamine is identical to that of the protective action of the compound against x-rays and HN₂, as claimed by Eldjarn and Pihl.¹⁰

Comparative studies on the protection against x-rays and HN₂ afforded by cysteamine, some cysteamine derivatives, tryptamine, tyramine and aminoethylisothiuronium-hydrobromide (AET) are reported, and the results are discussed.

It is emphasised that after a LD₉₈₋₅ of HN₂ death of the animals seems to be due to intestinal destruction, while the cause of death after a LD₉₇ of x-irradiation is apparently destruction of the bone marrow.

AMONG the compounds which have so far been found to afford protection against the effects of roentgen irradiation in experimental animals, cysteamine³ and S- β -aminoethylisothiuronium, 2 HBr (AET)⁸ are two of the most efficacious. Cysteamine (β -mercaptoethylamine) also gives good protection against nitrogen mustard (HN₂),^{16, 18} whereas the protective action of AET against HN₂ does not seem to have been studied.

The mechanism of the protective action of cysteamine against x-rays and HN₂ has not as yet been clarified. Among the theories offered in explanation of the protective effect, the one advanced by Eldjarn and Pihl¹⁰ has aroused great interest. These investigators claimed that both the radioprotective effect of cysteamine and its protective action against HN₂ may be explained by a temporary blockage of vital SH groups in the cells, by which these SH groups are protected against the effect of ionizing radiation and HN₂. Cysteamine is bound to these SH groups under the formation of mixed disulphides.¹⁴

Eldjarn and Pihl¹⁰ also expressed the view that the toxic action of cysteamine on isolated cells and its lethal effect on experimental animals may be explained by the same mechanism, since the blockage of the SH groups is lethal when it is not abolished sufficiently rapidly by excretion or break-down of the cysteamine.

The present writer has previously shown¹⁹ that glutathione (GSH) seems to be able to reduce the toxic effect of cysteamine on cells in tissue culture; this reduction may be explained on the basis of the theory of Eldjarn and Pihl, since it is well known

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that SH-bearing enzymes which are inactivated by blockage or oxidation of the SH group may often be reactivated by addition of GSH.⁴

However, if the mechanism of the lethal effect of cysteamine on experimental animals and of its protective action against x-rays and HN2 is identical with that of its cytotoxic effect, it should be expected that GSH would reduce all the effects of cysteamine.

Accordingly, the influence of GSH on the lethal action of cysteamine on mice and its influence on the protective effect of cysteamine against x-rays and HN2 has been studied. Comparative studies on the protection against x-rays and HN2 afforded by cysteamine, some cysteamine derivatives, tyramine, tryptamine and AET have also been undertaken.

It was hoped that these studies would reveal evidence suggesting whether or not the protection against x-rays and HN2 is due to the same mechanism. In a previous study,¹⁸ certain points of resemblance in the protective effects of some cysteamine derivatives against x-rays and against HN2 have been demonstrated.

MATERIALS AND METHODS

As in previous experiments on the toxicity of cysteamine and related compounds,¹⁸ the animals used in the study on the influence of GSH on the lethal effect of cysteamine were inbred "street" mice, divided into groups of 6 with an equal number of males and females; their weights ranged from 21 to 27 g and averaged about 24 g in the individual groups.

In the experiments on the protection against x-rays and HN2, groups of 10 inbred C₃H mice, aged about 2 months, were used; each group consisted of an equal number of males and females weighing from 17 to 23 g, the average weight of the animals in the individual groups being about 20 g.

The chemical compounds used in the experiments were: cysteamine from the pharmaceutical factory "Fluka", Buchs, Switzerland; hydrochlorides of the cysteamine derivatives and aminoethylisothiuronium-hydrobromine (AET) synthesized by the Institute of Chemistry of the University of Copenhagen (Professor K. A. Jensen); glutathione from Hoffmann-la Roche & Co., Basel; and hydrochlorides of tryptamine and tyramine supplied by E. Merck, A.G., of Darmstadt.

Before use, all SH compounds were titrated iodometrically by the method of Woodward and Fry.²⁰

Immediately before use, the compounds were dissolved in physiological saline; if necessary, the solutions were neutralized by addition of NaOH or HCl.

The nitrogen mustard used was "Erasol", supplied by A/S Ferrosan, of Copenhagen. Each ampoule contained 10 mg of methyl-bis-beta-chloroethylamine-hydrochloride (HN2) dissolved in 1 mg of absolute alcohol. Before use, the desired dilution was prepared by adding physiological saline to the contents of the ampoule.

The x-irradiation was performed at the Radium Centre for Jutland, Aarhus Municipal Hospital, with a Philips 250 kV unit (250 kV, 15 mA; filter 0.25 mm Cu; distance 60 cm).

All doses were measured in air by means of a Philips universal dosimeter; the dosage rate was usually about 85 r/min., but lower in a few experiments. In cases where a lower dosage rate was used, this is stated in the tables. During the exposure, the mice were placed in perforated plastic tubes of a length of 10 cm and a diameter

of 3 cm. Eleven plastic tubes were arranged radially on a circular board, and 10 animals were irradiated at a time; the ionization chamber of the dosimeter was inserted into the eleventh tube.

RESULTS

A. Influence of glutathione on the lethal effect of cysteamine in mice

The effect of only one dose of glutathione, viz, 36 mg, which are equimolar to 9 mg of cysteamine base, was studied in these and subsequent experiments. After dissolution in 0.5 ml of physiological saline and neutralization with NaOH, GSH was injected intraperitoneally. After dissolution in 0.2 ml of physiological saline and neutralization with HCl, cysteamine was injected, likewise intraperitoneally, 15 min later.

In parallel experiments, the effect was studied when the same amount of GSH was mixed with the same amount of cysteamine *in vitro* and injected intraperitoneally after standing for 15 min. In a control group, an injection of 0.5 ml of physiological saline was given 15 min before the cysteamine injection.

Each group consisted of only six "street" mice. The LD₅₀ had previously been determined as about 6.5 mg of cysteamine base/mouse.

The results are shown in Table 1. It is seen that GSH gives good protection against the lethal effect of 7, 8 and 9 mg of cysteamine base, while it does not afford any definite protection against a dose of 10 or 11 mg. It should be noted that 1 out of 6 mice survived a dose of 9 and even of 10 mg, which was quite unexpected.

TABLE 1

Intraperitoneal injections	Survivals out of 6 mice after cysteamine						Total number of surviving mice
	7 mg	8 mg	9 mg	9 mg	10 mg	11 mg	
36 mg GSH 15 min before cysteamine	6	5	6	5	1	0	23
36 mg GSH mixed with cysteamine <i>in vitro</i>	6	6	3	5*	1	0	21
0.5 ml physiol. saline 15 min before cysteamine	1	0	0	1	1	—	3

* 5 out of 5.

However, a comparison of the total of survivors in the first group (23 mice) and the corresponding figure in the unprotected group (3 mice) in Table 1 shows that the protective action of GSH is strongly significant ($\chi^2 = 24.5$; $P < 0.001$).

B. Influence of glutathione on the protective effect of cysteamine against nitrogen mustard (HN2) in mice

In these experiments, 36 mg of GSH was injected intraperitoneally into 10 C₃H mice, 10–15 min before the injection of 3 mg of cysteamine base. After the lapse of another 10–15 min a subcutaneous injection of HN2 was given in the nuchal region. The LD_{98.5} of HN2 had previously been determined as 0.11 mg in animals of the same strain, age and weight distribution.¹⁸

In all experiments, the protective effect of cysteamine alone, of GSH alone, and the survival rate after injection of the dose of HN2 used without preceding protection, were

also studied. Thus, each experiment comprised 4×10 mice, and four experiments with the following HN2 doses were performed: 0.11, 0.125, 0.143 and 0.167 mg. All animals were given three injections, the cysteamine and/or GSH injection being replaced by an injection of the same amount of physiological saline.

The results appear from Table 2, in which the numbers of surviving animals in each group of 10 are stated.

It is seen that GSH plus cysteamine give a protection which is significantly better than that afforded by cysteamine alone. (Application of the χ^2 test "corrected for discontinuity"¹¹ to the totals of the two groups (32 and 21 animals) gives $\chi^2 = 5.6$, corresponding to $0.02 > P > 0.01$.)

TABLE 2

Intraperitoneal injections before HN2	Survivals out of 10 mice after HN2				Total number of surviving mice
	0.11 mg	0.13 mg	0.14 mg	0.17 mg	
36 mg GSH 10 min before 3 mg cysteamine	10	8	9	5	32
0.5 ml physiol. saline 10 min before 3 mg cysteamine	9	7	4	1	21
36 mg GSH 10 min before 0.2 ml physiol. saline	8	6	1	2	17
0.5 ml physiol. saline 10 min before 0.2 ml physiol. saline	1	1	0	0	2

Thus, GSH increases the protection afforded by cysteamine against HN2. Furthermore, it appears that 36 mg of GSH gives roughly the same protection against HN2 as 3 mg of cysteamine, i.e. GSH must be given in molar amounts which are three times as high as that of cysteamine in order to yield the same protection against HN2.

C. Influence of glutathione on the protection of cysteamine against X-rays in mice

The experimental set-up was the same as that mentioned under (B), the only difference being that the HN2 injection was replaced by total-body irradiation given 3–8 min after the last of the two intraperitoneal injections.

However, the protective effect of GSH alone and the survival rates after no preceding treatment other than saline injections were studied only for the two lowest x-ray doses, since all animals in the two groups would probably have died on exposure to the higher doses.

The results appear from Table 3, in which the numbers of surviving animals in groups of 10 are shown. Doses ranging from 775 to 1000 r were given; as the dosage rates varied to some extent, these are stated in all the experiments. It is seen that a total of 51 out of 80 animals survived in the group which had been pretreated with cysteamine plus GSH, as against 55 out of 80 which had been given cysteamine only.

There is no significant difference between the two groups, i.e. GSH does not seem to exert any influence on the radioprotective action of cysteamine.

In addition, it appears from the experiments that 36 mg of GSH does not afford any definite protection against a LD_{100} of x-rays ($800 \text{ r} = LD_{98}$); thus, GSH seems to give less protection against x-irradiation than against HN2.

TABLE 3

Intraperitoneal injections before irradiation	Survivals out of 10 mice after:							Total number of surviving mice
	775 r	825 r	875 r	925 r	925 r	975 r	1000 r	
36 mg GSH 10 min before 3 mg cysteamine	8	10	10	2	9	1	6, 5	51
0.5 ml saline 10 min before 3 mg cysteamine	10	8	9	1	10	5	7, 5	55
36 mg GSH 10 min before 0.2 ml saline	1	0	—	—	—	—	—	
0.5 ml saline 10 min before 0.2 ml saline	0	0	—	—	—	—	—	
Dosage rate in r/min	82	80-84	74	59	85	67	81-83	

D. Protective action of some cysteamine derivatives against X-rays and HN2 in mice

In a previous paper,¹⁸ the results of a study on the protective effect of a number of cysteamine derivatives against a LD_{98.5} of HN2 were published. It was found that it would be of interest to compare the protection against x-rays and HN2 afforded by some of these derivatives.

The results of some of these experiments are shown in Table 4. While the preliminary investigations¹⁸ suggested that the protective effects of N-dimethylcysteamine, N-isopropylcysteamine and N-propylcysteamine are superior to that of cysteamine, it is seen from Table 4 that the four compounds, when used in equimolar amounts, have almost the same protective effect against HN2. Koch and Hagen¹⁴ claimed that the protective effect of cysteamine is increased by substitutions with methyl, ethyl or propyl groups on the amino group. This does not seem to hold good as far as the protection against HN2 is concerned. On the basis of the present experiments, it is impossible to say whether the statement holds for the radioprotective effect, but neither N-isopropylcysteamine nor N-propylcysteamine seems to have a better radioprotective effect than cysteamine. (However, the molar dose of N-propylcysteamine used was slightly lower than that of cysteamine.)

TABLE 4

Compound	Dose		Survivals out of 10 mice 30 days after:		
	(mg/mouse)	(μ moles/mouse)	HN2 (0.11 mg)	750 r	800 r
Cysteamine	1.0	13	4, 6, 4	10	8, 10, 4
S-methylcysteamine	15.0	120	0	8	1
N-dimethylcysteamine	1.82	13	5, 3	—	—
N-isopropylcysteamine	2.0	13	6, 5, 6, 8	6	4
N-propylcysteamine	1.75	11	5, 6, 6	—	0. 8
N-butyl- α -methylcysteamine	1.5	8	3	2	4*
N-hexyl- α -methylcysteamine	1.0	5	1	6	0
N-benzyl-cysteamine	2.0	10	0	9	5*
N- β -mercaptoethylpiperidine	0.625	3.5	0, 2	4	—
N- β -mercaptoethylmorpholine	4.0	22	0	9	5†
Controls	—	—	0, 1, 0, 0, 0, 0, 0, 1	4, 4, 3	1, 0, 0, 1, 0

* 1 mouse died during irradiation.

† 2 mice died during irradiation.

The radioprotective effect against two doses of roentgen irradiation was studied, viz. 750 r, which is somewhat lower than the LD₁₀₀, and 800 r, which is very close to LD₁₀₀ (67 out of 70 mice died). The relatively few experiments do not permit definite conclusions, but the results presented in Table 4 suggest that the compounds which afford protection against HN2 (cysteamine, N-isopropylcysteamine, N-propylcysteamine and N-butyl- α -methylcysteamine) also give some protection against x-irradiation, while the compounds which do not protect against HN2 (S-methylcysteamine, N-hexyl- α -methylcysteamine and N- β -mercaptoethylpiperidine) do not afford any protection against x-irradiation either. Exceptions from this rule are formed by N-benzylcysteamine and N- β -mercaptoethylmorpholine, which do not protect against HN2, but nevertheless seem to afford some protection against x-rays. However, in this connexion it should be noted that the doses of these two compounds used in the experiments were on the verge of the lethal dose, since 1 and 2, respectively, of the 10 mice died already during the irradiation; it is therefore possible that the effect against x-rays may have been excited through an influence on the circulation with ensuing tissue anoxia.

Four survivors in a group of 10 irradiated mice may be taken as an expression of a statistically significant protection. In a total of 7 control groups of 10 mice each, which were irradiated with 800 r, 1 animal in each of 3 groups survived, while all animals died in the remaining 4 groups. Thus, as 3 out of 70 animals survived after irradiation with 800 r, the probability of the survival of 4 mice in a group of 10 can be calculated from the formula:

$$\sum_{x=0}^3 \frac{\binom{70}{x} \left(\frac{10}{7-x} \right)}{\binom{80}{7}}$$

which gives $P = 0.0038$. As previously shown,¹⁸ 3 survivors out of a group of 10 animals denote statistically significant protection against a dose of 0.11 mg of HN2.

E. Protective effect of tyramine, tryptamine and aminoethylisothiuronium-hydrobromide (AET) against x-rays and HN2 in mice

The results of comparative studies on the protection against x-rays and HN2 afforded by these three compounds are shown in Table 5. Tyramine and tryptamine were studied in the dosage used by Bacq,¹ i.e. 10 μ moles/mouse, while AET was studied at the dosage level of 4 mg or 14 μ moles/mouse.

TABLE 5

Compound	Dose		Survivals out of 10 animals 30 days after:						
	(mg/ mouse)	(μ moles mouse)	725 r	775 r	850 r	950 r	1050 r	1150 r	HN2 (0.11 mg)
Tyramine-hydrochloride	1.73	10	6	3	0	—	—	—	0,0
Tryptamine-hydrochloride	1.96	10	10	10	9	9	3	4	0,0
S- β -aminoethyliso- thiuronium, 2HBr (AET)	4.0	14	—	10	10	9	10	6	3
Controls	—	—	3	0	0	—	—	—	0,0

The results of these studies may be summarized as follows:

(1) In the dosage used (10 μ moles/mouse), tyramine gives a slight protection against x-rays, but no protection at all against HN2.

(2) In the same dose (10 μ moles/mouse), tryptamine gives very good protection against x-rays, but no protection at all against HN2.

(3) Administered in a dose of 14 μ moles/mouse, AET gives a better protection against x-rays than tryptamine and affords only slight protection against HN2.

The protective effect of AET against HN2 was also studied when given in a dose of 6 mg/mouse in one experiment; in this, 8 mice out of 10 survived a LD_{98.5} of HN2.

DISCUSSION

If, as claimed by Eldjarn and Pihl,¹⁰ the biological effects of cysteamine are produced by a blockage of protein-bound SH groups, it might be conceived that GSH would reduce these biological effects, since it is well known that GSH may reactivate SH-bearing enzymes after blockage or oxidation of the SH group.⁴ In addition, Pihl and Eldjarn¹⁴ have shown that GSH *per se* forms mixed disulphides with cysteamine.

Accordingly, if the toxic effects of cysteamine on isolated cells or on mice consist in a blocking of vital SH groups, it might very well be conceived that GSH reduces these effects. In a previous paper,¹⁹ results which suggest that GSH reduces the toxicity of cysteamine in tissue-culture experiments have been published, and the present results seem to show that GSH reduces the lethal effect of cysteamine in mice.

As GSH does not influence the protective effect of cysteamine against x-rays, and increases its protective action against HN2, the investigations just mentioned suggest that the toxic and protective effects of cysteamine are not due to the same mechanism.

In this connexion, it must be stated that we have no definite evidence in support of the assumption that the principal point of attack of HN2 is the SH group in the cells, and whether inactivation of SH-bearing compounds plays a principal part in the biological effects of the x-rays is another question which is open to discussion.²

Several facts suggest that the principal point of attack of both nitrogen mustard and ionizing radiation is to be sought in the synthesis of nucleic acids,^{6, 12} even though the point of attack in this mechanism of synthesis may be different.

At the present time, it is impossible to say whether the chemical protection against HN2 and that against x-rays are due to the same mechanism. It was hoped that comparative studies on the protective effect of a number of chemical compounds against x-rays and HN2 would contribute to the solution of this problem.

The only unquestionable difference in the protection against HN2 and x-rays so far observed was revealed in the three amines in group E; specifically, tryptamine showed a great difference, with a pronounced radioprotective effect and no protection at all against HN2. However, it is possible, as stated by van Bekkum,⁵ that the biological amines exert their radioprotective action through a systemic effect; they may impair the circulation and thus cause a reduction in the oxygen tension of the tissues. If this be the case, such amines would give protection against x-rays but not against HN2, since the oxygen tension affects the radiation effect (fewer oxidizing radicals are formed), but not the action of HN2.

That AET does not only protect against x-rays, but also affords some protection against HN2 is in good agreement with the fact that this compound cannot be

conceived as a pure amine, since, in solution, it is found as a mixture of various forms, including one with a free SH group.¹⁷

The protective effect of N- β -mercaptoethylmorpholine and N-benzylcysteamine against x-rays may possibly be explained in the same way as that of tryptamine, since these two compounds were given in doses on the verge of the lethal one.

As already mentioned, the other cysteamine derivatives showed good agreement with regard to their protective effect against x-rays and HN2.

Moreover, after comparative studies the author has concluded that complete agreement could scarcely be expected between the protective action against HN2 and that against x-rays which is exerted by a series of compounds, even if it is presupposed that the mechanism of action is the same.

As shown in Fig. 1, a conspicuous difference was demonstrated in the survival times of the mice after a LD₉₇ of x-rays and one of HN2. Following the administration of a LD_{98.5} of HN2, the animals showed an average survival time of 4.9 days, and severe diarrhoea usually developed. After a LD₁₀₀ of x-irradiation, the average survival time was 11.5 days, and diarrhoea did not occur.

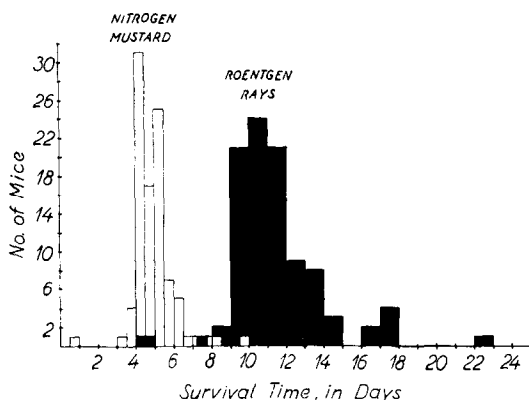


FIG. 1. Showing the survival time of 96 out of 99 C₃H mice dying after subcutaneous injection of 0.11 mg HN2 and the survival time of 97 out of 100 C₃H mice dying after total-body irradiation with doses ranging from 775 to 825 r. The deaths were registered every 12 hr after HN2, but only every 24 hr after irradiation.

It is well known that animals which are given x-irradiation may die from various causes, the most frequent being "intestinal death", which occurs after 3–5 days,¹⁵ and "marrow death", which occurs after 9–15 days, with a peak on the eleventh day.⁷ The findings shown in Fig. 1 correlated with the premortal symptoms (presence or absence of diarrhoea) strongly suggest that in the C₃H mice used, the cause of death after administration of HN2 is to be sought in intestinal destruction, while after x-irradiation death seems to be due to destruction of the bone marrow. If, for example, a given compound affords better protection against HN2 than against x-rays, the explanation might possibly be that the compound is unevenly distributed in the organism, resulting in a higher concentration in the intestinal tract than in the bone marrow.

It has been shown⁹ that cysteamine shows an uneven distribution in the organism in rats.

It must therefore be emphasized that a difference disclosed in a comparative study on the protective effect against x-rays and against HN2 exerted by a number of compounds cannot straightaway be interpreted as a manifestation of a difference in the mechanism of the two protective effects.

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EFFET DE LA MESCALINE, DU LSD 25 ET DE DERIVES DE L'ADRENOCHROME SUR LA DECARBOXYLYASE GLUTAMIQUE DU CERVEAU

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Abstract—The inhibitory effect of adrenochrome on glutamic decarboxylase from brain homogenates *in vitro*, does not occur with mescaline and LSD 25. Certain stable derivatives of adrenochrome such as MSCA and AHA, in which the quinonic function is blocked, have an opposite effect, and behave like activators of brain glutamic decarboxylase. This activation seems to be indirect; MSCA and AHA do not constitute new coenzymes.

L'EFFET inhibiteur exercé par l'adrénochrome et certains de ses précurseurs, comme l'oxytyramine et l'adrénaline, sur la décarboxylation de l'acide glutamique en acide γ -aminobutyrique par les extraits cérébraux, ont incité Holtz et Westermann^{1, 2} à postuler une relation éventuelle entre cet effet et le pouvoir hallucinogène attribué à l'adrénochrome par Osmond, Smythies et Hoffer.^{3, 4}

Quoique l'adrénochrome puisse également inhiber d'autres processus enzymatiques cérébraux tels que la glycolyse aérobie,⁵ l'hypothèse suggérée par Holtz et Westermann semble particulièrement intéressante étant donné le rôle de plus en plus important attribué à l'acide γ -aminobutyrique dans la biochimie et la physiologie du système nerveux central.

L'effet hallucinogène de l'adrénochrome étant très controversé, nous avons voulu voir quel serait l'effet sur la décarboxylase glutamique cérébrale:

(1) D'agents typiquement hallucinogènes comme la Mescaline ou la diéthylamide de l'acide lysergique (LSD 25).

(2) De substances ne différant de l'adrénochrome que par le blocage d'une fonction quinonique, et pour lesquelles il n'a jamais été observé d'effet hallucinogène, même à très forte dose.

MATERIEL ET METHODES

L'adrénochrome, la monosemicarbazone de l'adrénochrome (MSCA) et l'acéthydrazone de l'adrénochrome (AHA) répondant aux formules figurées ci-contre ont été préparés dans notre Laboratoire de Chimie par MM. Beaudet et Hénaux. Toutes les solutions sont préparées extemporanément malgré la très grande stabilité de MSCA et la relative stabilité de AHA.

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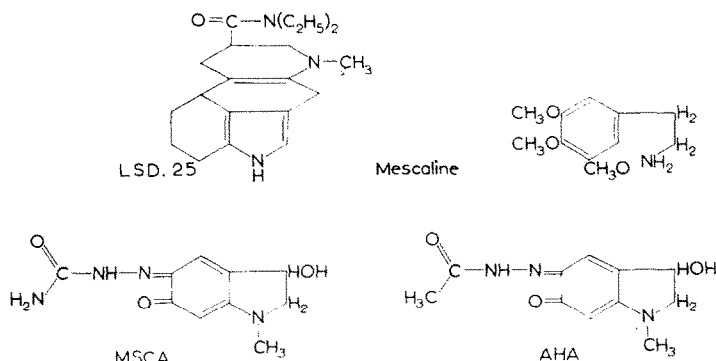


FIG. 1

Les cerveaux ont été prélevés sur des souris blanches, adultes, tuées par dislocation de la vertèbre cervicale, et des homogénéisats sont réalisés en tampon phosphate 0,05 M pH 6,2. La vitesse avec laquelle s'effectue la décarboxylation de l'acide glutamique par ces homogénéisats, additionnés, isolément ou non suivant le cas, de phosphate de pyridoxal (PP), des substances hallucinogènes ou des adrénochromes bloqués, est mesurée par la méthode manométrique de Warburg, à 38°C et en atmosphère d'azote, selon la technique décrite par Roberts et Frankel.⁶

Dans chaque cas, le volume contenu dans la fiole manométrique est de 2,5 ml, dont 2 ml sont constitués par le tampon phosphate.

Dans certains cas spécifiés, la décarboxylase cérébrale a été purifiée par salting-out au sulfate ammonique et dialysée. A cet effet, à un volume d'homogénéisat clarifié par centrifugation, on ajoute trois volumes d'une solution saturée de sulfate ammonique. Le précipité recueilli est remis en solution dans un volume d'eau égal à celui de l'homogénéisat centrifugé. La solution est dialysée contre eau distillée, puis concentrée en plaçant le sac de dialyse dans un courant d'air froid. La solution concentrée est enfin ramenée à la moitié du volume de l'homogénéisat centrifugé, par un tampon phosphate tel que la concentration finale en phosphate soit 0,05 M. Toutes ces opérations, excepté la concentration, sont réalisées à 2°C.

RESULTATS

Effets in vitro

Aux concentrations de $2 \cdot 10^{-6}$, $2 \cdot 10^{-5}$, $2 \cdot 10^{-4}$ M, le LSD 25 et la mescaline sont sans effet sur la vitesse de décarboxylation observée lors de l'incubation des extraits cérébraux et d'acide glutamique (0,004 et 0,04 M), éventuellement associé à 30 γ /ml de PP.

Le MSCA et l'AHA augmentent la vitesse de décarboxylation de l'acide glutamique par les extraits cérébraux. Le tableau 1 indique le dégagement d'anhydride carbonique par incubation de 500 mg de cerveau frais en présence d'acide glutamique et de quantités variables de PP ou d'AHA ou de MSCA pendant 210 minutes.

Ajoutés séparément à l'extrait cérébral, le MSCA, le AHA et le PP apparaissent comme étant des activateurs de la décarboxylation de plus en plus efficaces, L'un et l'autre présentent une concentration limite à laquelle correspond une vitesse maximale de la réaction et qui se situe aux environs de 20 γ /ml pour le PP et le MSCA et de 5 γ /ml pour le AHA. A ces concentrations limites et dans les conditions expéri-

TABLEAU I

Concentration de l'activateur (10 ⁻⁶ M)	Nature de l'activateur		
	Phosphate de pyridoxal	Acéthydrasone de l'adréno- chrome	Monosemicarba- zone de l'adré- nochrome
0	87	100	96
1,6	141	—	—
1,75	—	—	155
2	—	158	—
4	202	—	—
8	274	—	—
16	339	—	—
17,5	—	—	149
20	—	238	—
40	403	—	—
80	428	—	—
87,5	—	—	196
100	—	250	—
160	422	—	—
175	—	—	198
200	—	237	—
1000	—	225	—

Décarboxylation de l'acide glutamique, mesurée en μl de CO_2 dégagé après 210 minutes d'incubation de 500 mg de cerveau additionnés d'acide glutamique (0,04 M) et de quantités variables d'activateur.

mentales présentes, les quantités de CO_2 dégagé en présence de PP, de AHA et de MSCA sont respectivement 4,9, 2,5 et 2 fois supérieures à celle observée en l'absence d'activateur autre que la quantité de PP normalement contenue dans l'homogénéisat cérébral brut.

En représentant par c la concentration (en 10^{-6}M) en PP ajouté à l'homogénéisat cérébral et par $\mu\text{l}_{(0)}$, $\mu\text{l}_{(\text{PPvar})}$, $\mu\text{l}_{(\text{adr})}$ et $\mu\text{l}_{(\text{PPvar} + \text{adr})}$ les quantités de CO_2 , en μl , dégagées respectivement, en l'absence de tout activateur volontairement ajouté, et en présence de concentrations variables en PP, d'une concentration fixe en adrenochrome bloqué à $175 \cdot 10^{-6}\text{M}$ et des associations (adrenochrome bloqué ($175 \cdot 10^{-6}\text{M}$) + PP (concentrations variables)), la fonction :

$$y = (\mu\text{l}_{(\text{PPvar} + \text{adr})} - \mu\text{l}_{(0)}) - (\mu\text{l}_{(\text{PPvar})} - \mu\text{l}_{(0)}) - (\mu\text{l}_{(\text{adr})} - \mu\text{l}_{(0)}) = f(c)$$

représente la différence, exprimée en μl de CO_2 , entre d'une part l'activation provoquée par l'association de l'adrenochrome bloqué et de PP, et d'autre part, par la somme des deux activations individuelles.

L'expérience nous a montré que y est toujours négatif. Les activations par les associations (PP + MSCA) et (PP + AHA) sont compétitives. Chacun des trois activateurs étudiés agirait donc au niveau des mêmes centres actifs de la decarboxylase. D'ailleurs, lorsque l'extrait cérébral est additionné d'une quantité de PP telle que l'enzyme soit saturé, l'addition supplémentaire de MSCA par exemple, n'augmente plus la vitesse de la decarboxylation de l'acide glutamique. Dans ces conditions, y a atteint une valeur négative limite :

$$y = - (\mu\text{l}_{(\text{adr})} - \mu\text{l}_{(0)})$$

La cinétique de la décarboxylation de l'acide glutamique a été étudiée en présence de AHA et de MSCA. Fig. 2 montre, en particulier, le dégagement de CO_2 en μl , en fonction du temps lorsque l'acide glutamique (0,04M) est incubé-présence d'extraits cérébraux en quantités variables, additionnés ou non d'une quantité fixe de AHA égale à $100 \cdot 10^{-6}\text{M}$.

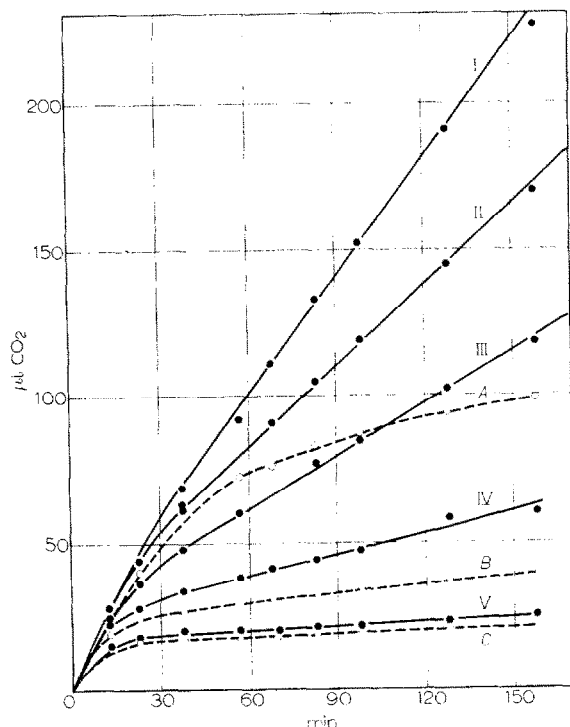


FIG. 2

Pour une quantité donnée d'adrénochrome "bloqué", l'activation dépend de la quantité d'extrait cérébral. Associé à 100 mg de cerveau frais, le AHA est pratiquement sans effet sur la vitesse de la réaction (courbes V et C). Associée à 200 et 500 mg de cerveau frais, la même quantité de AHA multiplie la quantité de CO_2 dégagé après 160 minutes d'incubation par un facteur respectivement égal à 1,7 et 2,5.

L'effet activateur du AHA et du MSCA ne se manifeste qu'après un certain temps d'incubation. Si l'on excepte ces premiers temps de réaction, la cinétique de la décarboxylation de l'acide glutamique représentée sur la Fig. 2 peut s'exprimer par

$$\Delta \mu\text{l} = 5,9 \cdot 10^{-6} \cdot \Delta t \cdot x^2$$

où Δt est l'intervalle de temps en minutes et x le poids de cerveau frais en mg. La réaction est d'ordre 1 par rapport au temps d'incubation. Elle est du second ordre par rapport à la concentration en homogénisat cérébral.

Après salting-out et dialyse, l'apoenzyme inactif par lui-même, est activable par le PP, mais ne l'est pas par le AHA ou le MSCA.

Effets *in vivo*

L'activation de la décarboxylase glutamique cérébrale par les adrénochromes bloqués semble être réalisable *in vivo*. En effet, les homogénéisats de cerveau prélevés une heure après une injection intrapéritonéale de 500 mg/kg de AHA chez la souris, manifestent une activité décarboxylante supérieure à celle des cerveaux de souris non traitées, les deux homogénéisats étant ou non additionnés de PP. De plus, une telle injection protège, de façon faible mais significative, les souris contre la mort par convulsion que provoque l'injection intrapéritonéale de thiosemicarbazide et dont le mécanisme résiderait, selon Killam et Bain^{7, 8} dans une inhibition du système décarboxylase glutamique-phosphate de pyridoxal.

Deux lots de 100 souris ont reçu par voie intrapéritonéale 15 mg/kg de thiosemicarbazide. Deux heures auparavant, un des lots avait reçu 500 mg/kg de AHA. Le temps s'écoulant entre l'injection de la carbazide et la mort a été déterminé individuellement. Le temps moyen de survie a été de 87 minutes (erreur standard corrigée: 4) pour le lot non traité, et de 106 minutes (erreur standard corrigée: 2,5) pour le lot traité par le AHA.

DISCUSSION

L'étude de l'influence exercée éventuellement par la mescaline et le LSD 25 sur la décarboxylation de l'acide glutamique par les extraits cérébraux, nous a été suggérée par l'analogie proposée par de nombreux auteurs⁹⁻¹² entre l'effet de ces substances et ceux de l'adrénochrome sur les mécanismes neurohumoraux adrénergiques centraux.

Nos expériences montrent que ni la mescaline ni le LSD 25 ne provoquent l'inhibition de la décarboxylase glutamique cérébrale, que Holtz et Westermann constatent avec l'adrénochrome, et à laquelle ils suggèrent que l'activité hallucinogène peut être rattachée.

En ce qui concerne l'adrénochrome proprement dit, il ne nous a pas été possible d'effectuer une expérience dans laquelle nous soyons assurés que l'adrénochrome ne se soit pas oxydé pendant le laps de temps nécessaire pour obtenir un dégagement de CO₂ mesurable. Il reste donc un doute quant à l'attribution de cette inhibition à l'adrénochrome même ou à l'adrénochrome seul.

À l'inverse de ce que l'on observe avec l'adrénochrome, les adrénochromes stabilisés par le blocage de la fonction quinonique, activent la décarboxylation de l'acide glutamique par les homogénéisats de cerveau. Ces dérivés de l'adrénochrome ne semblent pas constituer de nouveaux coenzymes, car l'apoenzyme purifié n'est pas réactivable en leur présence.

L'activation doit se faire par une voie indirecte. La cinétique de la décarboxylation, en présence de AHA, par exemple, étant du deuxième ordre par rapport à la concentration en extrait cérébral, peut s'interpréter par la présence dans celui-ci de deux activateurs: le PP, coenzyme naturel, et un autre dont la manifestation de l'activité requiert la présence de AHA ou d'une substance de structure analogue. Cette activation se constatant aussi *in vivo*, nous disposons là d'un moyen d'augmenter la rapidité de la conversion de l'acide glutamique en acide γ -aminobutyrique, fait qui ouvre de nouvelles possibilités expérimentales.

Toute considération théorique mise à part, peu après notre communication préliminaire,¹³ nous avons voulu nous rendre compte de l'effet hallucinogène de l'adrénochrome. L'un de nous a reçu par voie intraveineuse, 10, puis 20 mg d'adrénochrome fraîchement préparé, sans ressentir aucun trouble subjectif ni présenter le moindre signe objectif. Notre manque de compétence dans le maniement des tests permettant d'extérioriser des modifications de la perception ou de l'idéation, explique peut-être la divergence de nos résultats avec ceux de l'école canadienne;^{3, 4} ils sont cependant en accord avec ceux de Sobotka,¹⁴ qui a utilisé des doses encore plus élevées que nous, sans effet apparent.

Enfin rappelons à ce sujet que les adrénochromes bloqués, comme le MSCA et l'AHA, qui peuvent être administrés en quantités considérables en raison de leur très large index thérapeutique, ne provoquent aucun phénomène du type hallucinatoire.

RESUME

Le LSD 25 et la mescaline sont sans effet sur la décarboxylation de l'acide glutamique par les extraits cérébraux *in vitro*.

La monosemicarbazone (MSCA) et l'acéthydrazone (AHA) de l'adrénochrome activent cette décarboxylation. Ils ne constituent pas des coenzymes nouveaux. Les centres de l'apoenzyme indirectement activés par l'un et par l'autre, semblent être identiques à ceux qu'active le phosphate de pyridoxal.

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THE EFFECTS OF FLUOROCOMPOUNDS ON OXIDATIVE PHOSPHORYLATION

I—EFFECTS ON P/O RATIOS WITH FUMARATE AND WITH REDUCED DIPHOSPHOPYRIDINE NUCLEOTIDE*

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Abstract—The effect of fluoroinhibitors on the oxidative phosphorylation of rat liver mitochondria has been studied. Fluoroacetate *per se* had no effect, while synthetic fluorocitrate and fluoropyruvate directly interfered with phosphorylation. This interference is partially due to an increase in nucleotide destruction. The phosphate esterification during the oxidation of DPNH was depressed while respiration was stimulated by fluorocitrate without concomitant swelling of the particles. However, the respiratory stimulation showed no correlation with the phosphorylating efficiency of the mitochondria. In pretreated mitochondria, incapable of phosphorylation, fluorocitrate further increased the oxidation rate of DPNH indicating an effect on the permeability of mitochondrial membranes.

INTRODUCTION

IT HAS BEEN reported previously¹ that the addition of fluoroacetate, fluoropyruvate or fluorocitrate to preparations of rat liver mitochondria lowers the phosphorylating ability of the particles, and evidence has been presented to show that an increased dephosphorylation of adenine nucleotides accompanies and may be related to this effect.

The present study deals in detail with the effects of these fluorocompounds on the P/O ratios associated with the oxidation of fumarate and of DPNH† by rat liver mitochondria and suggests an explanation for the previously conflicting observations on the effect of fluoroacetate on the processes of oxidative phosphorylation.²

EXPERIMENTAL

Mitochondria were prepared from the livers of male white Wistar rats of 200–300 g by the method of Smith³ modified by using 0.25 M sucrose in place of mannitol. In all cases animals were fasted overnight for 15–18 hr prior to sacrifice.

Oxygen consumptions were measured by the direct Warburg technique at 37°C in air. The standard reaction mixture used in the determination of oxidative phosphorylation was: 25 μ moles of potassium phosphate buffer at pH 7.28, 30 μ moles of KF, 120 μ moles of KCl, 6 μ moles of MgCl₂, 40 μ moles of Tris at pH 7.28, 750 μ moles of

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† The following abbreviations will be used throughout: DPNH, reduced diphosphopyridine nucleotide; ATP, adenosine triphosphate; Tris, tris(hydroxymethyl) aminomethane.

sucrose, 0.5 or 5 μ moles of ATP and substrate, as indicated; all materials were adjusted to pH 7.28 before they were added to the flasks. The trapping system consisted of 50 μ moles of glucose and 0.5 mg (82 K.M. units) of hexokinase, and was added from the sidearm. Mitochondria from approximately 300 mg of rat liver were added to give a final volume of 3 ml. The fluorocompounds were added either directly to the flasks initially or from a second sidearm, as indicated in the text. After 5 min equilibration, except where otherwise indicated, the manometers were closed, the trapping system tipped and the respiration measured for 15 min. The flasks were then rapidly placed in an icebath and 0.5 ml of 20 per cent trichloroacetic acid added to stop the reaction. After centrifugation, inorganic phosphate determinations were made on the supernatant fractions by the method of Gomori;⁴ zero-time controls were made in the usual manner.

The adenine and pyridine nucleotides were obtained from the Pabst Laboratories; sodium fluoroacetate was purchased from the Tull Chemical Company, Oxford, Alabama and was further purified. Fluoropyruvic acid was synthesized according to the method of Gal, Peters and Wakelin⁵ and was found to react with L-cysteine as described by Avi-Dor and Mager,⁶ but did not inhibit crystalline lactic dehydrogenase, as has been reported by Busch.⁷ Fluorocitrate was synthesized by the method of Rivett.⁸

RESULTS

Effect of fluoroacetate on the P/O ratio with fumarate

The effect of fluoroacetate on the P/O ratio associated with the oxidation of fumarate can be seen in Table 1. It must be emphasized here that in these experiments the fluoroacetate was not placed in the sidearm, but was added initially to the flasks;

TABLE 1. EFFECT OF FLUOROACETATE ON P/O RATIO

Additions to system	Incubation time (min)	Oxygen (μ atoms)	Uptake Phosphorus (μ moles)	P/O
None	10	3.2	8.5	2.45
Fluoroacetate	10	2.4	3.7	1.56
None	20	7.2	16.2	2.25
Fluoroacetate	20	5.4	2.9	0.53

Vessels contained 0.5 μ moles of ATP, 25 μ moles of fumarate and other constituents as in the standard reaction mixture (see "Experimental"). Sodium fluoroacetate at a final concentration of 3.3×10^{-3} M was added to the iced flasks initially. The trapping system was added after 5 min equilibration.

after 10 min incubation under these conditions the respiration was somewhat depressed by fluoroacetate with a concomitant marked decrease in the phosphorus esterified, and after 20 min the effects became even more marked. During the course of very many experiments in which the effect of fluoroacetate on respiration, rather than on phosphorylation, was studied, a considerable variation occurred in the time taken for the fluorocompound, when added initially to the flasks, to produce appreciable inhibition of the respiration; this variation has not been actively investigated. It should be mentioned also that although fluoroacetate has consistently produced a depression in

the P/O ratio in the fumarate systems, occasionally the interference with phosphorylation occurred before any noticeable inhibition of respiration was observed;¹ this indicated that in some mitochondrial preparations the phosphorylation system had a greater sensitivity to the fluoro compound than did the respiratory enzymes.

Since it had been reported that fluoroacetate had no effect on oxidative phosphorylation,² further experiments were performed to ascertain the possible relationship of the time of addition of the fluoroacetate to the observed action of this compound (Table 2).

TABLE 2. EFFECT OF FLUOROCOMPOUNDS ADDED PRIOR TO AND SIMULTANEOUSLY WITH HEXOKINASE, ON P/O RATIO

Additions to system	Oxygen (μ atoms)	Uptake Phosphorus (μ moles)	P/O
None	7.6	15.9	2.12
Fluoroacetate, added simultaneously with hexokinase	5.6	8.4	1.49
Fluoroacetate, added 5 min prior to hexokinase	4.5	2.8	0.62
Fluoroacetate, added 10 min prior to hexokinase	4.3	3.3	0.74
None	3.9	8.7	2.23
Fluorocitrate, added simultaneously with hexokinase	1.5	0.9	0.61
Fluorocitrate, added 5 min prior to hexokinase	1.7	1.2	0.70
Fluorocitrate, added 10 min prior to hexokinase	1.9	1.1	0.60

Conditions as in Table 1 except that hexokinase tipped in all cases after 10 min preincubation. Final concentration fluoroacetate was 3.3×10^{-3} M; fluorocitrate, 5×10^{-4} M.

In these, fluoroacetate was added to the iced flasks initially with the substrate, or added from the sidearm 5 or 10 min after the flasks were placed on the bath; here a 10 min equilibration period was employed, at the end of which time the trapping system was tipped and the respiration and phosphorylation were measured. It can be seen that the introduction of fluoroacetate to the system prior to the addition of the glucose-hexokinase trap is necessary for appreciable action of this compound on the phosphorylation processes; it may be noted also that the effect is the same whether the fluoroacetate is allowed to react for either 5 or 10 min prior to the addition of the trapping system. These results suggest that the competition between fluoroacetate and the trapping system for the available ATP is an important factor in determining the effect produced by fluoroacetate, and imply that fluoroacetate *per se* has little effect on phosphorylation, prior to its activation and subsequent condensation to fluorocitrate. In keeping with these views are the data (Table 2) showing that the effect of fluorocitrate on such systems has little relationship to the sequence in which this compound is introduced into the flasks. Similar results (unpublished) have also been obtained with fluoropyruvate, which likewise appears to act directly on phosphorylation pathways.

Effect of concentration of fluorocompounds on P/O ratio

Studies on the effect of various concentrations of fluorocitrate and fluoropyruvate on oxidative phosphorylation (Table 3) indicate that with fumarate as substrate, fluorocitrate at 10^{-3} M has an appreciable effect on both the oxygen and phosphorus uptakes, and results in a marked depression of the P/O ratio. The effect on the P/O

TABLE 3. EFFECT OF CONCENTRATION OF FLUOROCOMPOUNDS ON P/O RATIO

Additions to system	Oxygen (μ atoms)	Uptake Phosphorus (μ moles)	P/O
None	6.9	14.3	2.08
Fluorocitrate, 10^{-3} M	2.7	1.6	0.58
Fluorocitrate, 10^{-4} M	4.4	6.7	1.54
Fluorocitrate, 10^{-6} M	4.3	9.7	2.24
None	5.7	10.6	1.87
Fluoropyruvate, 3.3×10^{-4} M	2.6	0.6	0.24
Fluoropyruvate, 3.3×10^{-5} M	4.3	6.4	1.49
Fluoropyruvate, 3.3×10^{-6} M	5.1	9.0	1.74

Conditions as in Table 1 except that fluorocompounds tipped from sidearm simultaneously with addition of hexokinase.

is smaller at 10^{-4} M and is almost absent at 10^{-6} M. Since occasionally the use of fluorocitrate at 10^{-4} M failed to produce a clearly evident effect on the P/O, a standard concentration of 5×10^{-4} M fluorocitrate was employed and was found consistently to produce a marked effect on the P/O. It can also be seen from Table 3 that fluoropyruvate at a concentration of 3.3×10^{-4} M inhibited respiration and almost completely suppressed phosphorus uptake, while at 3.3×10^{-5} M it had only a small effect.

Effect of level of added ATP on the fluorocitrate action

Since fluoropyruvate and particularly fluorocitrate have been shown to increase the destruction of added adenine nucleotides by rat liver mitochondria,^{1, 9} it was of interest to examine the effect of exogenous ATP concentration on the action of fluorocitrate. It can be seen (Table 4) that in the system where 5.0μ moles of ATP were added, the controls show a somewhat higher P/O than do the corresponding systems containing 0.5μ moles of ATP. When fluorocitrate was introduced, the P/O obtained

TABLE 4. EFFECT OF ATP ADDITION ON THE FLUOROCITRATE ACTION

Additions to system	ATP added (μ moles)	Oxygen (μ atoms)	Uptake Phosphorus (μ moles)	P/O
None	0.5	9.2	19.0	2.07
Fluorocitrate	0.5	6.6	7.7	1.16
None	5.0	8.3	19.2	2.30
Fluorocitrate	5.0	5.9	10.8	1.81

Conditions as in Table 1 with ATP as indicated. Fluorocitrate at a final concentration of 5×10^{-4} M added simultaneously with hexokinase.

is disproportionately higher in the series with 5μ moles of ATP, indicating that the higher concentration of ATP partially suppresses the fluorocitrate effect on phosphorylation. It thus appears possible that the increased concentration of added ATP raises somewhat the intramitochondrial level of phosphate acceptor and thus reduces the disturbance in phosphorylation caused by fluorocitrate.

Effect of fluorocitrate on the P/O with DPNH

In all the foregoing experiments the effect of fluorocitrate on the oxidative phosphorylative mechanisms has been obscured to some degree by the inhibition of respiration produced concurrently by this agent. To avoid these complications, the effect of the fluorocompound on the P/O associated with the oxidation of DPNH was studied.

Preliminary experiments disclosed that a brief exposure of the mitochondria to distilled water or the addition of cytochrome *c* to the systems produced an increased rate of oxidation of DPNH, but that the associated phosphorylation was not greatly increased. Therefore, the standard mitochondrial preparation was used, and some representative results are shown in Table 5. It can be seen that the P/O values show a wide variation and in no instance approach the theoretical value of 3. However, in all experiments the absolute phosphorus uptake is depressed by the addition of fluorocitrate while respiration is consistently increased; thus the action of this compound on the phosphorylation process is clearly demonstrated. The stimulation of respiration consistently produced in these systems by fluorocitrate was thought to occur as a result of the uncoupling of phosphorylation. However, the fact that the occurrence of such stimulation showed no correlation with the phosphorylating efficiency of the mitochondria suggested that fluorocitrate might have an additional action. The action

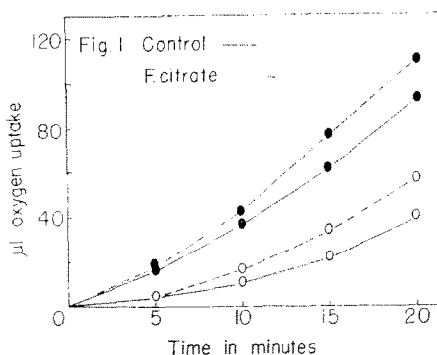


Fig. 1. Effect of fluorocitrate on the oxidation of DPNH by normal and by damaged mitochondria. Warburg flasks contained the standard reaction mixture with 10 μ moles of DPNH as substrate; incubated at 37° C; —○— normal mitochondria; —●— damaged mitochondria (see text).

TABLE 5. EFFECT OF FLUOROCITRATE ON P/O RATIO WITH DPNH

Experiment no.	Additions to system	Oxygen (μ atoms)	Uptake Phosphorus (μ moles)	P/O
1	None	5.2	3.9	0.75
	Fluorocitrate	6.1	2.4	0.38
2	None	5.2	1.9	0.38
	Fluorocitrate	5.9	0.2	—
3	None	3.7	3.0	0.80
	Fluorocitrate	4.9	0.6	0.12

Conditions as in Table 1 except that 10 μ moles of DPNH replaced fumarate as substrate. Final concentration of fluorocitrate was 5 $\times 10^{-4}$ M.

of this compound on the oxidation of DPNH by twice frozen and thawed mitochondria was then examined, since such treatment effectively destroyed the phosphorylating ability of the particles; the phosphorylation occurring during the oxidation of fumarate was measured to ensure that such damage was complete. These results indicate (Fig. 1) that while the oxidation of DPNH is much faster in the pretreated mitochondria, the addition of fluorocitrate further increases the oxidation rate. This suggests that fluorocitrate produces some change in the mitochondrial membrane over and above that produced by the freezing treatment, such that the entry of DPNH is further facilitated. That further damage during the incubation does indeed occur is indicated by the continued increase in the rates of oxidation seen in the damaged mitochondria. However, although fluorocitrate may act to alter the permeability of the mitochondria to DPNH, such effects cannot be correlated with changes in the optical properties of the mitochondria, since fluorocitrate has not been found to induce swelling of the particles under these conditions.

DISCUSSION

Although much work has been done in various laboratories on the mode of action of fluorine-substituted metabolites, little attention hitherto has been paid to the possible effects of these compounds on processes related to oxidative phosphorylation. The experiments presented here show that fluoropyruvate and fluorocitrate affect phosphorylation when added *in vitro* to rat liver mitochondria. Fluoroacetate *per se* appears to have no activity but must undergo a "lethal synthesis" before exerting its effect. This is in accord with the present finding that the action of this compound depends greatly on the sequence in which it is introduced into the system, wherein competition between the fluoroacetate-activating enzyme and the hexokinase of the trap for the available ATP determine the extent to which activation occurs. Judah and Rees² found no effect of fluoroacetate on oxidative phosphorylation. However, these workers used rabbit kidney mitochondria and presumably added fluoroacetate to their systems simultaneously with the trapping system. It is conceivable that these mitochondria differ from those of rat liver to the extent that aconitase becomes affected by the enzymatically produced fluorocitrate earlier than do the phosphorylation mechanisms. Furthermore, it is possible that fluorocitrate does not become homogeneously distributed within the mitochondria; a suggestion of this appears from experiments in which the phosphorylative mechanisms were affected before any inhibition of respiration could be seen.¹

It has been shown⁹ that fluoropyruvate and fluorocitrate cause a reduction in the intramitochondrial levels of adenine nucleotides, as well as a stimulation of nucleotidase activity.¹ Since presumably the intramitochondrial levels of such nucleotides are of prime importance in governing the rate of phosphorylation, it may well be that the addition of larger amounts of ATP, as described here, serves to suppress partially the effects of fluorocitrate by stabilizing the intramitochondrial levels of these nucleotides. It would be expected that the extent of such suppression will depend upon the facility with which the endogenous and exogenous nucleotides can exchange.

The effect of fluorocitrate on oxidative phosphorylation is clearly seen when DPNH is employed as substrate. In those experiments this fluorocompound consistently reduces the amount of phosphorus esterified, with a concomitant stimulation in oxygen consumption. This latter effect is seen whether the mitochondria are a

fresh, phosphorylating preparation or one damaged specifically to render the particles incapable of phosphorylation. Thus, the increase in respiration in such damaged mitochondria is apparently due, not to an uncoupling action of fluorocitrate, but more probably to the action of this compound on the mitochondrial membrane resulting in a more rapid entry of DPNH. In normal mitochondria, then, it would appear that the increased oxygen uptake produced by fluorocitrate in the presence of DPNH may be due to this increased permeability to the DPNH, in addition to any direct action which may occur by an uncoupling of oxidative phosphorylation.

SUMMARY

Fluoropyruvate and fluorocitrate have been shown to affect oxidative phosphorylation in rat liver mitochondria, and evidence is presented that fluoroacetate *per se* has not such action. The effect of fluorocitrate on the P/O associated with DPNH oxidation has been examined. Phosphorus esterification is depressed while oxygen consumption is stimulated; however, the latter effect is ascribed, at least in part, to an effect of fluorocitrate on the permeability to DPNH.

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THE EFFECTS OF FLUOROCOMPOUNDS ON OXIDATIVE PHOSPHORYLATION II—EFFECTS ON THE BREAKDOWN OF NUCLEOTIDES BY LIVER MITOCHONDRIA*

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Abstract—The fluorocompounds generally increased the dephosphorylation of adenine and pyridine nucleotides. A definite quantitative and qualitative difference, however, existed with respect to the pattern of breakdown produced by the fluorocompounds from those resulting through the action of DNP. Enzyme assays demonstrated an enhancement of adenine nucleotide dephosphorylase activity by fluorocitrate and fluoropyruvate while DPN-ase was not affected. Examination of the inorganic phosphate released showed a marked stimulation of AMP dephosphorylation by the fluorocompounds at 10^{-5} M concentration, while DNP exerted its effect mainly on the dephosphorylation of ATP. The "pre-aged" mitochondria displayed a reduced response to fluorocitrate and DNP. An interpretation of the analytical results is presented.

IT HAS BEEN shown previously¹ that fluoropyruvate and fluorocitrate affect the phosphorylation processes when added *in vitro* to rat liver mitochondria, and a preliminary investigation has shown that these fluorocompounds affect the level of adenine nucleotidase activity of the particles.²

The work reported here shows that fluoropyruvate and fluorocitrate produce alterations in the intramitochondrial levels of both adenine and pyridine nucleotides, and that while adenine nucleotide dephosphorylase activity is enhanced, that of DPN-ase† is unaltered. Some comparisons are made between the action of fluorocitrate and of DNP, and the mechanism of action of the fluorocompounds on oxidative phosphorylation and related processes is discussed.

EXPERIMENTAL

Mitochondria were prepared from the livers of male white Wistar rats of 200–300 g, as described previously.¹ All animals were fasted overnight for 15–18 hr prior to sacrifice. Pre-aged mitochondria were prepared by the method of Lardy.³ Nucleotide dephosphorylation activity was measured in a Dubnoff metabolic shaking incubator

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† The following abbreviations will be used throughout: AMP, ADP and ATP, adenine mono-, di- and triphosphates; DPN, diphosphopyridine nucleotide; DNP, 2 : 4-dinitrophenol; DPN-ase, diphosphopyridine nucleotidase; Tris, tris(hydroxymethyl) aminomethane; adenine nucleotide dephosphorylase, the enzyme or enzymes releasing inorganic phosphate from adenine nucleotides.

at 30°C in air; the reaction system contained 40 μ moles of Tris at pH 7.28, 750 μ moles of sucrose, 120 μ moles of KCl, 6 μ moles of $MgCl_2$, and 10 μ moles of adenine nucleotide neutralized to pH 7.28. The reaction was started by the addition of mitochondria from approximately 300 mg of liver, suspended in 0.25 M sucrose, to give a final volume of 3 ml. After incubation for 20 min, for which time the dephosphorylation rates were found to be essentially constant, the flasks were placed in an icebath and 0.5 ml of 20 per cent trichloroacetic acid was added. After centrifugation, inorganic phosphates were determined in the supernatant fractions by the method of Gomori.⁴ Paper chromatographic analyses of the supernatant materials were performed with a solvent system of isobutyric acid, ammonia and water.⁵

Diphosphopyridine nucleotides were assayed by first extracting the mitochondria by the method of Jedeikin and Weinhouse⁶ and then determining the fluorescence of the methyl-ethyl ketone derivative⁷ using the Aminco-Bowman spectrophotofluorimeter. Material which proved susceptible to the action of the DPN-ase of *Neurospora crassa*⁸ was taken to be oxidized pyridine nucleotide. DPNH was determined after oxidation to DPN with oxaloacetic acid and crystalline malic dehydrogenase. In the determination of DPN-ase activity of the mitochondria the DPN was estimated spectrophotometrically after conversion to DPNH with ethanol and crystalline alcohol dehydrogenase.

Quantitative measurements of adenine nucleotides were performed spectrophotometrically after perchloric acid extraction of the mitochondria, followed by separation on Dowex-I columns.⁹ Reagents used were those previously described.¹

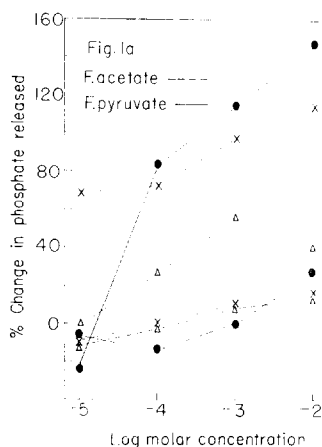
RESULTS

Effects on the dephosphorylation of AMP, ADP and ATP

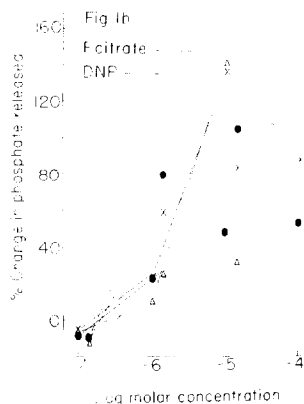
In Fig. 1 (a) and 1 (b) the increase in the release of phosphate from AMP, ADP and ATP by fresh mitochondria is shown, in the presence of fluoroacetate, fluoropyruvate, fluorocitrate and DNP. To facilitate the comparison of data from several experiments, the results shown have been corrected for the controls in the absence of the added fluorocompounds, and the phosphate values represent percentage changes over the respective controls. It can be seen that fluoroacetate has little effect on the release of phosphate from any of the nucleotides examined and probably requires activation and condensation to fluorocitrate before affecting the phosphorylation processes. Fluoropyruvate, however, has a marked effect on the release of phosphate from AMP, with smaller percentage stimulation of the ADP and ATP systems.

The effects of fluorocitrate were also compared with those of DNP in these systems, inasmuch as the latter compound has long been known to stimulate the dephosphorylation of ATP. It can be seen from Fig. 1 (b) that both agents evoke an increased phosphate release compared with the controls. Fluorocitrate has a marked percentage stimulation on the AMP system, with smaller effects on the ADP and ATP systems; essentially maximal effects are obtained at concentrations of about 10^{-5} M and increasing the concentration above this value produces little further effect. It should be noted also in Fig. 1 (b) that, at a concentration of 10^{-6} M, DNP produces somewhat less effect than does an equivalent concentration of fluorocitrate. A major difference between the effects produced by the fluorocompounds and by DNP appears in that the former exert their greatest action on AMP, whereas DNP produces the greatest stimulation of dephosphorylation in the ATP system. Another indication that the

dephosphorylation systems cannot be maximally stimulated by fluorocitrate has been shown by other experiments in which this compound was added together with DNP to a system containing ATP; under these conditions the phosphate released was always in considerable excess of that released by fluorocitrate alone.



(Fig. 1a)

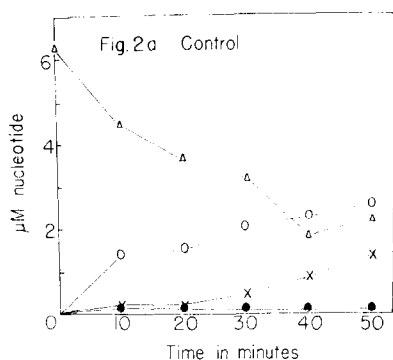


(Fig. 1b)

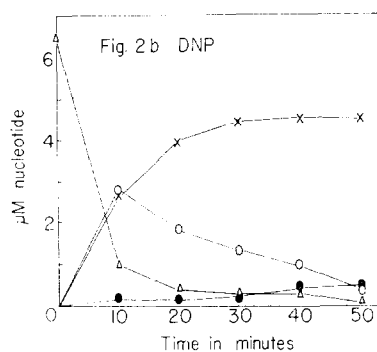
FIG. 1(a), (b). The dephosphorylation of adenine nucleotides by mitochondria. Flasks contained the standard reaction system with 10 μ moles of adenine nucleotide as indicated. Reaction stopped after 20 min at 30°C. AMP, $\bullet$ — $\bullet$; ADP, $\times$ — $\times$; ATP, $\triangle$ — $\triangle$. Concentration of fluorocompounds and DNP as indicated. Phosphate released from nucleotides by mitochondria in the control flasks were (μ moles): AMP, 1.20; ADP, 3.31; ATP, 5.88.

A further comparison of the effects of fluorocitrate and DNP is shown in Figs. 2 (a), (b) and (c). In these experiments mitochondria were incubated for various periods with ATP in the presence of fluorocitrate or DNP, and the reaction mixtures were analysed for the adenine nucleotides and adenosine, using the Dowex column fractionation technique.⁹ The addition of DNP greatly accelerates the dephosphorylation of ATP and ultimately a high level of AMP remains. Addition of fluorocitrate produces a much less marked effect; however, the conversion of ATP to ADP is accelerated initially. It would appear difficult to interpret these data in terms of the possible sites of action of fluorocitrate, since the resynthesis of nucleotides is known to occur, e.g. by myokinase activity; however, the results show clearly enough that the pattern of nucleotide interconversion is somewhat altered by fluorocitrate.

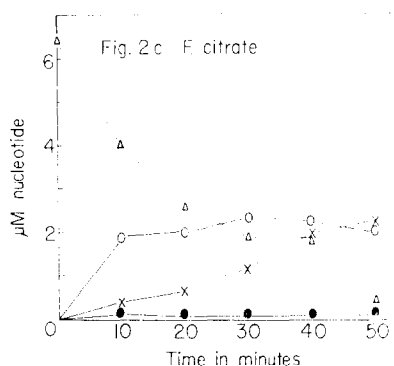
It would be expected from these results that some alterations in the endogenous adenine nucleotide levels might also be induced by the fluorocompounds, and from Table 1 it is evident that both fluoropyruvate and fluorocitrate reduce somewhat the endogenous levels of AMP, ADP and ATP. Since the intramitochondrial levels of these nucleotides play a major role in controlling the phosphorylation and respiration of the particles, it is possible that such alterations produced by the fluorocompounds are associated with the impaired phosphorylating ability induced by these agents.^{1, 2}



(Fig. 2a)



(Fig. 2b.)



(Fig. 2c)

FIG. 2(a), (b), (c). The interconversion of adenine nucleotides by mitochondria. Each flask contained mitochondria from 1.95 g of rat liver, 6.5 μ moles of ATP and standard concentrations of Tris, MgCl_2 , KCl and sucrose, in a total volume of 30 ml. Incubated at 30°C and 3 ml aliquots removed at indicated times for nucleotide assay. Final concentration of fluorocitrate, $5 \times 10^{-5}\text{M}$; DNP, $2 \times 10^{-5}\text{M}$. Adenosine, ●—●; AMP, ×—×; ADP, ○—○, ATP, Δ—Δ.

TABLE 1. EFFECT OF FLUOROCOMPOUNDS ON INTRAMITOCHONDRIAL LEVELS OF ADENINE NUCLEOTIDES

Additions to system	Intramitochondrial nucleotides		
	AMP (μ moles)	ADP (μ moles)	ATP (μ moles)
None	0.25	0.68	0.64
Fluoropyruvate, $5 \times 10^{-4}\text{M}$	0.15	0.44	0.38
Fluorocitrate, $5 \times 10^{-4}\text{M}$	0.15	0.60	0.36

Mitochondria (25.4 mg N) incubated at 30°C for 30 min with additions as indicated. After incubation, the nucleotides were extracted from mitochondria with perchloric acid, then separated on Dowex I columns.

Action of fluorocitrate and DNP on fresh and aged mitochondria

It is known¹⁰ that fresh mitochondria prepared in an isotonic medium have minimal dephosphorylase activity. However, such activity can be markedly increased by a

variety of treatments which appear, in general, to disrupt the structural integrity of the particles. In this connexion it was of interest to examine the effect of fluorocitrate on mitochondrial preparations which had been damaged by the "pre-aging" method of Lardy.³ In Table 2 the effects of fluorocitrate and of DNP are shown on normal and pre-aged mitochondria. The endogenous dephosphorylase of the particles is increased by this pre-aging treatment, but the responses to fluorocitrate and to DNP are reduced. This would suggest that in the freshly prepared mitochondria fluorocitrate may act primarily by inducing some alteration in the structure of the mitochondria upon which the level of the dephosphorylase activity depends, rather than by acting on the dephosphorylase itself. Additional evidence which could be interpreted as supporting this view comes from experiments in which it was found that neither fluorocitrate nor DNP affects the activity of the 5'-nucleotidase of rattlesnake venom.¹¹

TABLE 2. EFFECT OF FLUOROCITRATE AND DNP ON FRESH AND AGED MITOCHONDRIA

System	Fresh mitochondria		Aged mitochondria	
	Phosphate released (μ moles)	Stimulation (%)	Phosphate released (μ moles)	Stimulation (%)
Mitochondria + ATP	3.7	—	5.8	—
Mitochondria + ATP + DNP (10^{-4} M)	16.5	440	14.8	270
Mitochondria + ATP + fluorocitrate (10^{-4} M)	6.5	71	6.6	10

System as under "Experimental".

Further indications that fluorocompounds may produce structural changes within the mitochondria derive from analyses of the DPN, TPN and DPNH present in mitochondria which have been incubated with fluoropyruvate or fluorocitrate. It can be seen from Table 3 that fluoropyruvate induces a lowered level both of DPNH and oxidized pyridine nucleotides, with fluorocitrate having somewhat smaller effects. However, as the data in Table 4 show, the DPN-ase activity of the mitochondria is not enhanced by these agents; indeed, fluorocitrate suppresses DPN-ase activity somewhat. These findings would appear to indicate, therefore, that the fluorocompounds produce some alteration in the mechanisms whereby the nucleotides are bound to the mitochondria.

TABLE 3. EFFECT OF FLUOROPYRUVATE AND FLUOROCITRATE ON INTRAMITOCHONDRIAL PYRIDINE NUCLEOTIDE LEVELS

Additions to system	DPNH (μ g flask)	DPN (μ g flask)	TPN
None	145	31	
Fluoropyruvate (10^{-4} M)	28	6	
Fluorocitrate (2×10^{-4} M)	123	16	

All flasks contained 4.22 mg of mitochondrial N, with standard amounts of $MgCl_2$, KCl, Tris and sucrose. Incubated 20 min at 30°C, then reaction stopped by chilling flasks. Nucleotides assayed fluorometrically after extraction from mitochondria.

TABLE 4. EFFECT OF FLUORO COMPOUNDS ON MITOCHONDRIAL DPN-ASE ACTIVITY

Additions to system	DPN destroyed in 30 min (μ moles)
None	1.37
Fluoropyruvate (2×10^{-4} M)	1.35
Fluorocitrate (10^{-4} M)	1.00

All flasks contained 4.65 μ moles of DPN with standard amounts of $MgCl_2$, KCl, Tris, sucrose and mitochondria from approximately 100 mg of liver. DPN assayed after 30 min incubation at 30°C.

Mechanism of action of fluoropyruvate and fluorocitrate

It has been demonstrated in model systems¹² that fluoropyruvate undergoes a reaction with the sulphydryl group of compounds such as cysteine, leading to a loss of fluorine, and we have shown further¹³ that such sulphydryl enzymes as pyruvic dehydrogenase and alcohol dehydrogenase are also inhibited by this agent. It would appear likely that fluoropyruvate combines with sulphydryl groups within the mitochondria and thereby gives rise to some alteration which presumably results in an increased dephosphorylase or nucleotidase activity. This view is supported by the data shown in Table 5, from which it can be seen that both iodoacetate and *p*-chloromercuribenzoate increase the dephosphorylase activity.

TABLE 5. EFFECTS OF IODOACETATE, *p*-CHLROMERCURIBENZOATE, VERSENE AND FLUOROCITRATE ON THE DEPHOSPHORYLATION OF ATP

System	Phosphate released (μ moles)
Mitochondria + ATP	3.5
Mitochondria + ATP + iodoacetate (10^{-3} M)	3.8
Mitochondria + ATP + PCMB (10^{-3} M)	5.4
Mitochondria + ATP	4.0
Mitochondria + ATP + fluorocitrate (10^{-4} M)	5.4
Mitochondria + ATP + versene (10^{-3} M)	3.8
Mitochondria + ATP + versene (10^{-4} M)	3.7

System as under "Experimental".

The mechanism of action of fluorocitrate appears more difficult to characterize. This compound has been found not to react with sulphhydryl groups in model systems, presumably because of the stability of the C—F bond. Since citrate itself is an effective chelating agent, the possibility was also examined as to whether fluorocitrate acts by combining with some metal within the mitochondria. A comparison of the action of a chelating agent, Versene, and fluorocitrate on the dephosphorylation systems (Table 5) indicates that, in contrast to fluorocitrate, edathamil (Versene) fails to stimulate dephosphorylation.

DISCUSSION

It may be concluded from these studies that fluoropyruvate and fluorocitrate have effects on the systems involved in adenine nucleotide balance in liver mitochondria,

in addition to their well documented effects on respiratory enzymes.^{12, 13, 14} This finding necessarily implies that studies on the respiratory mechanisms of complex tissue preparations, in which these compounds are used as specific inhibitors, may well be complicated by additional effects on phosphorylation and on other systems involving these nucleotides.

The low activity of fluoroacetate in these systems suggests that this compound *per se* has little activity but that conversion to fluorocitrate is necessary to produce effects; the lack of oxalacetate in these substrate-free systems would severely limit the formation of fluorocitrate in this manner. Further indications that fluoroacetate itself is inactive have been obtained from studies on the conditions necessary to show activity of this compound on oxidative phosphorylation.¹

The observation that the fluorocompounds stimulate the dephosphorylation of AMP relatively more than that of ATP is of interest in that DNP does not show this characteristic pattern. It must be emphasized, however, that the fluorocompounds accelerate the absolute destruction of added ADP and ATP in considerable measure. The dephosphorylase activity of the mitochondria cannot be maximally stimulated merely by increasing the concentration of added fluorocitrate, because subsequent addition of DNP to the same system always evokes a further response. This, with foregoing observations, clearly indicates that a difference exists in the modes of action of fluorocitrate and DNP.

The further observation that the intramitochondrial levels of ADP and ATP are somewhat reduced in the presence of fluorocitrate suggests that this compound may interfere with oxidative phosphorylation by reducing the endogenous phosphate acceptor level within the mitochondria; such an hypothesis is in keeping with the finding that the effect of fluorocitrate on phosphorylation can be alleviated to some extent by increasing the amount of ATP added to the system.¹

It is significant that fluoropyruvate and fluorocitrate produce lowered intramitochondrial levels of both oxidized and reduced pyridine nucleotide, yet do not enhance the DPN-ase activity of the particles, as measured by the destruction of added DPN. This suggests that the mechanisms whereby the nucleotides are bound to the mitochondrial structure are altered, such that the nucleotides then become susceptible to the action of the DPN-ase of the particles.

While the mechanism of action of fluoropyruvate is most probably that of combining with sulphydryl groups within the mitochondria, that of fluorocitrate is evidently different, for it has failed to undergo such a sulphydryl interaction in the model systems tested. The fact that Versene does not produce increased dephosphorylase activity as does fluorocitrate may indicate that the latter compound does not act by binding with metals in the mitochondrial matrix; however, it may well be that fluorocitrate can penetrate to metal sites which are not accessible to the Versene. Although there thus appears to be a divergence in the action of fluoropyruvate and fluorocitrate, it appears that both agents give rise to alterations in the mitochondrial structure which then lead to an increased nucleotide breakdown and an impaired binding of pyridine nucleotides.

It is not known to what extent the effects of fluoropyruvate and fluorocitrate described in this and the previous report¹ contribute to the convulsive state produced in the intact animal by these agents. Small variations in the amounts of phosphate esters in rat brain have been shown to occur after fluoroacetate or fluorocitrate

administration,¹⁵ but it appears possible that these changes are not the primary ones responsible for the convulsive activity.¹⁶ On the other hand, the parallel seen here between the action of fluoropyruvate and fluorocitrate on mitochondria and the ability of both these agents to produce convulsions must not go unmentioned. Although essential differences exist in the nature of these convulsions, in that those induced by fluorocitrate are invariably followed by death, whereas those effected by fluoropyruvate often are not fatal, one may speculate that the effects *in vitro*, described here, may have a direct bearing on the actions of these compounds *in vivo*.

SUMMARY

Fluoropyruvate and fluorocitrate have been found to increase the dephosphorylation of AMP, ADP and ATP by rat liver mitochondria. Damaging the mitochondria by ageing reduces the response to the fluoro compounds. Both agents produce lowered levels of intramitochondrial pyridine nucleotides but do not increase the mitochondrial DPN-ase activity; they also depress the intramitochondrial levels of adenine nucleotides. The mechanism of action of these compounds is discussed.

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THE CYTOCIDAL ACTION OF BARBITURATES ON LYMPHOCYTES *IN VITRO*

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Abstract—Rat lymph nodes were maintained *in vitro* in a synthetic medium by an organ culture technique. The toxic effect of barbiturates was studied quantitatively by counting the number of dead lymphocytes after exposure to various concentrations for various times. Seconal was found to be the most toxic, followed by Nembutal, Amytal and Pentothal, in that order. Barbitone itself was non-toxic under the same conditions. Evidence that barbiturates produce a specific biochemical lesion (in other tissues) is examined and some proposed antagonists, namely succinate, ascorbic acid and megimide were tested. Apart from a small effect of succinate, none of these protected lymphocytes at all. It was also found that, compared with barbiturates, colchicine and cortisone were more toxic, cyanide was about equally toxic, while ethanol, acetone and urethane were relatively non-toxic.

THE SPECIFIC sensitivity of lymphocytes to barbiturates was first observed by Lajtha.¹ Using suspension cultures of human bone marrow cells in serum, he found that 1 mM Nembutal killed most of the lymphocytes without visibly affecting any of the other cell types present.

Using rat lymph nodes maintained *in vitro* in a synthetic culture medium, the author has confirmed the specific cytotoxic effect of Nembutal on lymphocytes and has measured it quantitatively. The effects of other barbiturates in common clinical use have been similarly measured and the influence of some supposed barbiturate antagonists has been investigated. Comparative data on the lymphocytotoxic action of cortisone, colchicine, urethane, ethanol, acetone and cyanide are also included.

METHODS

The lymph nodes were maintained *in vitro* for a total period of 4 days using an organ culture technique which has been described in detail elsewhere.² The basis of the method is that the cultures are supported at the surface of a fluid culture medium on a perforated metal grid, so that they are maximally exposed to the gas phase which is 5 per cent CO₂ in oxygen. The synthetic medium used is called T8; its exact composition and preparation have been described elsewhere² and it represents a modified Tyrode solution plus 12 essential amino acids, thiamine, *p*-aminobenzoic acid, insulin, phenol red and chloramphenicol.

The lymph nodes used were usually the lumbar and sacral from 4 week-old Wistar rats weighing 50–70 g, but in a few experiments the cervical nodes from similar animals were used instead. Control experiments showed that, in the case of Nembutal

at any rate, the lumbar, sacral and cervical nodes were equal in sensitivity. Each culture chamber contained 15–20 lymph node cultures, each weighing about 2 mg, in 5–6 ml of medium.

Experience with this system has shown² that over the first 6 days *in vitro* the only deleterious changes in the culture medium are the falling glucose concentration and the rising acidity (lactic acid). These can be counteracted by addition of appropriate volumes of isotonic glucose and isotonic NaHCO_3 solutions at suitable intervals, a procedure which we call "reconditioning" the medium.

In the experiments to be reported here the cultures were first maintained for 2 days in the normal T8 medium. This gives them a chance to settle down and allows time for disappearance of the few cells which die (from trauma) in the first few hours after explantation. After 2 days *in vitro* the medium was reconditioned by adding 0.2 ml of 5.83 per cent glucose and 0.5 ml of 1.36 per cent NaHCO_3 per 5 ml of medium and, after mixing, removing 0.7 ml to restore the original volume. At this point the barbiturate was added as a small volume (about 0.05 ml) of concentrated solution. The barbiturate solutions were made up in distilled water just before use and sterilized by passage through no. 5 porosity sintered glass filters. The sodium salts taken from freshly opened ampoules were used in each case.

The lymphocytes could not of course be examined during the experiment, they had to be fixed and stained at the end. At a determined time after the addition of barbiturate, ranging from 5 to 48 hr in different experiments, all the cultures were removed from the chamber and the lymphocytes from each separate culture were quickly fixed, stained and preserved by the following procedure. The culture is placed in a small drop of serum on a microscope slide; it is then cut up and teased out with fine knives to make a free suspension of the lymphocytes; the cell suspension is spread out to make a rather thick film which is immediately fixed (wet) by plunging the slide into Susa fixative. When all the films have been fixed they are washed, stained with haemalum, dehydrated in 2-ethoxyethanol and mounted in Euparal.

When lymphocytes die, from any cause, the nuclei undergo pyknosis and these pyknotic (dead) lymphocytes persist, in cultures, for many hours before they finally disappear by autolysis or phagocytic removal. In the film preparations the pyknotic lymphocytes are readily distinguishable from the normal ones and so the percentage of the lymphocytes which were dead at the conclusion of the experiment can be accurately counted. In normal cultures less than 2 per cent of the lymphocytes are pyknotic, but after addition of barbiturate the number increases indicating that barbiturate has killed some of the cells, and the percentage of dead cells found at standard times after standard doses gives an accurate measure of the effect. A few medium and large lymphocytes are present in these cultures but over 99 per cent are small ones.

The percentage of pyknotic lymphocytes in each culture (film) was determined from a total count of 500 lymphocytes. The figures for the fifteen to twenty cultures from the one chamber were averaged to give the final figure for the percentage of dead lymphocytes present at the end of the experiment. In most of the results which follow, each point on the curves was determined in this way from a single experiment employing one culture chamber. But in the case of some of the more important points the experiment was repeated once or twice, as a test of consistency, which was found to be good, and in these cases the average of all the results is given (Table 1).

RESULTS

Effect of barbiturates

Figure 1 shows that after addition of 0.3 mM Nembutal (pentobarbitone) the percentage of dead lymphocytes increases linearly with time for the first 48 hr and presumably longer. It will be noted that the controls—cultures at the time of adding Nembutal and cultures 48 hr after no addition—contain less than 2 per cent of dead cells (Table 1). After 48 hr 87 per cent of the lymphocytes are dead though none of the reticulum cells are visibly damaged. Some of the lymphocytes show a great ballooning and watery vacuolation of the cytoplasm and an irregular lobulation of the nucleus just before the onset of pyknosis. It is possible that all the dying lymphocytes go through this phase, but not many are seen in the films because the phase is short-lived, being terminated by rupture and dissolution of the cytoplasm and pyknosis of the nucleus.

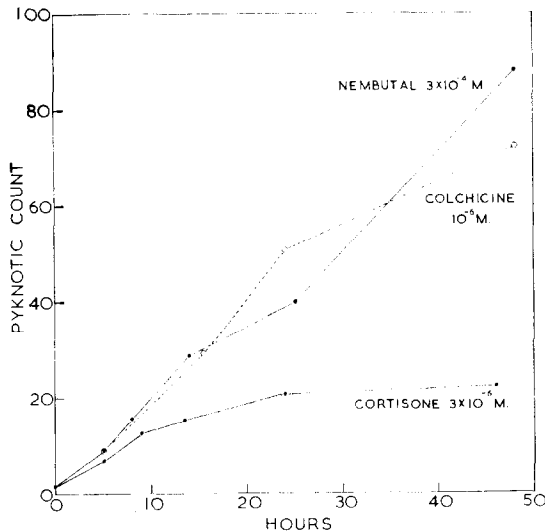


FIG. 1. Percentage of dead lymphocytes (pyknotic count) in lymph-node cultures at time intervals after addition of Nembutal, colchicine or cortisone.

The same phenomenon has been seen after poisoning lymphocytes *in vitro* with cyanide, but not to any extent after cortisone, colchicine or ionizing radiation.

Different concentrations of Nembutal were now compared, and 48 hr was chosen as a convenient (but arbitrary) end point at which to measure the effect. The dose-effect curve is shown in Fig. 2 together with similarly determined curves for Seconal (quinalbarbitone), Amytal (amylobarbitone), Pentothal (thiopentone) and barbitone. The numerical data are given in Table 1.

A certain number of reticulum cells are always present in the film preparations and it should be mentioned that some of these cells showed damage 48 hr after addition of mM Nembutal, Seconal, Amytal or Pentothal, but not to any extent after lower concentrations. Damage appeared as cytoplasmic vacuolation with nuclear karyolysis or pyknosis.

TABLE 1. PERCENTAGE OF DEAD (PYKNOTIC) LYMPHOCYTES IN LYMPH NODE CULTURES AFTER ADDITION OF VARIOUS CONCENTRATIONS OF VARIOUS BARBITURATES. COMPARABLE DATA FOR SOME OTHER CELL POISONS ARE ALSO GIVEN
(Number of cultures in brackets)

Addition	Hours after addition	100mM	1mM	0.3mM	0.2mM	0.1mM	0.03m M	0.01mM	0
Nembutal	0								1.7(15)
	5			8.8(17)					
	8			15.5(20)					
	14			28.7(20)					
	25			39.8(20)					
Seconal	48		97.9(15)	88.2(39)	44.2(47)	13.5(36)	1.5(17)		1.2(37)
			99.1(20)	96.9(40)	93.6(20)	45.0(59)	2.4(18)	1.2(19)	
			98.5(40)	56.7(20)		7.8(15)	2.3(18)		
			99.0(40)	41.7(57)	11.4(39)	6.3(33)			
			1.6(18)	1.6(17)		1.8(20)			
Amytal			1.7(31)						
Pentothal									
Barbitone									
Urethane									
Ethanol	48	1.6(20)							
Acetone		1.9(17)							
Cyanide			99.1(20)	84.6(40)	66.3(39)	8.5(38)	1.9(40)		
Cortisone							46.1(11)	30.4(12)	
Colchicine							91.4(20)	84.5(20)	

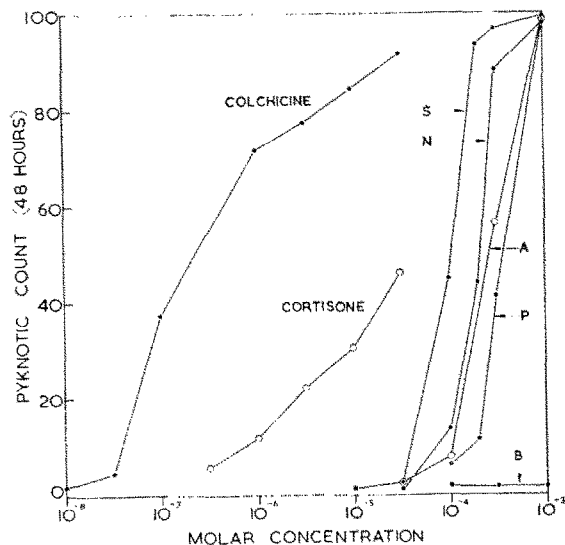


Fig. 2. Percentage of dead lymphocytes (pyknotic count) in lymph-node cultures 48 hr after addition of various concentrations of colchicine, cortisone or barbiturates: S = Seconal, N = Nembutal, A = Amytal, P = Pentothal, B = barbitone.

Effect of some other poisons

Figure 1 shows the time course of the effect of two other rather specific lymphocytocidal agents—colchicine and cortisone. The concentrations used were so chosen that the initial effects roughly matched that of 0.3 mM Nembutal. In the case of

colchicine the effect is linearly progressive for 48 hr, as after Nembutal, but in the case of cortisone the effect reaches a maximum after 24 hr.

The dose-effect curves in Fig. 2 show that on this 48 hr assessment the barbiturates are less toxic to lymphocytes than colchicine or cortisone. Lymphocytes are not specifically sensitive to cyanide, they are killed by concentrations of 0.1 mM upwards in the same way as other cells. It is interesting to note therefore that the barbiturates are just about as toxic to lymphocytes as is cyanide, in fact the figures for Nembutal and cyanide were so similar (Table 1) that it was not possible to plot a separate curve for cyanide in Fig. 2. Seconal was slightly more toxic than cyanide, Amytal and Pentothal slightly less so. Lymphocytes are not specifically sensitive to other narcotics: mM urethane, 0.1 M ethanol and 0.1 M acetone were shown to be quite harmless (Table 1) and higher concentrations poisoned lymphocytes and reticulum cells about equally.

Barbiturate antagonists

1. *Succinate*. Addition of barbiturate to tissues *in vitro* inhibits the metabolic oxidation of glucose, pyruvate and α -ketoglutarate, but not that of succinate. This has been shown for the action of mM Luminal on rat brain and liver slices,³ 0.3 mM Nembutal on rat brain homogenate,⁴ and 0.45 mM Amytal on rat liver mitochondria.⁵ Now it is known that the oxidation of succinate does not require coenzymes, whereas the oxidative steps in glucose metabolism prior to succinate are all dependent on coenzymes I and II. It seems therefore that barbiturate blocks these coenzymes and prevents the transport of hydrogen to FAD.

So if this metabolic block is in fact responsible for the pharmacological action of barbiturate, and if cells can utilize the energy derived from succinate oxidation for maintenance of their life processes, then succinate should prove an effective antagonist to barbiturate. Not surprisingly, it has been claimed that succinate does shorten the duration of barbiturate anaesthesia in animals,^{6, 7} though this is denied by others.^{8, 9} It has also been claimed that intravenous succinate is an effective antidote to barbiturate poisoning in man.^{10, 11}

It was therefore of interest to see if succinate would antagonize the action of barbiturates on lymphocytes. The results (Table 2) show that succinate could partly

TABLE 2. EFFECT OF BARBITURATE ANTAGONISTS. PERCENTAGE OF DEAD (PYKNOTIC) LYMPHOCYTES IN LYMPH NODE CULTURES 48 HR AFTER ADDITION OF ANTAGONIST ALONE (A), BARBITURATE ALONE (B), ANTAGONIST AND BARBITURATE (AB)
(Number of cultures in brackets)

	A	B	AB
Succinate 1mM, Nembutal 0.1mM	1.7(17)	13.5(36)	4.9(29)
Succinate 3mM, Nembutal 0.2mM	—	44.2(47)	42.7(39)
Succinate 1mM, Seconal 0.1mM	1.7(17)	45.0(59)	47.1(19)
Ascorbic acid 1mM, Nembutal 0.2mM	2.2(23)	44.2(47)	43.3(40)
Megimide 0.3mM, Nembutal 0.2mM	2.3(19)	44.2(47)	45.6(20)

antagonize the small effect of 0.1 mM Nembutal but was unable to mitigate the larger effects of 0.2 mM Nembutal or 0.1 mM Seconal. The succinate may be removed fairly

rapidly by oxidation, so in a further experiment with 0.2 mM Nembutal and 2 mM succinate a second lot of succinate was added 24 hr after the first, but it had no effect. These results suggest a small protective effect of succinate but are not very conclusive.

2. *Ascorbic acid*. It has been shown in guinea pigs that the duration of barbiturate anaesthesia is inversely related to the ascorbic acid content of the blood, when the latter is experimentally varied.^{12, 13} Greig,¹⁴ accepting the theory that barbiturate blocks the pathway of hydrogen transfer, suggested that ascorbic acid might act as a reversible hydrogen acceptor bridging the gap caused by this block, and showed that the depression of glucose oxidation produced in rat brain homogenate by mM Nembutal could be reversed by 4 mM ascorbic acid. In the author's experiments (Table 2) mM ascorbic acid did not influence the effect of 0.2 mM Nembutal on lymphocytes.

3. *Megimide*. Megimide (β : β -methyl-ethyl-glutarimide) is a recently introduced barbiturate antagonist which has proved effective in the treatment of acute barbiturate poisoning in man.^{15, 16} There is no evidence that it acts at the biochemical level and it probably acts as a central nervous stimulant. In this case it would not be expected to protect lymphocytes and the author's experiments (Table 2) show that it does not do so.

DISCUSSION

Figure 2 shows that the descending order of toxicity is Seconal, Nembutal, Amytal, Pentothal, barbitone. This bears no relation to their order of anaesthetic potency. Not does it match any particular chemical configuration of their molecules, except that, since barbitone itself is entirely non-toxic in this concentration range, the two side chains appear to be particularly important. It must be remembered however that these results were read from 48 hr effects, and no allowance has been made for the possible instability of some of the barbiturates in the rather alkaline (pH 7.6) culture medium at 37°C. Pentothal, for example, is known to be rather unstable and on a shorter term assessment might appear relatively more toxic. Pomerat and Painter¹⁷ found that the concentrations required for complete suppression of outgrowth from chick spinal-cord fragments in tissue culture were—Seconal 0.8 mM, Nembutal 1.4 mM, Amytal 1.8 mM (figures calculated from their data), which is in the same descending order as found here. It has also been shown in tissue cultures that the outgrowth of fibroblasts is partially inhibited by 10 mM barbitone and that of iris epithelium by 3 mM.¹⁸ In these tissue culture experiments the cells were only inhibited not actually killed, for the effects were reversible. Geiger¹⁹ reported that addition of 0.1 mM barbitone to human nerve cells maintained in tissue culture caused fragmentation of the mitochondria in a few minutes, an effect which was equally rapidly reversible.

Figure 2 shows that the dose-effect curves for the barbiturates are remarkably steep. There is a critical concentration, about 0.1 mM, above which they become highly toxic to lymphocytes. Now the plasma concentrations of Seconal, Nembutal and Amytal required to maintain surgical anaesthesia in the rabbit lie within the range 0.1–0.3 mM,²⁰ and in man the plasma concentration of Seconal or Amytal

at the time of regaining consciousness after narcosis is about 0.1 mM.²¹ It seems therefore that during surgical anaesthesia the concentration of barbiturate in the blood must usually exceed the critical level of 0.1 mM, albeit for only a few hours, while in cases of acute barbiturate poisoning it must greatly exceed it for many hours. In acute barbiturate poisoning therefore one would expect to find evidence of death of lymphocytes in the lymphoid organs and a depressed blood lymphocyte count. It seems, however, that this does not occur, at any rate in man. Ravn²² reported the white blood cell counts in thirteen cases of acute barbiturate poisoning. In seven cases there was an absolute neutrophilia during the acute phase and in four cases an absolute lymphocytosis during the recovery phase, but none showed an absolute lymphopenia at any stage. Others have reported similar findings and there is no record in the literature of lymphopenia following barbiturate administration.

With regard to the primary biochemical lesion (if any) produced by barbiturate, the current hypothesis of a block in pyruvate oxidation is open to some doubt because it has been shown only with concentrations rather higher than those which produce narcosis or which kill lymphocytes. Lower concentrations (0.1 mM) have been shown to uncouple oxidative phosphorylation²³ and to activate ATPase^{24, 25} in isolated mitochondria, but whether this occurs in the intact cell remains to be seen. The mechanism of barbiturate action remains obscure and although the present results throw no light on it they do perhaps widen the field of enquiry. Drugs consumed at the rate of 2000 tons a year in the U.S.A. alone²⁶ must continue to merit attention.

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A PROPOS DE L'EFFET DELAPORTE

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Résumé—Selon Delaporte, lorsqu'une population microbienne dense est soumise à une irradiation ultra-violette, les germes survivants qui se divisent exercent une action revivifiante sur les microorganismes se trouvant dans leurs environs immédiats. C'est l'effet Delaporte.

S'il en est ainsi, les colonies qui apparaissent sur une gélose ensemencée et irradiée ont pratiquement toujours une origine poly-microbienne, dont il faudrait alors tenir compte pour interpréter de nombreuses expériences d'ordre génétique.

Toutefois, l'observation microscopique directe du développement bactérien après irradiation ultra-violette ne nous a pas permis de retrouver le phénomène de restauration en amas que B. Delaporte avait décrit d'après l'examen d'images nucléaires observées sur des préparations fixées et colorées.

I. INTRODUCTION

DISCUTANT le développement des bactéries après irradiation ultra-violette, Delaporte¹ écrit: "Il est exceptionnel de rencontrer des amas dans lesquels les cellules ne sont pas ou bien toutes en cours de croissance ou bien toutes stationnaires", et "dès que l'une des cellules d'un amas commence à pousser, elle entraîne la croissance simultanée des autres cellules de l'amas". Le phénomène a été découvert par examen microscopique de préparations par impression, fixées et colorées au Giemsa après hydrolyse ménagée. Delaporte a observé ultérieurement un phénomène analogue dans des populations microbiennes traitées par les rayons X.² Si l'effet Delaporte est la règle habituelle, les colonies qui se développent à partir de suspensions denses irradiées ont, à peu près toujours, une origine pluribactérienne. Or, parmi ces colonies, il en est qui sont constituées, à l'état pratiquement pur, de mutants déterminés: chimio-résistants, auxotrophes et autres.³ Ces deux observations sont difficilement conciliables à moins qu'on admette que le germe survivant muté "transforme",⁴ en même temps qu'il les "restaure", les micro-organismes voisins. C'est pourquoi nous nous sommes proposé de rechercher l'éventualité d'une restauration par voisinage de façon plus directe. Utilisant le microscope à contraste de phase, nous avons suivi pendant plusieurs heures, directement ou par photographies en séries, la croissance sur gélose de bactéries irradiées par les rayons ultra-violets.

II. MATÉRIEL ET MÉTHODES

Toutes nos observations sont faites sur *Escherichia coli* W 1364, souche prototrophe dérivée d'*E. coli* K 12. Nous avons employé comme milieu de culture, le milieu minimal de Davis⁵ et le bouillon peptoné, éventuellement gélosés.

Les irradiations ultra-violettes sont effectuées au moyen d'une lampe à vapeur de mercure Sun Kraft dont les caractéristiques sont décrites ailleurs.⁶ L'intensité du rayonnement incident est mesurée avec le dosimètre de Latarjet et Morenne.⁷

Dans une série d'expériences, nous avons utilisé des cultures en phase de croissance exponentielle en milieu minimal ou en bouillon. Ajustées à une valeur turbidimétrique donnée par dilution en solution saline tamponnée (milieu minimal sans glucose), elles ont été irradiées pour obtenir une mortalité de 90 à 99 par cent. Les suspensions ainsi irradiées ont été ensemencées sur gélose en boîte de Petri, pour dénombrement des germes survivants et pour examen microscopique de leur développement. Dans ce but, la culture irradiée est étalée à la surface d'une couche mince de gélose solidifiée et séchée. On place les boîtes ouvertes à 37° pendant 15 minutes pour assurer l'évaporation du liquide en excès.

Dans une autre série d'expériences, pour nous rapprocher des conditions choisies par Delaporte, nous avons utilisé des cultures ayant atteint la phase stationnaire. Dans ce cas, 0,5 ml d'une culture de 48 heures en bouillon, diluée au 1/5, est réparti à la surface d'un bouillon gélosé en boîte de Petri de 8,5 cm de diamètre et exposé à un rayonnement U.V. assurant une mortalité de 99 par cent. On évalue celle-ci en comparant la densité de la culture obtenue sur la boîte irradiée, avec celle de la culture fournie par une dilution au 1/100 de la culture-mère ensemencée dans les mêmes conditions.

Les cultures destinées à l'observation microscopique sont préparées comme suit. A partir des géloses en boîte de Petri portant les germes irradiés, on découpe des blocs de 1,5 cm de côté. On les place entre lame et lamelle de verre mince, rigoureusement propres, stérilisées par flambage, et on lute à la paraffine. Les préparations sont placées sur platine chauffante maintenue à 37°. Un champ microscopique convenable est repéré et sera examiné à fréquents intervalles pendant plusieurs heures. Selon le cas, nous en avons pris des croquis successifs ou des photographies.

III. OBSERVATIONS

(1) Le développement des cultures irradiées est aussi bon sur les blocs de gélose que sur les boîtes de Petri.

(2) La survie appréciée par l'observation microscopique est en moyenne supérieure (14 pour cent) à la survie mesurée par le dénombrement des colonies (6 pour cent) (Tableau 1). Cette différence est peut-être attribuable au fait que, dans le premier

TABLEAU 1. SURVIE DE CULTURES D'*E. coli* W 1354 SOUMISES À UN RAYONNEMENT U.V. PENDANT LA PHASE DE CROISSANCE EXPONENTIELLE

Expérience No.	Survie % appréciée par	
	Observation des microcolonies*	Dénombrement des colonies†
1	10,0	0,5
2	20,0	2,6
3	3,5	1,7
4	6,6	1,7
5	7,5	1,3
6	22,5	6,0
7	13,0	8,2
8	9,7	19,0
9	23,0	15,0

* Incubation sur gélose entre lame et lamelle de verre lutées à la paraffine.

† Incubation en boîte de Petri.

cas, la survie est calculée sur un petit nombre de bactéries, alors que dans le second, elle est calculée sur le nombre de colonies visibles sur une surface beaucoup plus grande. D'autre part, il s'agit peut-être d'un effet de photoréactivation par la lumière indispensable aux observations microscopiques.

(3) Une culture de 48 heures, examinée immédiatement après son irradiation, contient des éléments dont les dimensions varient en longueur et en épaisseur. Ils sont d'habitude optiquement homogènes ou, parfois, nettement plus réfringents dans certaines portions. Des bactéries en phase de croissance exponentielle, examinées immédiatement après l'irradiation, n'ont pas toutes la même longueur mais ont la même largeur et sont optiquement homogènes.

(4) Les bactéries irradiées peuvent se comporter de quatre manières lorsqu'elles sont ensuite mises en incubation :

- (a) rester rigoureusement stationnaires ;
- (b) s'allonger en très longs filaments ;
- (c) s'allonger modérément et s'épaissir très nettement. Dans ce cas, certaines semblent se fragmenter par formation d'une sorte de vacuole.
- (d) s'allonger, puis se diviser environ toutes les 30 minutes.

Les modifications de forme surviennent progressivement après une période de 3 heures au cours de laquelle les bactéries ne subissent pas de changements appréciables. Par contre, les divisions ne commencent que 4 ou 6 heures après l'irradiation et la mise en incubation. Les divisions les plus tardives ont été observées dans les cultures irradiées après 48 heures de séjour à 37° et dans celles qui, irradiées en phase de croissance exponentielle, sont mises à incuber sur gélose minimale.

(5) Nous n'avons jamais observé de bactérie irradiée entrant en division pour la première fois après 7 heures d'incubation. Dans les cultures qui, après ce délai, séjournent 15 à 17 heures à la température du laboratoire, on retrouve inchangées les bactéries qui avaient atteint les stades a, b ou c décrits au paragraphe (4), souvent déplacées par les colonies dont on avait, la veille, observé la naissance.

(6) Ces observations nous permettent d'adopter la technique suivante : observation ou photographie des champs microscopiques de 30 en 30 minutes, depuis la troisième jusqu'à la septième heure après l'irradiation.

(7) Nous avons suivi 548 bactéries irradiées en phase de croissance exponentielle, dont 279 isolées et 269 réparties en amas de 2 à 10 germes (Tableau 2). 36 germes isolés

TABEAU 2. OBSERVATION MICROSCOPIQUE DU DÉVELOPPEMENT D'*E. coli* W 1364 APRÈS IRRADIATION U.V. PENDANT LA PHASE DE CROISSANCE EXPONENTIELLE

Répartition des bactéries observées	Nombre de cas ou se divisent :	
	1 bactérie	2 bactéries
Nombre de bactéries isolées : 279	36	—
Nombre d'amas de 2 bactéries : 66	10	3
Nombre d'amas de 3 bactéries : 21	5	4
Nombre d'amas de 4 bactéries : 7	1	1
Nombre d'amas de 5 bactéries : 2	1	—
Nombre d'amas de 8 bactéries : 1	1	—
Nombre d'amas de 9 bactéries : 2	—	1
Nombre d'amas de 10 bactéries : 1	—	—

et 36 germes faisant partie d'amas se sont divisés. 100 amas de 2 à 10 germes ont donné 27 colonies dont 18 aux dépens d'un seul germe et 9 aux dépens de 2 germes. Dans aucun cas, nous n'avons observé la multiplication de plus de 2 bactéries.

(8) Nous avons suivi 18 amas par des clichés successifs (Fig. 1 à 6). Sept d'entre eux, composés de 2 à 12 germes, provenaient de cultures irradiées en phase de croissance exponentielle (Tableau 3). Les 11 autres, composés de 2 à 74 bactéries, provenaient de

TABLEAU 3. ENREGISTREMENT PHOTOGRAPHIQUE DU DÉVELOPPEMENT D'*E. coli* W1364 APRÈS IRRADIATION U.V. PENDANT LA PHASE DE CROISSANCE EXPONENTIELLE

Nombre de germes par amas	Nombre d'amas	Nombre de germes divisés par amas
2	1	1
4	1	1
6	1	1
7	1	1
8	1	1
8	1	2
12	1	1

TABLEAU 4. ENREGISTREMENT PHOTOGRAPHIQUE DU DÉVELOPPEMENT D'*E. coli* W 1364 APRÈS IRRADIATION U.V. PENDANT LA PHASE STATIONNAIRE

Nombre de germes par amas	Nombre d'amas	Nombre de germes divisés par amas
2	1	1
3	4	1
5	1	1
7	1	1
8	1	1
20	1	1
39	1	2
74	1	1

cultures de 48 heures irradiées sur gélose (Tableau 4). Un seul germe s'est divisé dans 16 amas. Deux bactéries se sont divisées dans 2 amas contenant initialement l'un, 8, et l'autre, 39 germes.

IV. DISCUSSION ET CONCLUSIONS

Nos documents photographiques et nos observations microscopiques directes ne nous ont jamais montré le phénomène de restauration par voisinage. Dans aucun des amas que nous avons suivis, nous n'avons assisté à la division de tous les germes, mais, assez souvent, deux bactéries d'un même amas se sont divisées.

On pourrait nous objecter que l'illumination des cultures, indispensable pour l'examen microscopique et la prise des photographies, serait suffisante pour expliquer la discordance existant entre nos observations et celles de Delaporte. Il n'en est cependant rien car cette dernière observe le phénomène de restauration en amas même dans des cultures volontairement soumises à la photo-réactivation.⁸

Les résultats de Delaporte sont basés sur l'analyse statistique des images nucléaires présentées par les bactéries étudiées. Notre technique exigeant une longue observation,

portant chaque fois sur un petit nombre de germes seulement, se prête mal à la collection de renseignements en quantité suffisante pour être ensuite soumis à l'analyse statistique. Par contre, étant basée sur une observation directe des bactéries vivantes, elle nous paraît fournir des faits dignes de foi en dépit du nombre relativement minime de germes et amas observés.

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THE INTRACELLULAR DISTRIBUTION OF 5-HYDROXYTRYPTAMINE (HT; SEROTONIN) IN THE RAT'S BRAIN*

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Abstract—Differential centrifugation of homogenates of rat's brain has revealed that approximately one-fifth of the endogenous 5-hydroxytryptamine (HT; serotonin) is present in the non-particulate fraction, while two-thirds resides in the granules. The administration of reserpine causes a shift of the particulate/non-particulate (bound/free) ratio from 3.5 to 1.5, by virtue of a much greater depletion of the particulate store of HT than of the HT present in the non-particulate fraction. Iproniazid causes a rise in the HT of both fractions, but yields a ratio of 1.4. The administration of iproniazid followed by reserpine produces a result similar to that found after iproniazid alone. Thus, it would appear that iproniazid inhibits the release of HT elicited by reserpine. It is emphasised that the data presented can only be considered in a relative sense because of two difficulties inherent in the technique, which have been elucidated: (1) the granules isolated from brain lose a large portion of their HT upon re-suspension and re-centrifugation; and (2) there is a certain affinity of particulate material of brain for HT added to the suspending medium.

ONE of the assumptions basic to the concept of chemical transmission in the nervous system is that the transmitter substance exists in a pre-formed store, held in an inactive ("bound") state in the neurone. This type of storage may be concerned not only with transmitters but with neuro-secretory substances in general; and, in fact, evidence is accumulating that subcellular particles may serve as the storage sites. For example, the fine structure of the neuro-hypophysis has revealed the presence of granules believed to originate in the hypothalamic nuclei.¹ Similarly, there is evidence that the cytoplasmic granule serves as the storage site for acetylcholine,² histamine,³ and the catechol amines.⁴ Some confusion exists, however, about the intracellular localization of 5-hydroxytryptamine (serotonin; HT). Walaszek and Abood⁵ have reported that about 60 per cent of the HT in brain cells of the rat resides in the "mitochondria". Udenfriend and co-workers⁶ failed to substantiate any specific localization, having found a rather uniform distribution of HT within the cell. Later, these workers indicated that relatively little HT was to be found in mitochondria and microsomes, and that a considerable portion is associated with the nuclear residue.⁷ On the other hand, Blaschko⁸ and Baker⁹ have reported a high concentration of HT in the granules of duodenal mucosal cells of the rat. Similarly, large amounts of HT and histamine have been found associated with particulate material of solid mastocytoma of the mouse.^{10, 11}

This report is intended to confirm the finding of HT-localization in cytoplasmic

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† Wallace Laboratories Pre-doctoral Fellow in Pharmacology.

particles of the brain of the rat and to reveal certain aspects of the "binding" and release of HT.

MATERIALS AND METHODS

These studies were done on freshly excised brains (without cerebella, olfactory lobes and pituitaries) of adult male rats, sacrificed by decapitation. All operations prior to the differential centrifugation were carried out in a refrigerated room at 4°C. Homogenates (1 : 10) of brain were prepared in a glass homogenizer with an aqueous medium containing sucrose ($2.5 \times 10^{-3}\text{M}$), edathamil ("Versene") ($2 \times 10^{-3}\text{M}$), and *p*-tolylether of choline (TEC; a monoamine oxidase inhibitor) ($1.5 \times 10^{-3}\text{M}$). The actual centrifugation procedures varied in certain experiments and will be described in appropriate sections of the text; however, all were carried out in a Refrigerated Spinco Preparative Ultracentrifuge.

The HT was extracted by the method of Amin *et al.*,¹² and assayed biologically on the heart of the clam (*Venus mercenaria*).

RESULTS AND DISCUSSION

1. Complete separation of granules

Because preliminary studies¹³ in this laboratory had indicated that nuclei and microsomes are almost completely free of HT, a procedure of centrifugation was devised to yield as complete a separation as possible of the granular materials (Fig. 1). The results obtained from five representative experiments are summarized in Table 1.

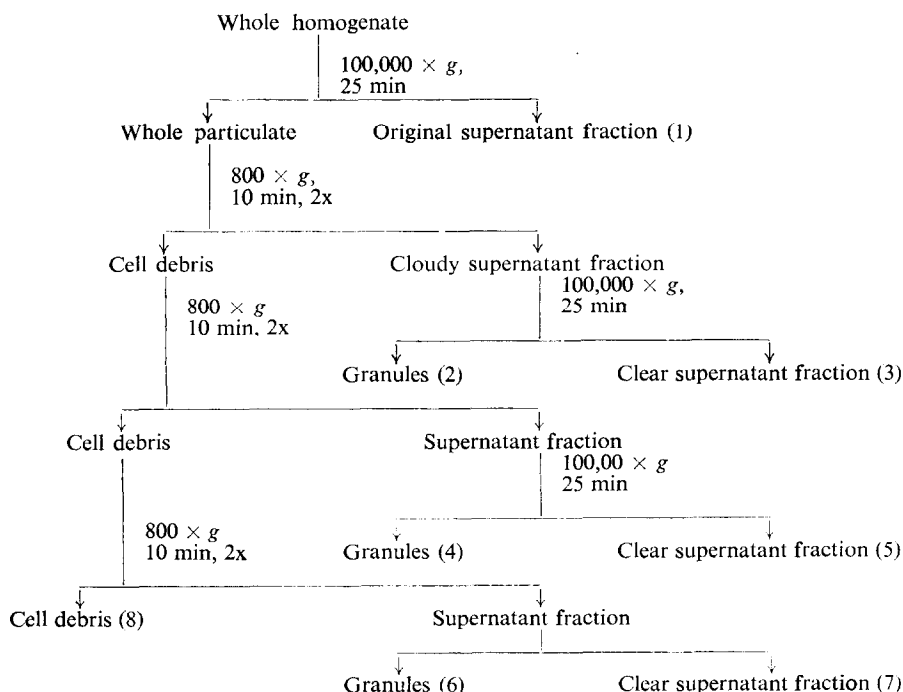


FIG. 1. Method for complete fractionation of cellular components for HT. Numbers in parentheses refer to fraction numbers found in Table 1.

The initial high speed centrifugation, in place of the traditional low-speed ($800 \times g$), for removal of cell debris, was an attempt to achieve a relatively unambiguous value for the HT in the non-particulate fraction of the cell. The supernatant fraction obtained more closely reflects the true unbound HT, which resides in the cytoplasm of the cells and, theoretically, in the interstitial fluid. It may be seen that 24 per cent of the total HT of the brain is found in this fraction under these conditions.

Another important point is made clear by these data. With each re-suspension and centrifugation of the particulate material, a considerable amount (approximately one-half) of the residual HT is leached out, despite the presence of edathamil, which

TABLE 1. DISTRIBUTION OF HT IN THE CELLS OF RAT BRAIN, SUBJECTED TO CENTRIFUGATION AS SHOWN IN FIG. 1

Fraction (numbers refer to those in Fig. 1)	Mean total HT (μg /whole brain)	Percentage of grand total
1. Original supernatant fraction	167	23.6
2. Granules	129	
4. Granules	79	
6. Granules	37	
Total	245	34.9
3. Supernatant fraction from (2)	93	
5. Supernatant fraction from (4)	103	
7. Supernatant fraction from (6)	85	
Total	281	39.9
8. Cell debris	30	
Grand total	703	

has been reported to inhibit this phenomenon.⁵ Thus, the proportion of the total HT which is found in the combined granules, i.e. approximately 35 per cent, is minimal and the true value most likely approaches twice this figure. In fact, a number of experiments which were done with two re-suspensions (instead of three) gave a result which indicated that at least 62 per cent of the total HT is found in the particulate material. These results are in good agreement with those of Baker.⁹ Microscopic examination of suspensions of these particles revealed that they were free of debris and consisted mainly of $1-4 \mu$ particles.

It is of some importance that very little HT was found associated with the final cell debris fraction, since Udenfriend *et al.*⁷ have reported 35-40 per cent in fractions precipitated by low-speed centrifugation. It is likely that these latter were contaminated with granules. However, microscopic examination of our final cell-debris fractions revealed relatively few granules. Earlier experiments demonstrated that there is a close relationship between the HT-levels of low-speed sediments and the numbers of granules contaminating them.

2. Effect of reserpine and iproniazid on the distribution of bound vs. free HT

Because of the leaching demonstrated in the above experiments, a more rapid

separation appeared to be essential to the study of the effect of reserpine (which releases HT) and iproniazid (which spares HT) on the relative distribution of free HT and its bound form(s). Therefore, a number of experiments were done by analysing the two fractions (residue = bound, and clear supernatant fluid = unbound) obtained after the first step shown in Fig. 1. Table 2 summarizes the results obtained from untreated rats and from those treated with reserpine or iproniazid, or both.

It may be seen, as before, that about 22 per cent of the HT of the brain is in the free form. It is of some interest, in this context, that 4 hr after a large dose of reserpine the percentage of free HT approximately doubled even though the absolute amount was halved. This is in agreement with the observation that reserpine impairs the

TABLE 2. EFFECT OF RESERPINE AND IPRONIAZID ON THE DISTRIBUTION OF HT IN RAT'S BRAIN
(Homogenates centrifuged at $100,000 \times g$ for 25 min)

Pre-treatment of animals	Dose (mg/kg)	Time of sacrifice (hr. after pre-treatment)	Number of animals	Mean HT-content (mg/g/whole brain)		Mean % in supernate	"Bound/free"
				Residue ("bound") Δ	Supernate ("free") Δ		
1. None	—	—	12	458	132	22	3.5
2. Reserpine	5	4	2	86 - 372	69 - 63	45	1.4
3. Iproniazid	100	12	12	667 - 209	439 + 307	39	1.5
4. (3) plus (2)	as in (2) and (3)	as in (2) and (3)	4	560 + 102	501 + 369	47	1.1

ability of cells to hold HT in a stored form.¹⁴ Moreover, it can readily be seen that reserpine depleted the granular pellet of about 80 per cent of the HT normally found there. Thus, the net effect of the action of reserpine is to reduce the ratio of bound to free HT from 3.5 to 1.4. In striking contrast to this is the finding by Weil-Malherbe and Bone¹⁵ that reserpine caused a more pronounced disappearance of catechol amines from the supernatant than from the particulate fraction of rabbit's brains. Such a difference is difficult to reconcile on the basis of the different amines involved.

Iproniazid inhibits the destruction of HT by monoamine oxidase, an enzyme which has been found in both particulate and non-particulate fraction of cells.¹⁶ Thus, iproniazid produced, as expected, a large increase in the level of HT in both components of the cell studied here. Since iproniazid is not known to alter the ability of the cell to bind HT, the much greater increase in the amount of free HT, and the similar decrease in the ratio of bound to free HT to that which is found after reserpine, might indicate:

(1) A saturation of the binding capacity of particulate sites and, therefore, a "spilling over" into the non-particulate compartment.

(2) Alternatively, non-particulate monoamine oxidase may be of greater importance in the oxidative deamination of HT than is the particulate enzyme.

The administration of reserpine following iproniazid produced a result which is closely similar to that obtained from iproniazid alone, with a large increase in both

compartments. Moreover, after ipronizid had been given, the release of HT by reserpine was not remarkable. Accordingly, these data support the suggestion of Pletscher¹⁷ that iproniazid inhibits the HT-releasing action of reserpine.

3. Binding of HT by particulate material of brain cells

In order to determine the validity of the values for free HT which have been presented in this report (Tables 1 and 2) the affinity shown by free HT for particulate material was investigated. This was done by adding HT to homogenates of brain cells (in a medium containing TEC ($1.5 \times 10^{-3}M$) and sucrose ($2.5 \times 10^{-3}M$)). These suspensions were kept in ice for 15 min prior to centrifugation at $100,000 \times g$ for 25 min. The resulting two fractions were then assayed for their content of HT. Table 3 summarizes these results.

TABLE 3. DISTRIBUTION OF HT ADDED TO HOMOGENATES OF RAT'S BRAIN
(Suspension kept at $0^\circ C$ for 15 min, then centrifuged at $100,000 \times g$ for 25 min)

Amount HT added (μg)	Number of replicates	Mean HT (μg)					
		Residue			Supernatant		
		Found	Increase	Percentage added HT	Found	Increase	Percentage added HT
0	12	0.46	—	—	0.13	—	—
2	8	0.91	0.45	23	1.13	1.00	50
4	4	1.45	0.99	22	2.28	2.15	54

The data show clearly that sub-cellular particles (and cell debris) have an affinity for HT added exogenously, and a certain capacity to adsorb it. When the amount of HT added was about $1\frac{1}{2}$ –3 times the level normally found in the whole brain of the rat (i.e. about 1.2 – $1.5 \mu g$), about 20 per cent became associated with the "high-speed residue". It is of interest that at the lower level of added HT ($2 \mu g$) the amine tended to become distributed in almost equal quantities in the two fractions. Thus, values reported above for levels of HT in non-particulate fractions are not absolute, and techniques such as this and those used by other workers in this field are productive of only relative values.

Baker⁹ has shown similarly that in homogenates of duodenal mucosa less than 30 per cent of added HT was taken up by the particulate material. On the other hand, this same worker demonstrated that suspensions of large granules from duodenal mucosal cells did not release more than 25 per cent of their HT into fresh suspending medium, as demonstrated by assay after recentrifugation at high speed.⁹ Our finding that a considerably greater portion of the HT in granules is lost might indicate that the granules of brain cells which contain HT are more fragile and possibly less specific than those isolated from duodenal cells. In this connexion, Gayet¹⁸ has recently demonstrated that granules obtained from cerebral cortex have different physical characteristics and reactivity than mitochondrial particles obtained from liver. Hebb and Whittaker² also believe that some of the particles of brain which contain acetylcholine are more fragile than others which contain it.

Another possible explanation for our observation of leaching of HT out of the granules on re-suspension lies in our centrifugation technique. It is conceivable that the first high-speed spin (Fig. 1) may injure some of the granules during the process of sedimentation and packing with larger particles.

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FURTHER STUDY OF 3-HYDROXYPHENYLDIMETHYLETHYLAMMONIUM (EDROPHONIUM) AND ITS CLOSELY RELATED ANALOGUES WITH RESPECT TO ACTIVITY AT THE NEUROMUSCULAR JUNCTION

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Abstract—(1) Using the indirectly stimulated frog sciatic nerve-sartorius muscle preparation *in vitro*, 3-hydroxyphenyldimethylethylammonium (edrophonium, Tensilon) and certain of its analogues have been studied with respect to action in augmenting muscle tension output, the time course of this augmentation effect, and anticholinergic activity.

(2) The order of effectiveness in augmenting muscle tension output was $3\text{-OH}\Phi\text{NMe}_2 > 3\text{-OH}\Phi\text{NMe}_2\text{Et} > 3\text{-OH}\Phi\text{NMe}_3 \cong 3\text{-OH}\Phi\text{NEt}_3$, at a drug concentration of 0.01 m-moles/l. At a concentration of 0.05 m-moles/l., $3\text{-OH}\Phi\text{NMe}_3$ becomes the most effective of the group in causing tension output augmentation, but this superiority is maintained for only a short period following drug application. With time, the augmentation effect of this compound is rapidly lost, and with higher concentration, muscle tension output falls below control levels. The significance of this result is discussed.

(3) The action of the phenolic hydroxyl group in the augmentation of muscle tension output was shown by demonstrating the superiority of $3\text{-OH}\Phi\text{NEt}_3$ over ΦNEt_3 .

(4) Using $3\text{-OH}\Phi\text{NEt}_3$ and $3\text{-OH}\Phi\text{NMe}_2\text{Et}$ over a concentration range of from 0.01 to 0.5 m-moles/l. and also $3\text{-OH}\Phi\text{NMe}_2\text{Et}$ over a range of from 0.01 to 0.05 m-moles/l. it was shown that the augmentation of muscle tension output is not maintained at the peak value reached. The slow fall which occurs in 1 hr amounts to 4-27 per cent of the peak tension attained. The possible significance of this result is discussed.

(5) At a concentration of 0.01 m-moles/l., $3\text{-OH}\Phi\text{NMe}_3$, $3\text{-OH}\Phi\text{NMe}_2\text{Et}$, and $3\text{-OH}\Phi\text{NMe}_2$ cause partial reversal of a neuromuscular transmission block produced by D-tubocurarine. The drug-produced improvement in transmission was maintained without loss for 1 hr, the total time of the test.

THE facilitatory action of 3-hydroxyphenyldimethylethylammonium (edrophonium, Tensilon) on neuromuscular transmission was demonstrated by Randall.¹¹ Under *in vivo* conditions he showed that neuromuscular block produced by D-tubocurarine can be reversed by edrophonium, and that edrophonium itself can produce a transient augmentation in the tension developed by skeletal muscle stimulated via a single nerve volley. Substantial evidence indicates that the facilitatory action of edrophonium depends mainly on its power to inhibit acetylcholinesterase. (4, 6, 13, 15, 16)

The factors which govern the time course of action of edrophonium have not yet been clarified. Under the conditions of Randall's¹¹ experiments *in vivo*, a single dose of edrophonium produces an augmentation of muscle tension output (often called twitch potentiation) which lasts about 7 min. This short time course of action might simply be due to a rapid reduction of drug concentration at the site of action and thus Randall's observation need not have fundamental significance as regards the mode of action of edrophonium. It would be quite another matter if, for example, the short time course resulted from inactivation of endplate membrane receptors. Clearly an

investigation of this question must begin with elimination of indirect influences by utilizing the superior control inherent in an experiment *in vitro*.

The few experiments *in vitro* reported earlier by Nastuk and Alexander⁶ served as a starting point for the work herein described. Over a range of concentration, we have studied the time course of action of edrophonium and certain of its analogues in producing augmentation or reduction of the tension developed by neurally stimulated skeletal muscle. We have also studied in a limited way the time course of anticholinergic action of edrophonium and two of its analogues.

In interpreting our results we argue that edrophonium and its related analogues possess, in varying proportion, two modes of action. The first involves a prompt inhibition of acetylcholinesterase, the second a process of activation-inactivation of end-plate membrane receptors. The argument is based on our own work and in addition, some critical results provided by others. A preliminary account of this study has been presented elsewhere.⁸

METHODS

The sciatic nerve-sartorius muscle preparation of the frog (*Rana pipiens*) was mounted for the recording of the isometric muscle tension developed subsequent to motor nerve stimulation. Details of the apparatus, method, composition of the Ringer solution, etc., have been fully described in another paper.⁹ Each nerve-muscle preparation was immersed in approximately 25 ml of fluid through which air was bubbled continuously. At 20 min intervals throughout the course of all experiments, the muscle chamber was drained and refilled with fresh solution of appropriate composition. The bath temperature was not controlled, but in individual experiments the difference between the minimum and maximum value never exceeded 3°C and generally it was less than this. The mean bath temperatures for all individual experiments were in the range 19–26°C.

Often in the investigation of the action of pharmacological agents either *in vivo* or *in vitro*, a single preparation is used for determination of the response to a range of drug concentrations or to compare the effects of a series of drugs which may or may not be related. In such experiments valid quantitation requires that the preparation make a complete recovery after each trial, i.e. return each time to the control state. In experiments where one measures the muscle tension developed by a neurally stimulated nerve-muscle preparation, the return of the twitch tension amplitude to the control value gives no assurance that the neuromuscular transmission process has also returned to control status.

To avoid the above complications, we used a fresh nerve-muscle preparation for each test of a particular compound at a given concentration. The value of such caution will become evident when results of our experiments are presented herein. To take into account the variation in drug sensitivity of individual preparations, the data has been treated statistically.

In practice, four nerve-muscle preparations were mounted and studied simultaneously, using myographs of the type fully described by Nastuk *et al.*⁹ About half of the experiments were performed with nerve-muscle preparations which were dissected in the morning, stored in Ringer solution at 4°C and used in the afternoon. It was sometimes more convenient to dissect the preparations in the evening, to store them overnight in Ringer solution at 4°C, and to use them in the morning. At first

we supposed that, despite the low temperature, the physiological properties of preparations stored overnight might show appreciable changes. However, the responses of both types of preparations to D-tubocurarine and to 3-OH Φ NMeEt₂ were indistinguishable. From these results we concluded that if any changes occur during overnight storage, they are not important in our experiments. In fact, there seemed to be some advantage in the use of overnight stored preparations in that they showed more rapid stabilization of twitch tension during the preliminary control period. Possibly the few muscle fibres which are inadvertently damaged during dissection deteriorate overnight, leaving only completely sound fibres functional.

Each nerve-muscle preparation, mounted and stretched to approximately 110 per cent of the body rest length, was maintained in Ringer solution at the start. Rectangular wave stimuli 0.1 msec in duration, and of strength $2\frac{1}{2}$ times the value required to produce a maximal twitch, were applied to the motor nerve. These values were chosen in order to avoid repetitive firing of motor axons and to insure that a liminal stimulus was applied to all axons at all times.

In setting a schedule for repetition of stimuli we had in mind the fact that these experiments would run for $2\frac{1}{2}$ hr or longer and that too frequent stimulation might fatigue the preparation. On the other hand we wanted some assurance of the consistency of the tension data collected at any particular time. With respect to the latter point we found, when taking three twitch tension readings 1 min apart, that the difference between the lowest and highest tensions was generally less than 5 per cent of the mean of all three.

The final procedure which we found to be satisfactory was to obtain, at 1 min intervals, three readings of the muscle tension developed (twitch tension). The preparation was then allowed to rest for 8 min before another set of three stimulations was repeated as indicated above. During the preliminary control period, readings taken at the ninth, tenth and eleventh minutes were averaged and plotted as representative of the 10 min point. A similar average value was plotted at the 20 min point, etc., continuing until the preparation had become stabilized. Our rule for judging achievement of stability was that for the last three 10 min periods, the difference between the highest and lowest average tensions must not exceed 5 per cent of the mean value for the three, and further that there should be no clear downward or upward trend indicated by these three 10 min periods. In all of our experiments, the preliminary control period was never shorter than 60 min.

When the experimental technique had been improved to a state where the above criteria could be consistently satisfied, five control experiments were conducted in which the nerve-muscle preparation remained in Ringer solution for a 4-5 hr period after stabilization. At the end of this extended period, the twitch tension was compared with the value obtained during the close of the preliminary stabilization period, and in individual experiments, the ending value deviated from the preliminary value by 2-6 per cent.

Experiments involving the tension augmentation effect were done as follows. After the nerve-muscle preparation was satisfactorily stabilized as described above, it was washed three times with Ringer solution containing a known concentration of drug, and then the preparation remained in solution of this composition. Tension readings were taken at the fourth, fifth and sixth minutes of the drug application period. The average of these three values was plotted at the 5 min point. Tension readings taken

at the fourteenth, fifteenth and sixteenth minutes were averaged and plotted at the 15 min point; average tension values for successive 10 min points were obtained in similar manner.

In practically all cases, the three readings which were taken at 1 min intervals did not deviate from their mean by more than 6 per cent. There were, however, a few exceptions to this statement. In obtaining data for the 5 min point, there were times when the 4 min reading was 10–30 per cent above the mean value, the 5 and 6 min readings usually being closer in agreement with each other. This effect was most evident at 0.1 and 0.5 mM drug concentrations, in particular with 3-OH Φ NMe₃. Part of the explanation for this effect is that it occurred when the muscle tension was falling rapidly and where, for this reason, readings taken 1 min apart could deviate from each other by as much as 10 per cent. Beyond this, there is some indication that, following drug application, the first stimulation of the preparation causes a diminution in the response to the next two stimulations. At later times in the drug test period, this effect becomes small and uncertain, to the point that it may often be obscured by other experimental deviations. The drug test period lasted 65 min in all cases, except three tests of 3-OH Φ NEt₃ (0.01 mM), in which the test period was terminated after 35 min because no change in tension amplitude was produced.

At the close of the drug test period, the preparation was rinsed three times with Ringer solution and allowed to remain in this medium during the terminal control period which lasted 35 min or longer. In general, the terminal control period was brought to a close when nearly equal muscle tension values were obtained for three successive 10 min intervals.

The magnitude of the changes in muscle tension which occurred during drug application were expressed in terms of the baseline value of muscle tension. By this is meant the tension which would have been measured at a specified time in the absence of drug. The baseline for the drug test period was generally obtained by interpolating between the stabilized tension values achieved during the preliminary and ending control periods. There were, however, certain exceptions to this rule. Those nerve-muscle preparations which were exposed to 0.1 and 0.5 mM 3-OH Φ NMe₃ and 3-OH Φ NEtMe₂, exhibited during the ending control period, stabilized tensions consistently above the preliminary control period tensions. In such experiments the measurements made during the preliminary control period were used to set the baseline throughout the drug test period.

We discarded as defective all experiments in which during the ending control period, the baseline stabilized at a value less than 85 per cent of the preliminary control period baseline tension.

In general, the accuracy of the tension measurements and the setting of the baselines was such that changes in muscle tension output produced during drug application were not considered significant unless they exceeded 5 per cent of the baseline tension.

Experiments concerned with anticurare action were done as follows. The preliminary treatment of the nerve-muscle preparation followed that previously described. When the preparation had been in a stabilized state for 30 min, D-tubocurarine (DTC) was applied in sufficient concentration to reduce the twitch tension appreciably. After some trials, we found that the quickest way to achieve and maintain such a partial neuromuscular block at a fixed level was first to immerse the preparation in Ringer solution containing 2 mg/l. DTC. After 8 min, this solution was replaced with Ringer

solution containing 1 mg/l. DTC. If the now depressed level of twitch tension was maintained within ± 5 per cent for 30 min., the solution in the chamber was washed out and replaced with Ringer solution containing 1 mg/l. DTC plus the drug to be tested at a concentration of 0.01 mM. At the end of 60 min exposure to the drug—containing curare—Ringer—the muscle was again returned to normal Ringer solution and maintained therein until the twitch tension had returned to the preliminary control value.

Drugs used

The following compounds were tested: 3-OH Φ NMe₃ bromide, 3-OH Φ NMe₂Et chloride, 3-OH Φ NMeEt₂ bromide, 3-OH Φ NEt₃ iodide, Φ NEt₃ iodide, and Φ OH. The concentrations ranged from 0.01 to 0.5 m-moles/l., a range in which the effects of iodide and bromide anions on the duration of the active state are apparently insignificant.³

RESULTS

Augmentation of the muscle tension output

The time course of the muscle tension augmentation effect produced by indicated concentration of four of the compounds studied is shown in Fig. 1(a-d). A similar plot for Φ NEt₃ at 0.5 m-moles/l. is also included in Fig. 1(e) for comparison with 3-OH Φ NEt₃. In all plots each point represents the average of five separate experiments. For each such experiment, the baseline tension measured during the last 30 min of the preliminary control period (stabilized state) was set at 100 units on an arbitrary tension scale. All subsequently measured tension values were plotted with respect to this scale. Drug was applied at zero time.

In individual experiments, the maximum measured value of muscle tension developed was usually that represented by the reading taken 5 or 15 min after drug application, but occasionally, as in the case of 3-OH Φ NEt₃, the peak reading was that reached in 25 min. We wished to compare the activity of the analogues with respect to both the extent of muscle tension augmentation and the maintenance of this effect with time following attainment of peak augmentation. With this in mind, the plots of muscle tension vs. time obtained from our experiments were, in effect, shifted along the time axis such as to bring the points representing maximum muscle tension developed into coincidence. The results obtained using this realignment procedure are presented in numerical form in Table 1. In this table, each value represents the average of the five individual experiments done at a specified concentration of a particular compound. For each such individual experiment the maximum measured value of muscle tension and the tension developed 30 min later were selected. The average of the peak values is listed under "maximum tension developed". To the right of this column are listed the averages of the developed tension values, in this case expressed as percentages of the corresponding maximum tension developed.

The rapidity with which the tension augmentation effect grows is shown by the plots of Fig. 1. For all concentrations of 3-OH Φ NMe₃ and for the 0.1 and 0.5 mM solutions of 3-OH Φ NMe₂Et and 3-OH Φ NMeEt₂, the maximum augmentation is that obtained at the five minute point which is the first reading taken. For the 0.01 mM and 0.5 mM solutions of 3-OH Φ NMe₂Et and 3-OH Φ NMeEt₂ and for the entire range of concentrations of 3-OH Φ NEt₃, maximum tension augmentation is reached in approximately 15 min.

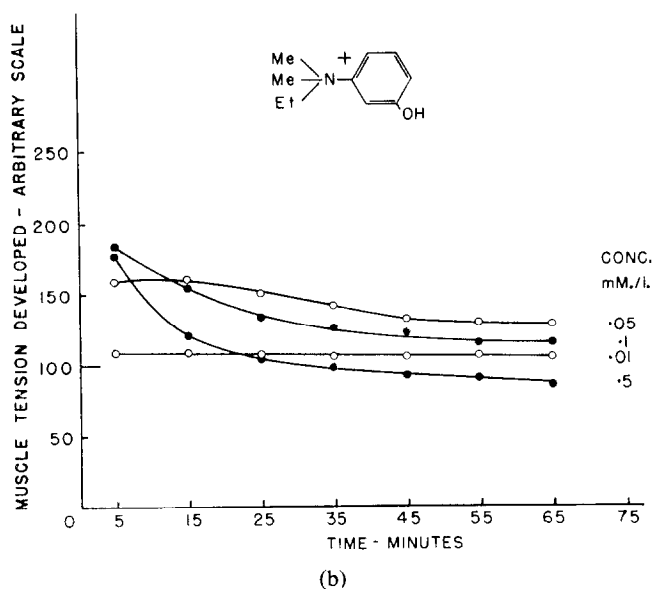
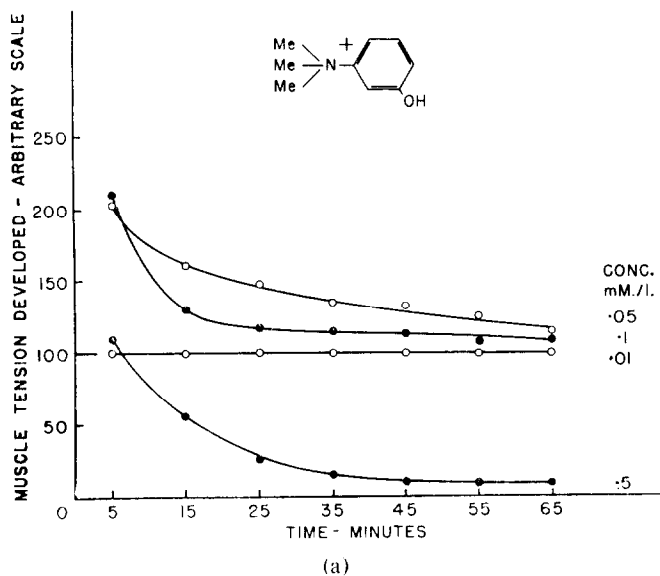
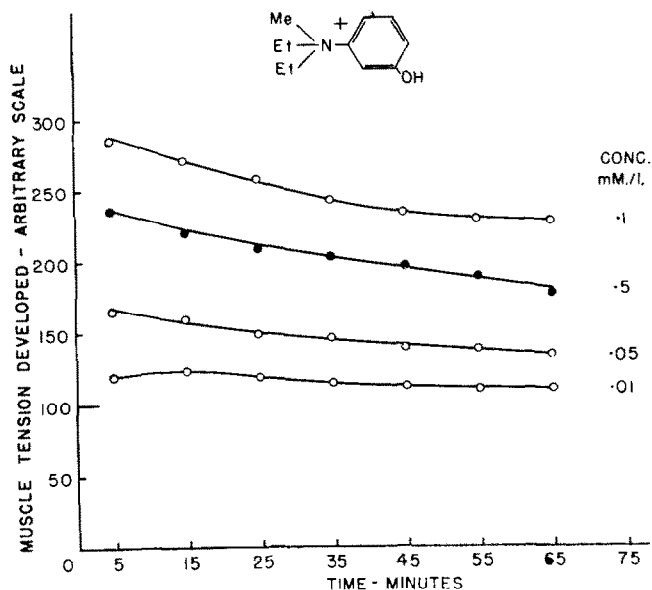
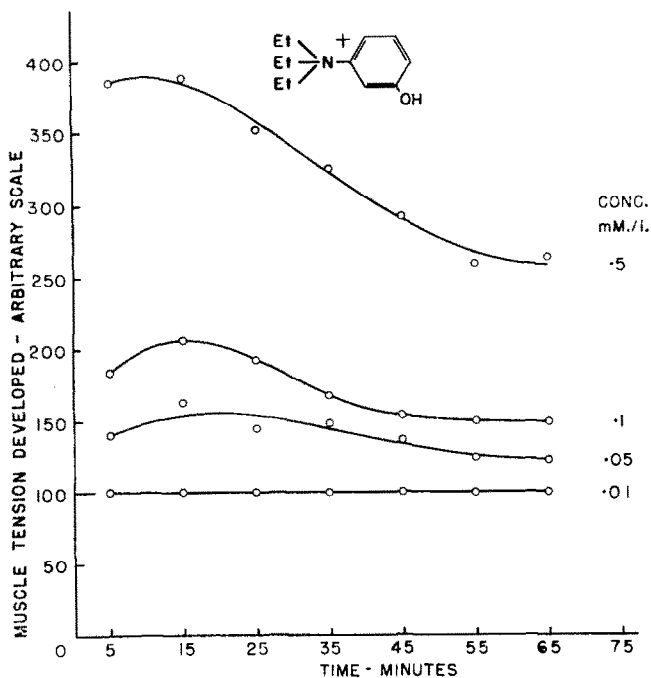


FIG. 1 (a)–(e). Showing, vs. time, the maximum muscle tension output of the indirectly stimulated frog sartorius muscle after application of the quaternary ammonium compound whose structural formula appears over each group of plots. Concentration of the applied drug in m-moles/l. is given to the right of the particular plot involved. Nerve stimulus—single rectangular pulse 0.1 msec in duration.



(c)



(d)

Muscle tension output during the stabilized state prior to drug application equals 100 units on the arbitrary tension scale. Open circles indicate that with progressive increase in drug concentration, output tension also increases. Closed circles indicate that tension output has declined below results obtained at the next lowest drug concentration tested. Each plot represents the average of five experiments. Further details in text.

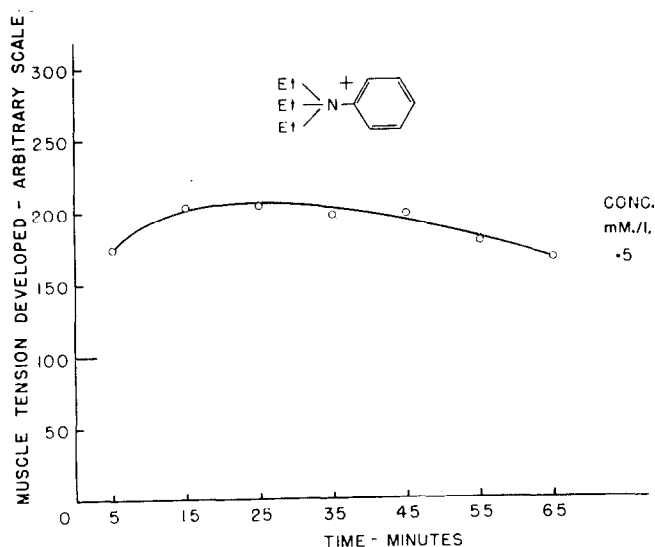


Fig. 1(e)

TABLE 1. MUSCLE TENSION OUTPUT AND ITS MAINTENANCE. EFFECT OF APPLICATION 3-HYDROXYPHENYLTRIALKYLAMMONIUM COMPOUNDS TO THE INDIRECTLY STIMULATED FROG SARTORIUS MUSCLE

Compound	0.01 m-moles/l.		0.05 m-moles/l.		0.1 m-moles/l.		0.5 m-moles/l.	
	Max. tension developed	Tension 30 min after max. (% max.)	Max. tension developed	Tension 30 min after max. (% max.)	Max. tension developed	Tension 30 min after max. (% max.)	Max. tension developed	Tension 30 min after max. (% max.)
3-OH Φ NMe ₃	100†	—	203 ± 18	67 ± 4	211 ± 27	56 ± 6	110 ± 6	14 ± 6
3-OH Φ NMe ₂ Et	111 ± 3*	96 ± 1	164 ± 19	84 ± 4	183 ± 14	70 ± 4	177 ± 17	56 ± 7
3-OH Φ NMeEt ₂	126 ± 5	91 ± 3	169 ± 18	87 ± 3	285 ± 28	85 ± 4	236 ± 17	86 ± 1
3-OH Φ NEt ₃	100†	—	167 ± 13	82 ± 4	214 ± 11	73 ± 5	396 ± 58	78 ± 3

* Standard error of mean.

† No significant change from tension value measured during "stabilized state" of preliminary control period.

Each datum represents average of five experiments.

The relative activity of the four compounds tested can be considered first in terms of the increment in muscle tension developed, and second, with respect to the maintenance of this augmentation effect.

On the first mentioned basis, the data of Table 1 show that at 0.01 m-moles/l., 3-OH Φ NMeEt₂ exhibits maximum activity. It should be mentioned that in the case of 3-OH Φ NMe₃ and 3-OH Φ NEt₃, there were some experiments at 0.01 m-moles/l. concentration in which we observed a small increase in muscle tension output which never exceeded 6 per cent of the control tension and was more frequently less than this. Since the overall accuracy of our method is estimated at 5 per cent we preferred

not to try to establish the significance of such small increments in tension. Hence at 0.01 m-moles/l., these two compounds are listed as having negligible effect.

At 0.05 m-moles/l., the mono, di and triethyl compounds are equally effective in raising the muscle tension output, but all are less effective than the trimethyl derivative. At 0.1 m-moles/l. the superiority of the trimethyl derivative is lost, the diethyl compound being again most active. Finally, at 0.5 m-moles/l. the triethyl compound becomes the most active member of the series.

Considered with respect to maintenance of the tension augmentation effect, there seems to be clear indication from Fig. 1 and Table 1 that there are two processes involved which have dissimilar time courses. For all concentrations of the tri and diethyl compounds and for the monoethyl compound at 0.01 and 0.05 m-moles/l. the reduction of the developed tension 30 min after attainment of the maximum value varies from 4 to 27 per cent of the max. developed tension. The tension loss is remarkably independent of concentration in the case of the diethyl compound. As one goes to higher concentrations, the maintenance of the tension augmentation becomes poorer with the monoethyl compound, and with the trimethyl compound this decline becomes marked. Thus, the trimethyl compound shows superior activity as regards tension augmentation only if one considers its early effect, and, in this case, the tension increment is not only rapidly lost with time, but, in fact, a blocking effect supervenes. That this reversal of activity is related with the number of methyl groups attached to the N atom can be readily appreciated from the curves shown in Fig. 1.

The preceding experiments indicate that substitution of ethyl for methyl groups attached to the nitrogen atom influences the magnitude of the tension augmentation and its maintenance with time. We also tested the effect of removal of the phenolic group on the activity of the triethyl derivative. The results shown in Fig. 1(e) indicate that this change causes a decrease in the tension augmenting activity of the triethyl compound. There is no doubt that activity depends to a considerable extent on the presence of the phenolic group, but at present we cannot specify the magnitude of its influence because in this set of five experiments, the tension augmentation produced by ΦNEt_3 varied widely, much more so than with the other compounds. The time course of action shown in these experiments also varied considerably.

We also attempted to test the direct action of the phenolic hydroxyl group by applying phenol itself to the nerve-muscle preparation at a concentration of 0.5 m-moles/l. An appreciable augmentation of muscle tension output was produced which, in two out of five experiments, was almost completely established within 5 min and which was maintained without loss for 55 min thereafter. The magnitude of the augmentation varied greatly. At one extreme there was no sign of augmentation; at the other, the tension rose to 220 per cent of the control value. Besides this variability, the phenol experiments showed another outstanding characteristic in that after removal of the phenol, in three experiments the tension did not return to the control value, but remained clearly above it at a new plateau, and in one experiment it fell far below the control value.

Experiments involving anticholinergic action

To secure additional evidence bearing on the physiological and pharmacological properties of this group of compounds, we proceeded to determine the action of certain of them in increasing the tension output of a nerve-muscle preparation in

which neuromuscular transmission was partially inhibited by D-tubocurarine. The principal point of interest in these experiments was the time course of anticurare action produced. The experiments were not designed to test critically the relative effectiveness of these compounds as anticurare agents.

Since the time course of anticurare action of the compounds was the point of principal interest, we had to set up experimental conditions in which the extent of neuromuscular block produced with D-tubocurarine remained constant with time. The results shown in Fig. 2 indicate that if nearly equal average tension readings are obtained at three successive 10 min intervals during the final application of D-tubocurarine at 1 mg/l., then a constant degree of neuromuscular block will be maintained for at least the next 60 min, the maximum period studied.

In Figs. 3(a) to 3(c) is shown the effect on muscle tension output of applying three of the compounds to a neuromuscular preparation in which a stabilized partial block was produced by D-tubocurarine. The plot of Fig. 3(c) represents the average of four experiments; those of Figs. 3(a) and (b) are the average of three experiments each. For 0.01 m-moles/l. 3-OH Φ NMe₃ and 3-OH Φ NMe₂Et the tension increase was practically complete 5 min after drug application, and this tension increment was maintained without loss for the next 60 min. For 0.01 m-moles/l. 3-OH Φ NMeEt₂, the tension increase was very largely established 5 min after its application; there was a small additional tension increment during the next 20 min, following which the tension remained constant at the new level for the next 35 min. Unfortunately, 3-OH Φ NEt₃ was not available for this series of experiments.

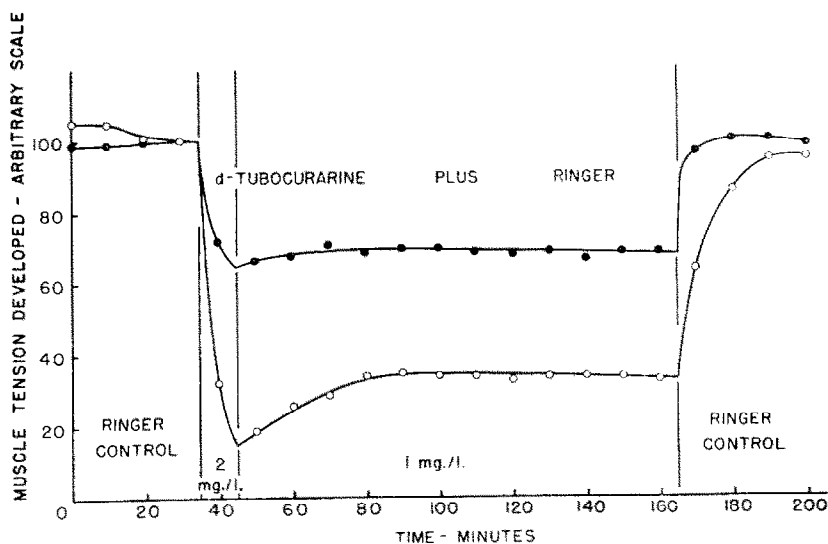


FIG. 2

FIG. 2. Two control experiments showing the muscle tension output versus time for an indirectly stimulated frog sartorius muscle. Nerve stimulus—single rectangular pulse 0.1 msec in duration. During the Ringer control period, tension output in the stabilized state is set at 100 units on an arbitrary scale. D-tubocurarine is applied at a concentration of 2 mg/l. for 8 min. Following this the D-tubocurarine concentration is reduced to 1 mg/l. The partial neuromuscular block, when stabilized, remains at a particular level for at least 60 min.

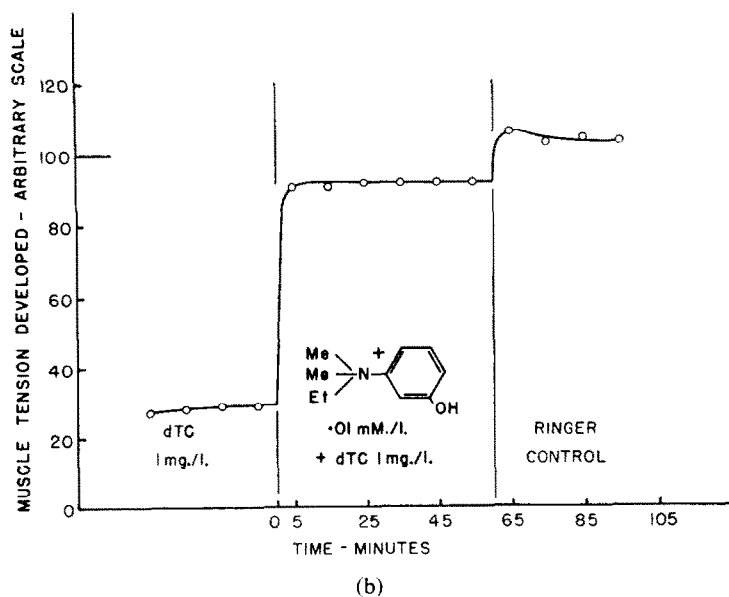
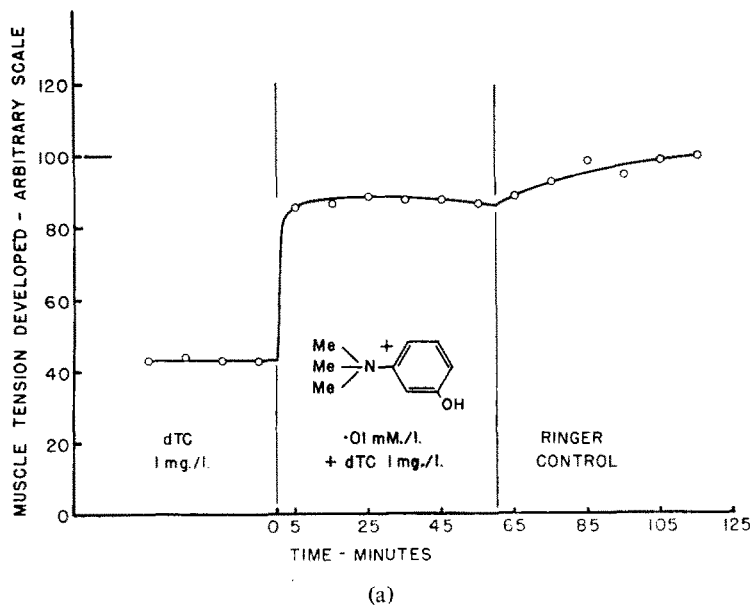


FIG. 3(a)-(c). Showing, vs. time, the muscle tension output of an indirectly stimulated frog sartorius muscle which has been treated with D-tubocurarine to produce a stable partial neuro-muscular block as shown in Fig. 2. Results show the anticholinergic action of three phenolic quaternary ammonium compounds at a concentration of 0.01 m-moles/l. Figs. 3(a) and 3(b) represent the average of three experiments; Fig. 3(c) is the average of four experiments.

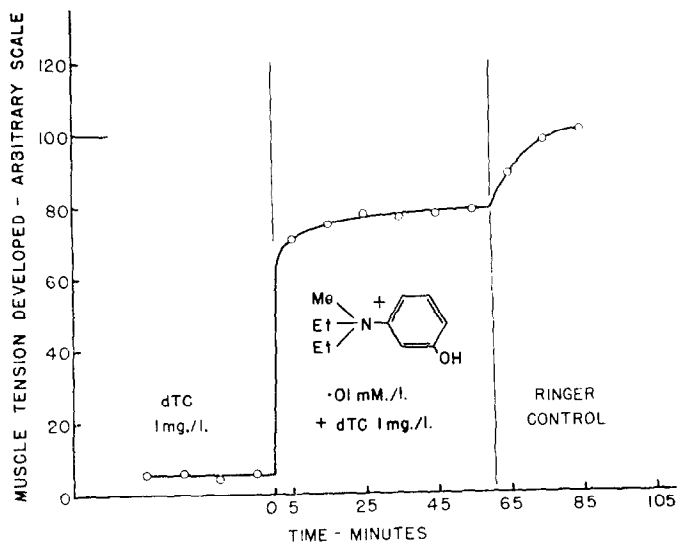


FIG. 3(c)

A final point brought out by this series of experiments is that for the compounds tested, the concentration required for appreciable anticholinergic activity is much less than that required for augmentation of the muscle tension output, indicating that the preparation in which neuromuscular transmission is partially blocked by D-tubocurarine is a more sensitive indicator of facilitation of neuromuscular transmission.

DISCUSSION

It is now clear that under *in vitro* conditions, wherein drug concentration is constant, edrophonium produces an augmentation of muscle tension output and anticholinergic action which are long-sustained compared with that seen *in vivo*. Therefore, *in vivo*, important but indirect processes must operate which reduce the drug concentration at the site of action.

Muscle tension augmentation

Even *in vitro*, the augmentation of muscle tension output produced by edrophonium and its analogues, falls with time. Our data indicate that there can be an early-produced rapid decline in augmentation. In addition there is a more generally present slow fall in tension augmentation.

In an attempt to explain both the magnitude and the time course of the tension augmentation produced by this series of quaternary ammonium compounds, we offer the following: Consistent with the information now available one may state that for these compounds, the primary site of action lies on or near the muscle endplate membrane. At this site, these drugs exhibit in varying degrees, two modes of action. In what we call mode one action, the drug molecule combines rapidly and reversibly with acetylcholinesterase thereby inhibiting the enzyme. In mode two, the drug molecule combines with endplate membrane receptor sites causing either (a) receptor

activation followed by inactivation, or (b) inhibition without activation. Process (a) would result at first in a reduction in membrane potential at the endplate, which in turn would facilitate neuromuscular transmission, perhaps leading to production of repetitive action potentials in the muscle fibre. With time, the endplate membrane receptors would become desensitized to molecules like acetylcholine, i.e. receptor activators, the resting membrane potential would return to normal values, and neuromuscular transmission would be inhibited. Process (b) is a simple inhibition involving no receptor activation or change in resting membrane potential.

That the members of this series of drugs act as anticholinesterases in physiological concentrations is indicated by the following evidence.

Smith *et al.*,¹³ using enzyme preparations from electric eel, bovine erythrocytes and frog rectus muscle have determined the anticholinesterase activity of the four members of this series. All were found to have appreciable inhibitory activity, although somewhat below that of neostigmine. The order of decreasing anticholinesterase activity was $3\text{-OH}\Phi\text{NMeEt}_2 > 3\text{-OH}\Phi\text{NMe}_2\text{Et} > 3\text{-OH}\Phi\text{NMe}_3 \cong 3\text{-OH}\Phi\text{NEt}_3$, with the last two compounds alternating in position, depending on the source of the enzyme used. Additional evidence bearing on the appreciable anticholinesterase activity of these quaternary ammonium compounds has been provided by Wilson¹⁵ and Wilson and Quan.¹⁶ It is worth mentioning once again that the rapid onset of activity of these compounds at the neuromuscular junction is not in itself a critical argument against the dependence of such activity on acetylcholinesterase inhibition.¹⁵

Using an entirely different approach, Katz and Thesleff⁴ showed that at the muscle endplate membrane, $3\text{-OH}\Phi\text{NMe}_2\text{Et}$ (edrophonium) potentiates the depolarizing action of acetylcholine, but has no such effect on the depolarizing action of carbachol, a compound not destroyed by acetylcholinesterase. These investigators showed further that the above mentioned potentiation of acetylcholine by edrophonium was lost if the experiment was carried out using a muscle which had been pretreated with sufficient neostigmine to inhibit accessible cholinesterase. They concluded that the principal mode of action of edrophonium is by acetylcholinesterase inhibition.

Experiments of Nastuk and Alexander⁶ link augmentation of muscle tension output with the anticholinesterase activity of edrophonium and provide evidence which explains the nature of the tension augmentation effect. They showed that subsequent to the arrival of a single motor nerve action potential at the telodendrites, there is produced an unusually prolonged endplate potential such that there is generated not one, but a burst of action potentials in the muscle fibre. Therefore, instead of the expected twitch, a brief tetanus results and for this reason, the tension output is raised above the twitch level.

As regards the magnitude of the muscle tension augmentation, in our present experiments (Table 1) results obtained at a low drug concentration (0.01 m-moles/l.) run parallel with anticholinesterase activity as determined by Smith *et al.*¹³ However, this relationship does not hold at higher drug concentrations at which mode two activity apparently becomes more effective.

The evidence which supports the argument, that among these compounds there is a second mode of activity which involves reaction of drug with endplate membrane receptors, is the following.

Direct measurements⁶ using intracellular microelectrodes show that $3\text{-OH}\Phi\text{NMe}_3$ at 0.01 m-moles/l. causes a reduction of endplate membrane potential averaging

8mV; 3-OH Φ Me₂Et at 1 m-moles/l. similarly causes a reduction averaging 12 mV. From these data it was concluded that, as a depolarizing agent, the trimethyl compound is six to seven times more active than the dimethylethyl derivative. It is important to note that this ratio is comparable to that found by Holton and Ing² who used stimulation of the rectus abdominis muscle as a gauge to study the effect of substituting an ethyl for a methyl group in acetylcholine. Katz and Thesleff⁴ have also shown that a depolarization of the endplate membrane amounting to a few mV follows the application of 3-OH Φ Me₂Et. Their experiments were not designed to fix the extracellular drug concentration, and hence the result can only be given in qualitative terms.

There are a number of papers in the literature in which the stimulating action of edrophonium and its analogues has been described. Using contracture of frog rectus abdominis muscle, Smith *et al.*¹³ found 3-OH Φ Me₃ to be the most potent compound of the series; progressive substitution of ethyl for methyl groups resulted in progressive diminution in activity. Expressed with reference to the contracture producing action of acetylcholine, these investigators showed that the relative order of effectiveness was 3-OH Φ Me₃ = 1, 3-OH Φ Me₂Et = 1/7, 3-OH Φ MeEt₂ = 1/400, and 3-OH Φ Net₃ = 1/360. The relative weakness of the diethylmethyl derivative compared with the dimethylethyl compound (edrophonium) is especially noteworthy. Results similar to those of Smith *et al.*,¹³ were obtained by Pelikan *et al.*,¹⁰ who studied production of contracture in avian muscle. Finally Randall¹¹ showed that application of edrophonium and certain of its analogues can produce contracture of chronically denervated mammalian muscle. Here again the activity is greatest with 3-OH Φ Me₃ and drops progressively as ethyl groups are substituted for methyl groups.

From the preceding evidence it seems safe to conclude that at some concentration, 3-OH Φ Me₃ will sufficiently depolarize the muscle endplate membrane such that propagated action potentials are initiated and thus muscle contraction occurs via direct stimulation. On this basis, therefore, immediately after its application, this compound would be expected to act synergistically with acetylcholine in neuromuscular transmission. From the work of Thesleff¹⁴ and also Katz and Thesleff⁵ it is known that the sustained application of certain membrane depolarizing agents, such as acetylcholine, with time leads to a desensitization and inactivation of endplate membrane chemoreceptors. Therefore, with respect to neuromuscular transmission, 3-OH Φ Me₃ and acetylcholine are synergists at the start, but with time the added drug produces receptor inactivation and thereby inhibition of transmission possibly to the point of blocking it completely. A synergistic relationship between edrophonium and acetylcholine which evolves into inhibition has been demonstrated by Katz and Thesleff.⁴

On the basis of the preceding, it would be predicted that the purest example of mode one activity should be shown by 3-OH Φ Net₃ and that as methyl groups are substituted for ethyl groups around the nitrogen atom an increase in mode two activity should occur. The data of Table I is consistent with such a prediction. When the concentration is raised to 0.05 m-moles/l., 3-OH Φ Me₃ becomes the most effective agent in producing an early augmentation of tension output, but as time elapses this relative increase in effectiveness is rapidly lost. At still higher concentration (0.5 m-moles/l.) the trimethyl compound rapidly inhibits and blocks neuromuscular transmission. Edrophonium like the trimethyl compound also shows mode two activity

but only at higher concentrations. Weak mode two action is shown by 3-OH Φ NMeEt₂ at 0.5 m-moles/l.

It is also worth noting that our results, obtained using the amphibian preparation, parallel those obtained in the mammal by Riker *et al.*¹² These investigators measured the peak augmentation of an isometric muscle contraction and using various doses of 3-OH Φ NMe₃ and 3-OH Φ NEt₃ they obtained results which we believe can be interpreted on the basis of the two modes of action outlined above.

In the preceding discussion we have argued that via activation of endplate membrane receptors (mode two activity), there is produced an additional early increase in tension augmentation but, as might be expected, this event is followed by a rapid decline in tension augmentation which under some circumstances may evolve into a neuromuscular block. Nonetheless with compounds such as 3-OH Φ NEt₃ and 3-OH Φ NMeEt₂, in which the power to activate endplate membrane receptors is weak or absent, there still appears a slow decline in tension augmentation. This slow decline in tension augmentation could conceivably represent a gradual deterioration of neuromuscular transmission in the *in vitro* preparation. A mild deterioration of this sort could exist undetected in our control experiments since the appreciable safety margin in the neuromuscular transmission process would act as a buffer against loss in tension output. By comparison, the maintenance of repetitive firing at the neuromuscular junction, in the presence of an anticholinesterase, could be a vulnerable process in which various minor interfering sources might play a detectable part. Their presence would then be signalled by a slow fall in tension augmentation.

While the above explanation may be correct, it does not seem to be an entirely sufficient one. From a recent study *in vitro*,⁷ we now know that the tension augmentation effect produced by neostigmine and its diethyl analogue (at 0.005 m-moles/l.) is, by comparison, appreciably better maintained over long periods of time, the rate of loss of the tension augmentation being less than 7 per cent/hr.

We have no additional explanation for this residual phase of the slow decline of tension augmentation as seen in our presently reported results and we are forced to let the matter rest until new evidence is available.

Anticurare action

As might be expected, the concentration of drug required to reverse a partial block in neuromuscular transmission was found to be much less than that required to cause augmentation of the muscle tension output. It is of salient importance, that all three compounds tested maintained anticurare action without any loss over the entire test period. Hence transience of anticurare action as seen *in vivo* must be the result of factors other than drug action *per se*.

Results of our anticurare experiments could be taken to mean that all members of the series which were tested are about equal in effectiveness. While in a rough way this is true, it must be kept in mind that our anticurare experiments were not designed to measure critically the relative effectiveness of the compounds tested, and they cannot be considered as providing such information.

It would seem that the anticurare action exhibited by these drugs depends primarily on anticholinesterase activity. Direct receptor activation is probably not an important consideration under the conditions of these experiments first, because the drug concentration was low, second, the D-tubocurarine present, by combining with

endplate membrane receptors, inhibits receptor activation by the later added drug. Since at the low concentration of D-tubocurarine used there is little inhibitory effect on acetylcholinesterase,¹ this enzyme will not be appreciably hindered from combining with any of the 3-hydroxyphenyltrialkylammonium compounds added subsequently. Therefore the drug-produced enzyme inhibition, and the consequences thereof will occur as usual.

Additional structure activity relationships

Molecular complementariness between acetylcholinesterase and certain phenolic quaternary ammonium and related compounds has been studied by Wilson and Quan.¹⁶ In the light of their work, inhibition of the esterase by ΦNMe_3 can be interpreted in terms of combination of the anionic site with the positively charged quaternary nitrogen. The augmentation of muscle tension output, shown in our work to be produced by ΦNEt_3 (Fig. 1(c)), can be interpreted on the same basis.

Based on their results obtained using acetylcholinesterase, Wilson and Quan¹⁶ state that the relative binding strength of 3-OH ΦNMe_3 is 120 times greater than that for ΦNMe_3 and forty times greater than that for 3-Me ΦNMe_3 . The difference is suggested as being due to formation of a hydrogen bond between the phenolic group and, most probably, the esteratic site of the enzyme. Our own experiments were not designed to test critically the relative effectiveness of 3-OH ΦNEt_3 and ΦNEt_3 , but we can state that the phenolic compound is certainly the more active of the two.

The fact that the hydroxy group plays an important part in the inhibition of acetylcholinesterase led us to test the action of phenol itself. As mentioned earlier, phenol was found to cause augmentation of muscle tension output, but the irreversible effects of the compound make an interpretation of its action hazardous. The physiological activity of phenol deserves more investigation.

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THE CONVERSION OF ^{14}C -LABELLED TRYPTOPHAN TO 5-HYDROXYTRYPTAMINE BY NEOPLASTIC MAST CELLS*

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Abstract—The conversion of tryptophan-3- ^{14}C to 5-hydroxytryptamine (5-HT) by neoplastic mouse mast cells in culture has been described. The labelled 5-HT formed was identified by paper chromatography and by recrystallization with carrier 5-HT. The results indicate that in these cells free tryptophan is the major, if not the sole, precursor of 5-HT. In preliminary experiments, this conversion was demonstrated in cell-free homogenates of mast cell tumours.

INTRODUCTION

IN RECENT years, 5-hydroxytryptamine (5-HT; serotonin), because of its presence in the tissues of a large variety of animal species and its striking pharmacological activities,¹ has been the subject of increasing interest, and its physiological functions, especially its role in the central nervous system, are still under intensive investigation.

Evidence has been presented that in mammalian tissues this amine is formed from tryptophan in two enzymatic steps: an aromatic hydroxylation, resulting in the intermediate 5-hydroxytryptophan (5-HTP), followed by a decarboxylation giving rise to 5-HT.² The decarboxylation of 5-hydroxytryptophan has been studied extensively, and the enzyme catalysing this reaction, shown to occur in many tissues,³ has been partially purified and characterized.^{4, 5} On the other hand, relatively little is known about the mechanism of the hydroxylation, the rate-limiting step in the overall conversion of tryptophan to 5-HT in mammalian systems; this transformation has been studied in the rabbit by Udenfriend *et al.*,⁶ using tryptophan-2- ^{14}C . The hydroxylation has been shown to occur in the toad⁶ and in *Chromobacterium violaceum*.⁷ Dalglish and Dutton⁸ have suggested that the argentaffin cells of the gastro-intestinal mucosa are the major site of tryptophan hydroxylation, a reaction which could not be demonstrated in rat liver.

The observation that mast cells of rats and mice, in contrast to those of many other species, contain large amounts of 5-HT,⁹ and the availability of two transplantable mouse mastocytomas,^{10, 11} have offered new possibilities of studying the formation of 5-HT. The cells of one of these experimental neoplasms (line P-815, developed by Dunn

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and Potter.¹⁰) when grown in culture in the presence of undialysed horse serum, actively formed this amine, and sublines characterized by a markedly increased production of 5-HT were obtained by selection and cloning procedures (Schindler *et al.*¹²) In the present paper, the conversion of ¹⁴C-labelled tryptophan to 5-HT by these neoplastic mast cells multiplying *in vitro* is described, and preliminary studies on the formation of 5-HT from tryptophan-3-¹⁴C by homogenates of mast cell tumours are reported.

EXPERIMENTAL

(a) Cell culture studies

A genetically pure mast cell line (designated P-815-X₁), derived from the ascitic mast cell tumour P-815¹⁰ by a selection and two consecutive cloning procedures¹² and characterized by an unusually high rate of 5-HT-production, was used in the experiments. The culture techniques employed and the medium have been described in detail previously.¹² Of the cells, 2×10^6 were suspended in 5 ml of medium containing, per ml, 8 μ g of DL-tryptophan-3-¹⁴C (instead of 4 μ g of L-tryptophan), and incubated at 37°C. After 24 hr the medium was renewed by centrifugation of the cell suspension, aspiration of the supernatant fluid and resuspension of the cells in 10 ml of fresh medium containing the same concentration of labelled tryptophan (a total addition of 294 μ -moles of L-tryptophan). After a second 24 hr incubation period the cell suspension was centrifuged, the medium removed, and the 5-HT extracted from the cells; for extraction, 95 per cent acetone (40 ml) was used as described by Amin *et al.*¹³ In parallel cultures with non-radioactive DL-tryptophan, the amount of 5-HT formed by the mast cells during the 48 hr period of incubation was determined from the increase in cell number and the 5-HT content of the cells before and after the incubation. 5-HT was measured by bioassay on the heart of the clam, *Venus mercenaria*.¹⁴ The values obtained in the two experiments described in Table 1 were: 0.014 μ moles of 5-HT per 2×10^6 cells and 0.018 μ moles of 5-HT per 2×10^6 cells, respectively, before the incubation; 0.045 μ moles of 5-HT per 8.0×10^6 cells and 0.058 μ moles of 5-HT per 7.3×10^6 cells, respectively, at the end of the incubation.

In order to separate the radioactivity of tryptophan from that of 5-HT, the material extracted by acetone was chromatographed on Whatman paper (No. 3), using as a solvent system a mixture of *n*-butanol and 25 per cent methylamine (8 : 3). In the chromatogram, two radioactive peaks with the *R_f*-values of tryptophan (0.39) and of 5-HT (0.65) were observed. The material with the *R_f* of 5-HT was eluted from the paper with formic acid (0.1 N) and the eluate was evaporated to dryness. Subsequently, non-radioactive 5-hydroxytryptamine-creatinine-sulphate (100 mg) was added and recrystallizations from water-acetone were carried out, as described by Rapport *et al.*,¹⁵ until constant specific activity (number of counts per unit of optical density at 275 m μ in acid solution) was obtained. From the specific activity of the recrystallized material the total number of counts incorporated into 5-HT was calculated and expressed as percentage of the radioactivity added as L-tryptophan-3-¹⁴C during the incubation. Similarly, the proportion of L-tryptophan which was converted to 5-HT, as measured by the bioassays, was determined. Since D-tryptophan presumably did not serve as a substrate for the formation of 5-HT, the amount and the radioactivity of L-tryptophan (e.g. half of that of DL-tryptophan-3-¹⁴C) was used as the basis (100 per cent) of these calculations.

In Table 1 the results obtained in two experiments are summarized; there is good agreement between the rate of conversion as measured by the bioassays and that calculated from the radioactivity found in 5-HT.

TABLE 1. THE QUANTITATIVE UTILIZATION OF DL-TRYPTOPHAN-3- ^{14}C FOR THE FORMATION OF 5-HT BY NEOPLASTIC MURINE MAST CELLS IN CULTURE

	Experiment 1		Experiment 2	
	(μmoles)	(c.p.m. $\times 10^6$)	(μmoles)	(c.p.m. $\times 10^6$)
Total L-tryptophan-3- ^{14}C added	0.294	3.2	0.294	3.2
5-HT formed according to bioassay	0.031	—	0.040	—
Total radioactivity of isolated 5-HT (carrier)	—	0.28	—	0.39
Conversion of L-tryptophan to 5-HT	($\%$)		($\%$)	
(a) by bioassay	10.6		13.6	
(b) by 5-HT radioactivity	8.7		12.2	
Specific activity of tryptophan used	c.p.m. μmole)		c.p.m. μmole)	
Calculated* specific activity of 5-HT formed	10.8×10^6		10.8×10^6	
	9.0×10^6		9.8×10^6	

$$* \frac{0.28 \times 10^6 \text{ c.p.m.}}{0.031 \mu\text{mole}} = 9.0 \times 10^6 \text{ c.p.m. } \mu\text{mole}$$

$$\frac{0.39 \times 10^6 \text{ c.p.m.}}{0.040 \mu\text{mole}} = 9.8 \times 10^6 \text{ c.p.m. } \mu\text{mole.}$$

(b) *Experiments with cell-free homogenates*

The cell line used (P-815- X_1) was the same as that employed in the culture experiments. However, in this case solid mast cell tumours were produced by subcutaneous injections of a suspension of mast cells into (AKR $\times$ DBA/2) F_1 hybrid mice. Approximately 2 weeks later the animals were killed and the tumours were excised and homogenized at 0°C in potassium phosphate buffer (0.01 M; pH 7.4), using 2 ml per gram of tissue. After centrifugation of the homogenate at approximately $950 \times g$ for 5 min, the supernatant fluid was decanted and an aliquot was examined in a haemocytometer and found to be free of undisrupted cells (less than 1000 cells per ml).

The following compounds were added to the cell-free supernatant fluid in the concentrations indicated:

	($\mu\text{g/ml}$)
Nicotinamide	120
DPN	200
Pyridoxal phosphate	2
<i>p</i> -Tolyl ether of choline chloride	
(as an inhibitor of monoamine oxidase)	30
DL-tryptophan-3- ^{14}C	8

The reaction mixture (volume: approximately 2.5 ml) in a 10 ml beaker was shaken for 30 min in air, at 37°C , on a Dubnoff shaking incubator. Subsequently, an extrac-

tion with 95 per cent acetone¹³ was carried out and the extracted material was chromatographed on Whatman paper (No. 3) with *n*-butanol–glacial acetic acid–water (4 : 1 : 5).⁹ The 5-HT (R_f , 0.47) was separated from an unidentified metabolite (R_f , 0.75) of tryptophan, not 5-HTP, but separation of 5-HT from tryptophan was not obtained in this system. The material characterized by the R_f of tryptophan and of 5-HT was eluted from the paper with formic acid (0.1 N) and chromatographed on Whatman paper (No. 1) with *n*-butanol–25 per cent methylamine (8 : 3) as solvent. By parallel chromatography of non-radioactive 5-HT the R_f -value of the amine was determined (u.v.-absorption and fluorescence). The radioactivity of the chromatogram at different R_f -values was measured following division of the paper into strips of 1 cm width, each of which was eluted with formic acid (2 ml; 0.1 N). Figure 1 shows that a high accumulation of radioactivity at the R_f of tryptophan and a second radioactive peak coinciding with the R_f of 5-HT were obtained. In two experiments, this second peak represented 0.3 and 0.15 per cent, respectively, of the total radioactivity added to the incubation mixture as L-tryptophan. In a parallel experiment with an homogenate which was heated for 30 min at 100°C prior to the addition of nicotinamide, DPN, etc., accumulation of radioactivity at the R_f of 5-HT was not observed.

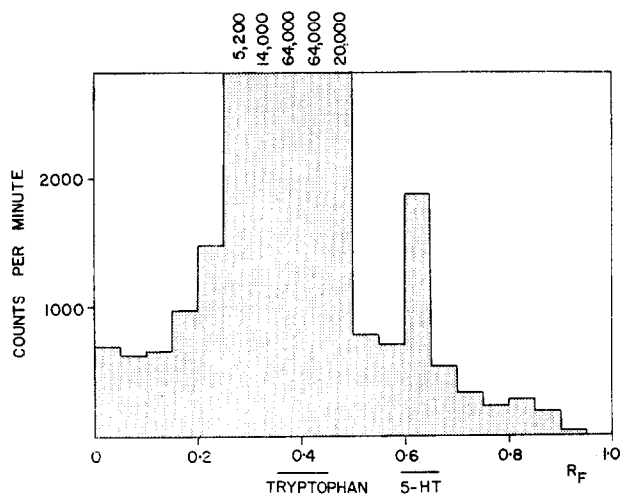


FIG. 1. The chromatographic identification of 5-HT formed from tryptophan-3-¹⁴C by a cell-free homogenate of mast cell tumours. Solvent: butanol–25 per cent methylamine (8 : 3). The R_f of 5-HT was determined by parallel chromatography of non-radioactive 5-HT.

DISCUSSION

The data of Table 1 demonstrate that neoplastic mast cells in culture (line P-815-X₁) efficiently use tryptophan as a precursor for the formation of 5-HT. Two reasons can be cited to account for the slightly lower specific activity of the 5-HT which was formed during the incubation, as compared to that of the precursor, tryptophan. One, the labelled tryptophan probably was diluted to some extent by tryptophan present in the undialysed horse serum in the medium;¹² and two, a small quantity of 5-HT may have been destroyed, prior to the addition of carrier 5-HT, by oxidation during paper chromatography with the alkaline solvent (butanol–methylamine). It appears, however, that these neoplastic mast cells form 5-HT mainly or exclusively

from free rather than the protein-bound tryptophan of the medium. Since the capacity to form 5-HT from tryptophan is quite specific for mast cells and cannot be demonstrated for other cell types (although it has been suggested⁸ that the argentaffin cells of the gastro-intestinal mucosa may carry out this conversion), the findings indicate that in the culture system used for these mast cells¹² this biochemical function is maintained without evidence of de-differentiation.

The relatively low rate of conversion of tryptophan-3- ^{14}C to 5-HT in cell-free homogenates of solid mast cell tumours is not surprising in view of the well-known lability of other enzyme systems which catalyse aromatic hydroxylations.¹⁶⁻²⁰ Because of the limited conversion in these preliminary experiments identification of 5-HT synthesized by the homogenate was restricted to paper chromatography with an alkaline as well as an acid solvent system. However, this chromatographic evidence for the formation of 5-HT is supported by the fact that the homogenate was prepared from a tissue the cells of which are known to carry out this reaction efficiently. In fact, had cell line P-815- X_1 (characterized by a production of 5-HT which may be up to twenty times that of the parent line) not been available, the quantities of 5-HT formed by an homogenate might have been too small for detection. It should be emphasized that the low rate of conversion of tryptophan to 5-HT by the homogenate cannot be attributed to the presence of a few undisrupted cells, since such contamination was excluded by microscopic examination prior to the incubation.

These preliminary studies of the enzymatic transformation of tryptophan into 5-HT in the cell-free system should serve as the basis for a further characterization of the enzymes responsible. It is hoped that purification procedures and possibly the appropriate use of 5-HTP as a trapping agent and of a suitable decarboxylase inhibitor will help to increase our knowledge of the properties and the mode of action of tryptophan-5-hydroxylase.

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STUDIES ON FLUORINATED PYRIMIDINES

VII—THE DEGRADATIVE PATHWAY*†

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Abstract—A study of the metabolic degradation of 5-fluorouracil has been carried out in mice and human cancer patients. 5-Fluoroorotic acid is not significantly degraded by mice. The first steps of 5-fluorouracil degradation, the conversion to dihydrofluorouracil and α -fluoro- β -ureidopropionic acid, are exactly analogous to those of uracil. In human subjects there is extensive conversion of FU to urea. Most normal tissues, except spleen, degrade FU *in vivo*. The liver is probably the main site of degradation, and studies with high-speed supernatant fractions have been carried out. Two mouse tumours have been shown to be incapable of degrading the drug. In human beings there is more extensive catabolism when the drug is administered orally than by intravenous injection. There is a higher concentration of radioactivity in a human malignant carcinoma and intestinal mucosa than in the normal tissues and a benign fibro-adenoma. The presence of α -fluoro- β -guanidopropionic acid as a metabolite of 5-fluorouracil has been suggested.

INTRODUCTION

IN CONJUNCTION with the previous studies reported from this laboratory on the metabolism and mechanism of action¹⁻⁴ of the series of fluorinated pyrimidines^{5, 6} that are potent inhibitors of the growth of several transplanted tumours^{1, 7, 8} and certain human solid tumours,⁹ an investigation of the metabolic degradation of these compounds in mice and human cancer patients has been undertaken.

The catabolism of uracil has been the subject of thorough investigation for several years in the laboratories of the Finks¹⁰⁻¹² Canellakis,^{13, 14} Grisolia,¹⁵⁻¹⁷ Rutman,¹⁸ Fritzon,^{19, 20} and Reichard.²¹ These studies have been carried out in rats *in vivo*, in micro-organisms, and in slices, homogenates, and enzyme extracts of rat liver and all agree in demonstrating that uracil catabolism follows the pathway: uracil (U) $\rightarrow$ dihydrouracil (DHU) $\rightarrow$ β -ureidopropionic acid (UPA) $\rightarrow$ CO₂ + NH₃ + β -alanine $\rightarrow$ acetic acid. Some urea has also been detected.¹² In the present study, using compounds labelled with ¹⁴C in the 2-carbon, we have shown that 5-fluoroorotic acid (FO) is not appreciably catabolized by mice, that 5-fluorouracil (FU) in mice and man follows a pathway exactly analogous to that of uracil for at least the first two steps (FU $\rightarrow$ FDHU $\rightarrow$ FUPA) with the direct production of variable quantities of urea, and that 5-fluorouridine (FUR) and 5-fluoro-2'-deoxyuridine (FUDR) are cleaved to FU, which is then degraded further.

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† Paper VI—*Proc. Soc. Exp. Biol. Med.* **97**, 470 (1958).

MATERIALS AND METHODS

Labelled compounds. The syntheses of 5-fluorouracil-2-¹⁴C and 5-fluoroorotic acid-2-¹⁴C have been reported previously.² We are greatly indebted to Dr. B. A. Koechlin of Hoffmann-La Roche, Inc, for converting the labelled fluorouracil into 5-fluorouridine-2-¹⁴C and 5-fluoro-2'-deoxyuridine-2-¹⁴C by the methods described by Duschinsky *et al.*⁶ Uracil-2-¹⁴C was obtained from New England Nuclear Corp., on allocation from the U.S. Atomic Energy Commission.

Animals. Female Swiss mice were obtained from Taconic Farms, Inc, Germantown, N.Y. and were studied without tumours and with 8-day-old transplants of Sarcoma-180 or Ehrlich ascites tumours.⁸

Human patients. Patients with advanced metastatic cancer were used in studies of the metabolism of the labelled drugs. The compounds were administered and frequent urine samples were collected either by voiding or catheterization. Respiratory carbon dioxide samples were collected in Douglas bags, precipitated and counted as barium carbonate. The total radioactivity expired was calculated from the integrated specific activity curve and the basal metabolic rate of the patient. Surgical samples were obtained freshly from the operating room and were minced finely and plated and dried for specific activity determinations and otherwise processed by previously published methods.² The clinical work on these patients was carried out by Dr. A. R. Curreri, Dr. F. J. Ansfield, Dr. E. L. Weston, and Dr. J. Strawitz of the University of Wisconsin Hospitals, and we are greatly indebted to them for this assistance.

Carrier compounds. 5-Fluorodihydrouracil and α -fluoro- β -ureidopropionic acid (FUPA) were synthesized by Dr. R. Duschinsky of Hoffmann-La Roche, Inc.²² and were generously made available to us.

Incubations in vitro. Ehrlich ascites cells were incubated for 30 min at 37°C in the medium described by Harbers and Heidelberger.²³ For each flask 0.25 ml of packed cells were suspended in 1 ml of ascites serum, 1 ml of a 10 per cent extract of chick embryo extract (Difco), and 0.5 ml of an isotonic saline solution containing 0.5 μ mole of the labelled precursor, penicillin, streptomycin and glucose in a final concentration of 3 mg/ml. At the end of the incubation in air the cells were centrifuged and rapidly washed (twice) in the cold with isotonic saline and suspended in isotonic saline; perchloric acid was added to a concentration of 4 per cent. This acid-soluble extract was neutralized with KOH, the KClO₄ removed by centrifugation, and the neutralized extract was processed chromatographically as described below.

Mouse liver high speed supernatant fraction. A high speed supernatant fraction was prepared from mouse liver essentially as described by Stone and Potter for rat liver.²⁴ Six mice were sacrificed by decapitation and their livers (9 g), were combined and homogenized in a medium (36 ml) of the following composition: 0.13 M KCl, 0.01 M MgCl₂, 0.015 M KH₂PO₄; the pH was adjusted to 7.2 with KOH. The homogenate was centrifuged at 100,000 $\times g$ for 1 hr in the preparative Spinco ultracentrifuge, and the supernatant fluid was used for the experiments.

For the incubation, Erlenmeyer flasks were used for the isolation of metabolites. Duplicate incubations at 37°C for 1 hr in air were carried out in Warburg flasks with

KOH in the centre wells for trapping of radioactive CO_2 , which was precipitated and counted as BaCO_3 . Each flask contained 4 ml of the liver supernatant solution and 1 ml of a solution containing 40 μmoles of potassium phosphoglycerate (from the barium salt), 30 μmoles of MgCl_2 , 20 μmoles of KH_2PO_4 , 3 μmoles of triphosphopyridine nucleotide (TPN), 120 μmoles of nicotinamide, and 1 μmole of the labelled precursor. The reaction was stopped by pouring the mixture into alcohol to give a final concentration of 80 per cent.

Ion-exchange chromatographic procedures. Some initial fractionation procedures were carried out with charcoal (Darco G-60) columns, in which pyrimidine bases, nucleotides, and nucleosides were adsorbed. Urea was not adsorbed, but variable results were obtained with FUPA, which was sometimes retained on the column and at other times was not. The ion-exchange chromatographic separations were carried out on 1×20 cm columns of Dowex-1-formate. The samples were collected on the automatic fraction collector in 10-ml volumes. In a typical run, the samples were adjusted to pH 11, which causes the pyrimidine bases and nucleosides to be retained on the column. The column was washed with 100 ml of water to remove the urea. Then they were eluted with 50 ml of 0.05 M formic acid, which removed the pyrimidine bases or nucleosides. Subsequently, a gradient elution was performed with 500 ml of water in the mixing flask and 4 M formic acid in the reservoir. Under these conditions, 5-fluorocytidylic acid was obtained in tubes 25–35, FUPA in tubes 41–53, FUMP in tubes 87–100, and 5-fluoroorotic acid (FO) in tubes 105–119. Nucleoside di- and tri-phosphates were then eluted with 0.5 M HCl. Individual tubes were plated to locate the radioactive peaks, and the tubes in the individual peaks were combined and the total radioactivity was determined.

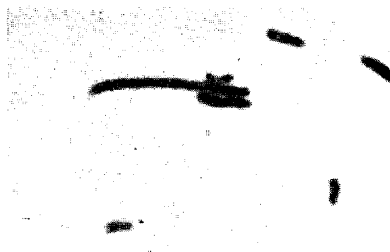
In experiments not involving FO a simplified hand column procedure was developed. Columns of Dowex-1-formate (1×10 cm) were used, the sample was put on at pH 11, and 10-ml fractions were collected. Water, 50 ml, was used to elute urea, followed by 50 ml of 0.05 M formic acid to remove bases and nucleosides. The FUPA was eluted with 100 ml of 1.5 M formic acid, FUMP with 50 ml of 2.5 M formic acid, and nucleoside di- and tri-phosphates with 0.5 M HCl. The radioactivity was located as described above.

Paper chromatographic separations. The numerous solvent systems described by Fink *et al.*^{11, 12} were explored. The most useful one was the upper phase of the following mixture: ethyl acetate : 90 per cent formic acid : water (60 : 5 : 35V/V/V). The R_f values in the above descending system were $\text{FU} = 0.57$, $\text{FUR} = 0.35$, and $\text{FUDR} = 0.51$. In the system *n*-butanol : water : formic acid (77 : 13 : 10) FUPA had an R_f of 0.48, while that of UPA was 0.55. The paper chromatographic systems were mostly used for the confirmation of the separations obtained by ion-exchange chromatography.

Identification of metabolites. Radioactivity present as urea was identified by actual isolation from human urine and crystallization to constant specific activity following initial ion-exchange fractionation. In other experiments, carrier urea was added and urea nitrate and urea oxalate were prepared and crystallized repeatedly until constant specific activity was obtained. The identification was confirmed by the liberation of radioactive CO_2 from samples with urea carrier by treatment with urease. It was also



1



2



3



4



5



6

PLATE 1

Développement d'*Escherichia coli* W 1364 après exposition à un rayonnement ultra-violet de 7,2 ergs par mm² par sec pendant 105 sec.

Les figures 1 à 6 sont des photographies successives d'un même groupe initial de 3 bactéries, prises respectivement: 1, 4, 5, 5½, 6¼, et 6½ heures après l'irradiation. On peut constater qu'un seul des trois individus se divise sans entraîner la multiplication de ses deux voisins. Incubation à 37°C.

Observation au microscope à contraste de phase. × 5610.

determined in urine by direct paper chromatography in the system ethyl acetate : formic acid : water (60 : 5 : 35), $R_f = 0.43$; and in the system *n*-butanol : water : formic acid (77 : 13 : 10), $R_f = 0.28$. The urea spot was located by spraying with a solution containing 100 ml of ethanol, 10 ml of concentrated HCl and 1 g of *p*-dimethylaminobenzaldehyde. A yellow spot was obtained after drying.¹¹

The radioactivity present as UPA and FUPA was identified by paper chromatography in the presence of the appropriate carriers, following ion-exchange fractionation. The spots were located by spraying with the same reagent as used for urea.

Dihydrofluorouracil is very labile to alkali, which readily hydrolyses it to FUPA. Hence, it was not determined as such in most experiments, since it is hydrolysed at the pH of urine. However, it was characterized in the homogenate experiments, which were determined by the addition of ethanol. Following the addition of DHFU as carrier, the radioactivity followed the carrier in the butanol : water : formic acid paper chromatographic system with an R_r of 0.41. This material was eluted, hydrolysed with mild alkali and rechromatographed to give FUPA.

Radioactivity present as 5-fluorocytidylic acid was identified by hydrolysis with 60 per cent perchloric acid of the characteristic peak from ion-exchange chromatography. The solution was neutralized with KOH, the $KClO_4$ was removed, and carrier 5-fluorocytosine was added. This was then chromatographed on Dowex-1-chloride as described for the purification of FU,² and all the radioactivity was contained in the 5-fluorocytosine peak.

RESULTS

Metabolism of FU in cancer patients

Throughout this programme of research it has seemed desirable to carry out metabolism experiments on the series of fluorinated pyrimidines both in animals and in human cancer patients receiving the drugs for therapeutic evaluation. Such studies in human patients of blood levels, excretion, tissue distribution, and metabolism might serve to guide the clinicians towards more effective regimens of therapy. We have previously reported data of this kind on a patient suffering from carcinoma of the lung.² Because we have consistently observed⁷ that these compounds were considerably less inhibitory towards the growth of transplanted rat and mouse tumours when given by stomach tube than by other routes or administration, clinical trials of 5-fluorouracil have thus far been carried out primarily by intravenous injection.⁹ Nevertheless, it was desirable to compare the blood level, excretion, and tissue distribution of the drug given intravenously and orally. Accordingly a female patient with recurrent benign fibroadenomas following mastectomy was studied. She was given, intravenously per kilogram, 15 mg (i.e. 955 mg; 138 μ C) of 5-fluorouracil-2-¹⁴C and frequent blood, urine and respiratory carbon dioxide samples were collected. Twelve hours later biopsy specimens were obtained of liver, tumour, and surrounding muscle, skin and fat. One week later she returned and received the same dose of labelled drug orally. A second liver biopsy was refused, but the other specimens were obtained.

The blood levels of radioactivity following both modes of administration are shown in Fig. 1. When the specific activity of the intravenous experiment was extrapolated to zero-time a quantity was obtained consistent with equal distribution throughout the body water. The blood level of radioactivity decreased rapidly for the first 2 hr and then more slowly. The figures represent drug equivalents of radioactivity. It will be

noted that following oral administration the drug is absorbed rapidly, since the maximum blood level was reached 20 min after the drug was swallowed in pineapple juice. After the first hour the blood levels were not significantly different with both routes of administration. The radioactivity was essentially all present in the plasma. Of the radioactivity in the blood 1 hr after the intravenous injection, 25 per cent was present as unchanged drug.

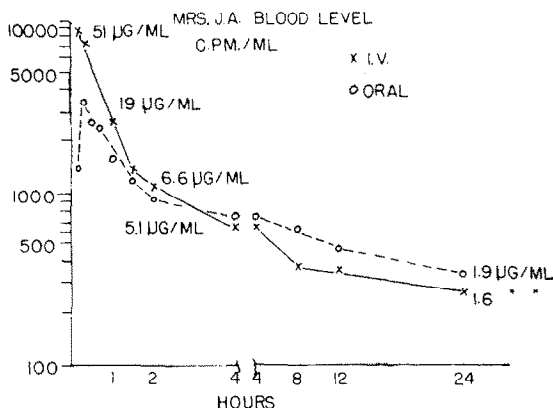


FIG. 1. The blood level of radioactivity in a cancer patient following intravenous and oral administration of 5-fluorouracil-2-¹⁴C. Counts/min per ml of plasma vs. time.

The data on excretion of radioactivity in the respiratory CO₂ and urine together with the specific activity of the tissues obtained on biopsy are given in Fig. 2. There was insignificant foecal elimination of radioactivity. Although the shape of the specific activity curves of the respiratory carbon dioxide was similar after both methods of administration, a higher specific activity was reached in the oral experiment. This is reflected in a higher total degradation of 5-fluorouracil to carbon dioxide, when the compound was administered orally than following its intravenous use. There was somewhat less urinary excretion when the drug was given orally. However, at 24 hr 90 per cent of the dose was excreted following oral administration as compared with 75 per cent by the intravenous route. This greater retention following parenteral administration is reflected in the somewhat higher specific activity of the tissue samples. In these benign lesions the specific activity was considerably less than that of the liver, in contrast to the previously reported higher specific activity of a human lung carcinoma than liver.² In order to gain further information on the correlation of drug uptake and malignancy of human tumours, labelled FU was given intravenously, 4 hr before surgery to a female patient with a malignant carcinoma of the coecum. The specific activities of various tissue samples are shown in Table 1. It was observed that both the tumour and normal intestinal mucosa had specific activities higher than that of liver, and considerably greater than those of the other tissue samples taken. This is in agreement with the findings of selective localization of radioactivity previously reported in Sarcoma-180 tumour of mice, although in the human subject there was a relatively greater concentration of label in the intestinal mucosa than in the mouse.² This finding explains the high toxicity of 5-fluorouracil to the gastro-intestinal tract of cancer patients,⁹ and taken together with the information on the other two patients,

shows that there is a higher specific activity in malignant tumours than liver, whereas benign tumours have a lower specific activity than liver.

Urinary excretion of metabolites

Because the fluorinated pyrimidines studied were labelled in the ureido(2)-carbon it was possible to study the degradation of the compounds as far as α -fluoro- β -ureidopropionic acid (FUPA). This has been shown to be cleaved to urea and carbon dioxide, but the fate of the remainder of the molecule could not be determined because it no longer contained the labelled carbon atom. Although in normal metabolic experiments the specific activity of the urea is known to be in equilibrium with that of respiratory carbon dioxide, the fact that in urine samples from patients treated with labelled FU the specific activity of the lowest sample of urea was fifty times higher than the most active sample of respiratory CO_2 indicates that there is a direct cleavage of the drug to urea.

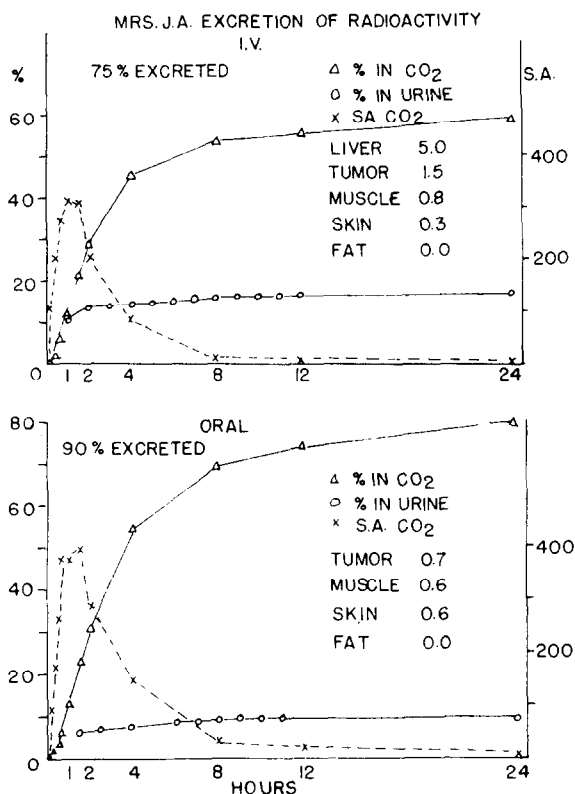


FIG. 2. The specific and total radioactivities of respiratory carbon dioxide and the total radioactivity in urine of the patient following oral and intravenous administration of FU-2- ^{14}C . The figures after the tissue samples are in counts/min per mg dry weight of tissue.

Urine specimens obtained 12 hr after intraperitoneal injection of tumour-bearing mice with FU-2- ^{14}C and FO-2- ^{14}C were fractionated as described under Methods. The results of typical experiments are given in Table 2. It will be noted that 5-fluorourotic acid is excreted in the urine largely unchanged. However, there is an appreciable

TABLE 1. HUMAN PATIENT RADIOACTIVITY DISTRIBUTION
FU
(14 mg/kg, 232 μ c 4-5 hr)

	Dry tissue (counts/min per mg)	RNA (counts/min per μ g)
Tumour	8.8	288
Intestinal mucosa	9.0	257
Intestinal muscle	3.2	
Skeletal muscle	1.5	
Liver	6.7	
Lymph node	4.4	
Bone marrow and blood	2.4	
Skin	2.5	
Fat	2.4	

TABLE 2. TOTAL COUNT/MIN AND PERCENTAGES OF ADMINISTERED FLUOROPYRIMIDINES
IN FRACTIONS OF MOUSE URINES 12 HR AFTER DOSAGE

Fractions	Precursor			
	FU-2- ¹⁴ C		FO-2- ¹⁴ C	
	(counts/min)	(%)	(counts/min)	(%)
Sample fractionated	7,500,000	100	1,100,000	100
Urea	50,000	0.7	0	0
FU	2,850,000	38	165,000	15
FCMP	67,000	0.9	0	0
FUPA	4,650,000	62	36,000	3.3
FUMP	10,000	0.1		
Unknown*			100,000	9.1
FO			680,000	62
Polyphosphates	20,000	0.3	2700	0.2
Total recovery	7,650,000	102	984,000	89

*An aliquot of this material lost 90 per cent of its radioactivity on hydrolysis with 15 per cent KOH for 12 hr.

conversion to FU. Although there was little FUPA present in these urines (3.3 per cent) there was 9.1 per cent of an unknown metabolite. The fact that the latter lost practically all its radioactivity after prolonged alkaline hydrolysis suggests that it is a ureido-acid different from FUPA, and considerably more acidic, as determined by its ion-exchange chromatographic behaviour. Thus we tentatively suggest that this metabolite might be α -fluoro- α^1 -ureido-succinic acid, the compound obtained by ring-opening of fluoroorotic acid, analogous to the FUPA obtained by cleavage of fluorouracil. In the case of FU, 38 per cent was excreted unchanged and 62 per cent was present as FUPA. Only minor amounts were converted into urinary FCMP, FUMP, urea and polyphosphates.

Similar experiments were carried out on mouse urines following administration of 5-fluorouridine (FUR) and 5-fluoro-2'-deoxyuridine (FUDR) and the results of the fractionations are shown in Table 3. In the case of FUR there was an appreciable conversion to urea, and 11 per cent was excreted unchanged, with no evidence of conversion to FU. There was extensive metabolism to FUPA (69 per cent). With

TABLE 3. TOTAL COUNTS/MIN AND PERCENTAGES OF ADMINISTERED MATERIAL IN MOUSE URINE FRACTIONS FOLLOWING FLUORONUCLEOSIDE TREATMENT

Fraction	Precursor			
	FUR-2- ¹⁴ C (24 hr)		FUDR-2- ¹⁴ C (4 hr)	
	(counts/min)	(%)	(counts/min)	(%)
Sample fractionated	382,000	100	10,000,000	100
Urea	20,000	5.2	50,000	0.5
FU	0	0	320,000	3.2
FUR	38,000	11	0	0
FUDR	0	0	5,010,000	50
FCMP	0	0	25,000	0.3
FUPA	265,000	69	3,900,000	39
FUMP	2000	0.5	20,000	0.2
Polyphosphates	1000	0.3	3000	0.03
Total recovery	326,000	85.4	9,340,000	93.4

FUDR, 50 per cent of the radioactivity in the urine was present as unchanged FUDR with some conversion to FU. FUPA accounted for 39 per cent of the label in the urine. Because of the possibility that the pyrimidine ring might have been cleaved before the loss of the ribose or deoxyribose, the FUPA fractions in these urines were examined by paper chromatography. However, in both solvent systems the radioactive spots migrated as FUPA. Therefore, it appears that these nucleosides were converted into the pyrimidine base before ring cleavage.

In order to obtain more extensive information as to the course of the metabolism of FU in man, the urine samples collected from the lung cancer patient, whose overall excretion was given in a previous paper,² were fractionated; the results are shown in Table 4. The greatest amount of radioactivity was excreted during the first 45 min after the intravenous injection of labelled FU; this was preponderantly unchanged drug.

TABLE 4. URINARY EXCRETION IN HUMAN PATIENT GIVEN 14 MG OF 5-FLUOROURACIL PER KILOGRAM (177 μ c)

Time of Collection	Total ¹⁴ C (%)	FU (%)	FUPA (%)	Urea (%)
0-45 min	12.5	91.5	8	0.5
45 min-1½ hr	2.5	23	70	7
1½-3½ hr	1.7	1.5	84	13.5
3½-5 hr	0.81	0	74	26
5-7 hr	0.55	0	55	45
7-8 hr	0.34	0	50	50
8-10 hr	0.26	0	41	59
10-12 hr	0.19	0	30	70
12-24 hr	0.54	0	28	71
24-48 hr	0.34	0	16	84
Total	19.8			

However, the amount of FU present in the urine samples rapidly decreased, so that no unchanged drug was detectable after 3½ hr. The proportion of FUPA in the urines increased to a maximum in the 3½ hr sample and then decreased with time. The metabolic conversion to urea increased with time, so that in the 24-48 hr specimen, 84 per

cent of the radioactivity was present as urea. Evidently the human being converts 5-fluorouracil more extensively to urea than does the mouse. Thus, it appears that shortly after injection of the drug, when the blood level is high, there is a spillage of the drug through the kidneys, and as time passes there is an increased excretion of metabolic degradation products.

When 5-fluoro-2'-deoxyuridine became available for clinical trial, some labelled compound was administered to the first patient that received the drug. Insufficient radioactive compound was available for tissue studies. The blood-level and CO₂-excretion curves seemed similar to those obtained with FU and will not be given here. However, the pattern of excretion in catheterized urine samples was studied and the data are given in Table 5. It was found that the greatest amount of radioactivity was excreted in the 10–20 min interval after intravenous injection. The total amount eliminated in the urine was comparable to that found with 5-fluorouracil itself. As in

TABLE 5. PERCENTAGE RECOVERY AND FRACTIONATION IN CARCINOMA PATIENT RECEIVING FUDR-2-¹⁴C INTRAVENOUSLY (8.2 mg/kg, 565 mg, 70,000,000 counts/min total)

Time interval	Dose (%)	Urea (%)	FU (%)	FUDR (%)	FUPA (%)
0–10 min	1.3	0.2	26	72	2.4
10–20 min	3.9	0.2	28	67	2.9
20–30 min	2.3	0.3	30	66	16.7
30–40 min	1.9	0.6	23	57	18.4
40–50 min	1.6	1.2	13.5	41	44.5
50–60 min	0.4	2.1	11.5	38.5	48
Total 0–1 hr	11.4		FU + FUDR		
1–2 hr	3.3	3.8	48		48
2–4 hr	3.0	6.4	38		55
4–8 hr	2.5	31.6	27		42
8–12 hr	1.0	70.5	8.8		21
12–24 hr	1.6	80	2		18
Total 0–24 hr	22.8				

the case of FU there was considerable excretion of unchanged drug at first, the FUPA reached a maximum at 2–4 hr, and the proportion of label present as urea steadily increased. When the 0.05 M formic acid fraction was separated by paper chromatography into FU and FUDR (no FUR being found), a constant ratio of about three parts of FUDR to one part of FU was found at all time intervals studied. Suitable control experiments with labelled FUDR showed that there was no decomposition to FU on standing in urine or during the isolation procedure. Therefore, this finding represents drug metabolism, although its significance is not clear.

Experiments in vitro

Because of the numerous demonstrations of the degradation of uracil in soluble systems derived from rat liver,^{13–17, 21} we decided to study the catabolism of FU under similar conditions. The system that we employed was similar to that of Stone and Potter,²⁴ and consisted of the high speed supernatant fraction from mouse liver fortified with phosphoglyceric acid and nicotinamide. Incubations were carried out in

Erlenmeyer flasks for 1 hr, except that duplicate runs were carried out in Warburg flasks for the collection of respiratory carbon dioxide. In the same experiments we compared equimolar amounts of FU-2-¹⁴C and uracil-2-¹⁴C. The results are shown in Table 6. It is evident that both substrates were extensively degraded. However, this system converted uracil predominantly to carbon dioxide, whereas the metabolism of FU was carried mostly to the FUPA stage. Perhaps the enzymes that degrade FUPA to carbon dioxide and urea are located elsewhere in the cell. In these experiments, in agreement with previous work,²⁵ there was relatively little conversion of uracil into nucleotides. It seemed likely, based on the analogy^{13, 18} of the situation with uracil, which is extensively catabolized in liver and anabolized in tumours, that the selective localization of radioactivity from FU in tumours^{2, 3} may have resulted from an inability of the tumour to degrade the drug. Accordingly, we investigated the acid-soluble fraction following incubation of Ehrlich ascites cells with FU-2-¹⁴C. The results given in Table 7 show clearly that there is no conversion of the drug into urea, and that only 2.5 per cent of the radioactivity in the sample was present as FUPA. On the other hand, there was an extensive conversion (45 per cent) into nucleotides. Thus, it is evident that these tumour cells have little, if any, capacity to degrade 5-fluorouracil and instead convert it efficiently into nucleotides.

TABLE 6. TOTAL COUNTS/MIN AND PERCENTAGES IN METABOLITES OF 5-FLUOROURACIL AND URACIL PRODUCED BY HIGH-SPEED SUPERNATANT FRACTIONS OF MOUSE LIVER

Fraction	Precursor			
	FU-2- ¹⁴ C		Uracil-2- ¹⁴ C	
	(count/min)	(%)	(count/min)	(%)
Sample fractionated	2,500,000	100	2,300,000	100
CO ₂	100,000	4	1,380,000	60
Urea	30,000	1.2	3200	0.1
DHFU	20,000	0.8		
FU	25,000	1.0		
FUR	50,000	2.0		
U + UR			8000	0.3
FCMP	30,000	1.2		
FUPA	1,920,000	77		
UPA			97,000	4.2
FUMP	33,000	1.3		
UMP			11,000	0.5
Polyphosphates	12,000	0.5	5600	0.3
Total recovery	2,220,000	89	1,505,000	66

TABLE 7. TOTAL COUNTS/MIN AND PERCENTAGES AS METABOLITES OF 5-FLUOROURACIL IN THE ACID-SOLUBLE FRACTION OF EHRlich ASCITES CELLS FOLLOWING *in vitro* INCUBATION

	(counts/min)	(%)
Acid-soluble fraction	80,000	100
Urea	0	0
FU + FUR	22,000	27
FCMP	3000	3.8
FUPA	2000	2.5
FUMP	16,000	20
Polyphosphates	20,000	25
Total recovery	63,000	79

Metabolism in vivo of FU in other tissues

In order to determine the amount of metabolic degradation products of 5-fluorouracil found in the tissues of normal mice 1 hr after intraperitoneal injection, the experiment described in Table 8 was performed. The extent of degradation is exemplified by the percentage of urea and FUPA found in the tissues, and the anabolism is measured by the amount of radioactivity present as FUMP and the di- and tri-phosphates. In this experiment, FCMP was not separated from the FU-fraction. It is evident that high amounts of degradation products were found in all the tissues examined except the spleen, and the greatest conversion of FU into nucleotides was observed in the spleen and small intestines. With this information at hand it became desirable to determine the amount of degradation products found in the liver and tumours of mice bearing Sarcoma-180 following injection of labelled FU. The data given in Table 9 show that appreciable amounts of degradation products were present in the tumour, although they were found to a considerably lesser extent than in the liver. In this experiment the FU- and FCMP-fractions were separated by paper chromatography, and a third component with an R_f of 0.73 was obtained from the tumour. The tumour showed a higher conversion into nucleotides than did the liver.

TABLE 8. THE DEGRADATION OF 5-FLUOROURACIL IN THE TISSUES OF NORMAL MICE *in vivo* 1 HR AFTER INTRAPERITONEAL INJECTION OF 25 mg/kg PER CENT OF RADIOACTIVITY IN COMPOUNDS

Tissue	Total (counts/min)	Urea	FU	FUPA	FUMP	di and tri-P's	Recovery
Liver	115,000	12.5	32.5	45	0.9	3.5	94.5
Kidney	53,400	6.0	12	59	0.6	2.0	79.6
Small intestine	38,400	2.5	16	44	11	11	84.5
Brain	9760	9	9	76	0	1	95
Lungs	8180	4	0	44	0	5	53
Spleen	7220	8	25	17	2	13	65
Heart	3530	7	1.8	70	0	6	84.8

In this experiment, FCMP was included in the FU fraction.

TABLE 9. THE DEGRADATION OF 5-FLUOROURACIL 1 HR AFTER INJECTION OF 25 mg/kg INTO MICE BEARING SARCOMA-180 OR EHRlich ASCITES CARCINOMA PERCENTAGE OF RADIOACTIVITY IN COMPOUNDS

Tissue	Total counts/min	Urea	FU	R_f 0.73	FUPA	FUMP	FCMP	di and tri-P's	Recovery
Liver	315,000	15	19	0	77	1	6	5	123
Sarcoma-180	87,900	3.8	61	2	20	6	0	8	101
Plasma	10,700	8.0	16.0		80	13		0.1	117
Ascites fluid	971,000	1.5	15.5	5.1	62.1	3.5	3.5	0.6	91
Ascites cells	291,000	3.9	14.4	0.7	17.3	16.4	2.7	28.0	83

In view of the fact that degradation had not been observed in Ehrlich ascites cells incubated *in vitro*, but was found to at least a small extent in Sarcoma-180 *in vivo*, an experiment was carried out in which FU was injected intraperitoneally into

mice bearing the Ehrlich ascites carcinoma. After 1 hr a sample of blood was obtained by heart puncture and the ascitic material was collected. The cells were obtained by centrifugation, were washed twice rapidly in the cold with isotonic saline, and the washes were combined with the ascites fluid. Acid-soluble extracts were prepared and chromatographed from the blood plasma, ascites fluid, and ascites cells. The results are shown in Table 9. Of the radioactivity in the plasma, 80 per cent was present as FUPA. In the ascites fluid there was considerable FUPA but small amounts of nucleotides. On the other hand, in the ascites cells there were relatively little FUPA and large quantities of nucleotides. With respect to degradation of FU by the cells, the question here is whether the FUPA in the cells was derived from the FUPA in the ascites fluid or vice versa. In this experiment the volumes of the ascites fluid and cells were 9.0 and 4.5 ml, respectively. When the concentrations of FUPA are calculated in the fluid and cells they are 66,800 and 11,000 counts/min per ml, respectively. Similarly, the concentration of FUPA in the blood plasma is 8600 counts/min per ml. These concentrations lead to the firm conclusion that the FUPA in the cells came from the ascites fluid. If the reverse had been true, and the cells were making FUPA, the concentration would have had to be higher in the cells than in the fluid, such as was found for the nucleotides, which are made in the cells. Thus it has been shown, both *in vivo* and *in vitro*, that Ehrlich ascites cells are incapable of degrading 5-fluorouracil.

These data may also pertain to the question as to the relationship of the ascites fluid and blood plasma. The similarity of the pattern of metabolites in the two fluids suggest a common derivation and rapid equilibration. However, the concentration of FUPA in the ascites fluid is considerably higher than in the plasma. This suggests that there may be a direct leakage of FUPA from the liver into the ascites fluid possibly due to irritation. This appears reasonable, because the concentration of FUPA in the liver at the same time is 92,000 counts/min per g. Thus it appears that the liver is the main centre of FU-degradation, and it probably supplies the majority of the FUPA found in the blood plasma, ascites fluid, and ascites cells.

The substance with an R_f of 0.73 in the butanol-formic acid-water system had an acidity comparable to that of FCMP, according to its behaviour on ion-exchange chromatography. It seemed likely that this compound might be α -fluoro- β -guanidopropionic acid. Accordingly, an aliquot eluted from the paper was hydrolysed with boiling 0.2 N KOH for 2 hr, and another aliquot was treated with nitrous acid at room temperature. As a result of the latter treatment there was some unchanged material, but the majority was converted into FUPA. In the alkaline hydrolysis, 50 per cent of the radioactivity was present in urea and the remainder in FUPA. These results strongly suggest, but in the absence of a carrier compound do not prove, that the compound with an R_f of 0.73 is α -fluoro- β -guanidopropionic acid.

DISCUSSION

It is clear from the present investigation that the initial steps in the degradation of 5-fluorouracil to dihydrofluorouracil and α -fluoro- β -ureidopropionic acid are exactly analogous to those established for uracil (Fig. 3); in addition, there is some further cleavage of FUPA to give urea, a process which is quantitatively important in the human being. Since the FU is labelled in the 2-carbon, which becomes the ureido-carbon of FUPA, it has not been possible to study the fate of the remainder of the

molecule. Studies which are now in progress with FU-6-¹⁴C are aimed at the elucidation of that problem. The probable demonstration of α -fluoro- β -guanidopropionic acid as a metabolite represents a new contribution to the field. 5-fluoroorotic acid is not appreciably degraded by the mouse.

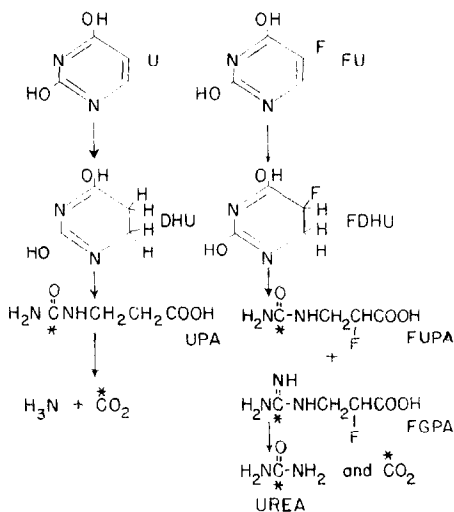


FIG. 3. The initial stages in the metabolic degradation of uracil and 5-fluorouracil.

The experiments on the degradation of FU with mouse liver supernatant fractions showed that this material was capable of degrading FU to the FUPA stage, and uracil primarily to carbon dioxide, in agreement with the work of others.^{13-17, 21} This degradation is so extensive that the liver is probably the main site of catabolism in the body, although we have found that degradation is carried out *in vivo* by normal tissues with the exception of spleen.

The inability of the Ehrlich ascites tumour to degrade FU has been demonstrated both *in vivo* and *in vitro*. Since the majority of the radioactivity circulating in the plasma is in the form of FUPA and Sarcoma-180 is highly vascular, it is possible that the FUPA found in this tumour *in vivo* may have been present in the blood in the tumour and not as a tumour metabolite. It has been suggested several times that the reason for the high utilization of uracil by tumours might reflect a lack of catabolism in the tumour,^{13, 18, 26} and the present study suggests that this is likely. It is of interest in this connexion that Potter has clearly demonstrated *in vivo* that thymidine is extensively degraded by rat liver, but not by the Novikoff hepatoma.²⁷ Skipper and Bennett have recently reviewed a number of other cases where tumours have shown a low catabolic activity for other nucleic acid precursors and metabolites,²⁸ and de Lamirande *et al.*²⁹ have demonstrated a lack of purine catabolic enzymes in the Novikoff hepatoma. Thus, it may be that this is a general biochemical characteristic of tumours. The selective localization of radioactivity from FU in tumours² and the selective biochemical effects of the compounds in tumours³ may be a result of this lack of catabolism in the tumours.

The study of the catabolism and tissue distribution of labelled FU in human cancer patients has been a contributing factor in setting up clinically effective dosage regimens.⁹

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ACRYLYLCHOLINE: A NEW NATURALLY OCCURRING PHARMACOLOGICALLY ACTIVE CHOLINE ESTER FROM *BUCCINUM UNDATUM*

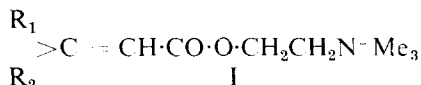
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Abstract—Extracts of the hypobranchial gland of the prosobranch marine gastropod *Buccinum undatum* (common whelk) has been found to have a very high acetylcholine-like activity (equivalent to about 0.3 μ moles acetylcholine/g tissue) when assayed on the frog rectus abdominis muscle. So far, high hypobranchial acetylcholine equivalences have been known to occur only in the dye-secreting family of prosobranch gastropods, Muricidae, to which *Buccinum* does not belong. The active principle has been identified as acrylylcholine and is thus related to the two esters which have been isolated previously from Muricidae, urocanylcholine (4(5)-imidazolylacrylylcholine) and β , β -dimethylacrylylcholine.

PROSOBRANCH gastropods possess, on the under-surface of their mantles and adjacent to their gills, a modified slime gland of unknown function known as the hypobranchial gland. The glands of one particular family of this order of snails, the Muricidae, are rich in a fluorescent indoxyl derivative which in the presence of crushed glandular material, light and air, is oxidized to the intense purple dye, 5,5'-dibromindigo, the much prized Tyrian Purple of the ancient world. Extracts of the glands are highly toxic to both cold- and warm-blooded animals,¹ producing paralysis. The glands also contain a very high content of an acetylcholine-like substance,² later identified as urocanylcholine³ (I, $R_1 = H$, $R_2 = 4(5)$ -imidazolyl-), a choline ester with neuromuscular blocking action,^{4, 5} Thus urocanylcholine is probably mainly responsible for the toxic properties of the glandular extracts.



Any new naturally occurring pharmacologically active ester of choline—particularly one possessing neuromuscular blocking action—is of interest not only in connection with the general problem of cholinergic function, but also in relation to possible clinical applications in human and veterinary medicine. Urocanylcholine has itself received clinical trial.⁶ Accordingly a survey was made of a number of marine gastropods and other molluscs.⁷ Two other choline esters were discovered. One of these occurs in place of urocanylcholine in the Muricidae *Thais floridana* (Southern oyster drill) and has been identified as β , β -dimethylacrylylcholine (I, $R_1 = R_2 = Me$); like urocanylcholine, this has neuromuscular blocking and ganglion-stimulating properties.⁸

The other new ester occurs in *Buccinum undatum* (the common whelk). The high choline ester content of the hypobranchial glands of this species, which is not a member of the Muricidae, is the first example of this phenomenon in a non dye-secreting snail.

This ester has now been identified as the parent substance of the series, acrylylcholine ($I, R_1 = R_2 = H$). The evidence for this is as follows. The active substance was alkali-labile, was destroyed by purified plasma cholinesterase, formed an acid hydroxamate in alkaline solution and yielded choline (identified by paper chromatography) on hydrolysis. The active material is therefore a choline ester. It took up approximately 1 mole of bromine/mole ester (Table 1) suggesting that it is the choline ester of an unsaturated fatty acid. The active material when chromatographed on columns of the ion-exchange resin Amberlite XE-97 buffered to pH 4.3 proved to have a retention volume lower than that of crotonylcholine or its higher homologues.⁷

TABLE 1. FORMATION OF VOLATILE FATTY ACID FROM, AND UPTAKE OF BROMINE BY HYDROLYSATES OF BUCCINUM ESTER

Ester estimated by the ferric hydroxamate method,¹⁰ choline spectrophotometrically,⁹ volatile fatty acid by steam distillation in a Markham still in the presence of excess magnesium sulphate at pH 3 and titration of successive fractions of distillate⁷ and bromine uptake by the method of Trappe.¹² Figures in brackets are percentage yields.

Ester (μ moles)	Choline (μ moles)	Volatile fatty acid (μ eq.)	Bromine uptake (μ moles)
32.8	22.5 (69)	—	—
14.2*	—	9.32 (66)	—
5.0	—	2.61 (52)	—
10.0	—	4.47 (47)	—
12.0*	—	4.83 (40)	—
44.0	—	14.60 (33)†	—
1.5	—	—	1.3 (87)
—	—	0.69	0.48 (70)

*Ester hydrolysed by incubating with 10 mg purified human plasma cholinesterase preparation (Kabi A, B., Stockholm) for 15 min at 37° and pH 7.0. This preparation released 120 μ moles acid/mg per hr from 30 mM acetylcholine in $\text{NaHCO}_3 - \text{CO}_2$ buffer, pH 7.4.

†Identified as acrylic acid by vapour phase chromatography (Fig. 1).

Since retention volumes increase with chain length in both the saturated and unsaturated series,⁷ this suggested that the new ester was acrylylcholine. On submitting hydrolysates of the ester to steam distillation (Table 1), a volatile fatty acid was recovered in 33–66 per cent yield which was identified (Fig. 1) as acrylic acid by vapour phase chromatography. Synthetic acrylylcholine had the same chromatographic properties (Fig. 2) and relative molar potency on the frog rectus abdominis muscle as the new ester.

EXPERIMENTAL

Techniques. The extraction of tissue, biological assays, chromatography and chemical analyses were carried out as already described.⁷ The unit of biological activity (u.) is that amount of extract eliciting a response from the test organ (frog rectus abdominis muscle) equivalent to that from 1 μ -mole acetylcholine.

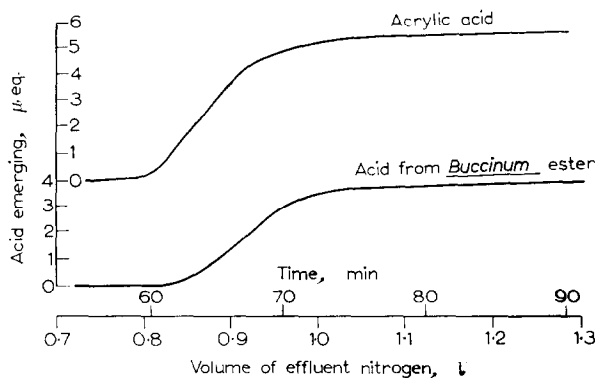


FIG. 1. Vapour phase chromatography of acrylic acid and volatile fatty acid from *Buccinum* ester. The graph shows the amount of fatty acid emerging from the column as a function of time or volume of effluent nitrogen.

Isolation of ester from Buccinum undatum. A typical preparation was as follows. The hypobranchial glands (wt. 413 g) from 62 live *Buccinum* (obtained from the Marine Biological Laboratory, Plymouth, with the kind co-operation of the Director, Dr. F. S. Russell, F.R.S.) were dissected out and dropped into ice-cold 10 per cent (w/v) trichloroacetic acid (900 ml). The tissue was finely comminuted in a blender and centrifuged; the precipitate was resuspended in more 10 per cent (w/v) trichloroacetic acid (200 ml) and recentrifuged. The combined supernatants were extracted with ether to pH 4.5, evaporated to a small volume (70 ml) and centrifuged to remove a small amount of insoluble material. This extract contained 120,000 u. of activity, corresponding to a specific activity ('acetylcholine equivalence') of the original gland of 290 u./g. The active ester was purified by precipitation as the water insoluble reineckate. Saturated aqueous ammonium reineckate (2.5 vol.) was added to the extract at 0°; the precipitate was collected, dissolved in a small volume of 50 per cent (v/v) aqueous acetone and the reineckate ion replaced by chloride by passing the solution through a column of De-Acidite FF (The Permutit Co., Ltd., London, 16 + 50 mesh) in the chloride form. The effluent was evaporated to dryness giving a crystalline residue containing 78,000 u. of activity (yield, 65 per cent).

Properties of active material. Activity was destroyed by heating for 10 min at 100° and pH 10–12 or by incubating with cholinesterase (details in Table 1). Choline was indentified in the hydrolysate as a compound giving a positive enneaiodide reaction under the conditions described by Appleton, La Du, Levy, Steele and Brody⁹ and having an R_F value (0.35) identical with that of authentic choline in *n*-butanol-ethanol-acetic acid-water (8 : 2 : 1 : 3). Approximately 1 mole of choline was recovered/mole ester (Table 1). On treating solutions of the active material with alkaline hydroxylamine under the conditions given by Hestrin,¹⁰ an acylhydroxamic acid was formed which was detected by the formation of a reddish-brown complex with ferric ions. A comparison of the ester content of solutions determined in this way with their biological activity using acetylcholine as a standard enabled their molar potency relative to acetylcholine to be determined. This was 20–40 per cent when the frog rectus was the assay preparation but less than 1 per cent when the guinea pig

ileum was used. This difference in the relative molar potencies as determined on these two test organs is also seen with substituted acrylic esters of choline.^{7, 8}

On submitting hydrolysates of active material to steam distillation at pH 3 in the presence of enough MgSO_4 to retain HCl ,⁷ a volatile acid was obtained in the distillate. The yield of acid was 33–66 per cent of the ester hydrolysed (Table 1). Acrylic acid was recovered in 63 per cent yield under identical conditions. The acid was concentrated and submitted to vapour phase chromatography¹¹ (Fig. 1). It had the same retention volume as acrylic acid and could not be separated from the latter when rechromatographed as a mixture.

The acid component of the ester was found to combine with bromine in chloroform under the conditions given by Trappe.¹² Chloroform solutions of the acid were prepared from steam distillates evaporated to dryness under alkaline conditions or from freeze-dried alkaline hydrolysates of the ester after acidification with a few drops of 30 per cent phosphoric acid. After extraction into chloroform, the solution was dried by passing it through a small column of anhydrous Na_2SO_4 and keiselguhr. The amount of bromine taken up (Table 1) corresponded approximately to that required for one double bond in the molecule.

Synthesis of acrylylcholine. Acrylylcholine was synthesized by the method of Fournau and Page.¹³ Acrylic acid (3.5 ml) was refluxed with β -bromoethanol (3.5 ml) in dry benzene (10 ml) in an apparatus with a water trapping device. The presence of an acid catalyst caused the polymerization of acrylic acid. It was therefore omitted, although this resulted in a low (30 per cent) yield of ester. After 1 hr the benzene was evaporated, the residue taken up in ether, freed from unesterified acrylic acid by extracting with 5 per cent (w/v) NaHCO_3 and washed with water. The ether layer was dried, evaporated and the residue heated with excess anhydrous trimethylamine

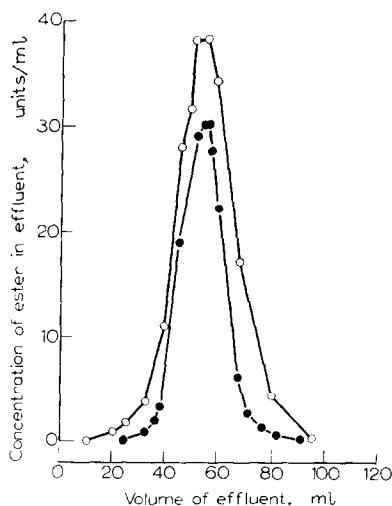


FIG. 2. Column chromatography of synthetic acrylylcholine (filled circles) or natural ester (open circles) on columns of the weak acid ion exchange resin Amberlite XE-97 (0.6 cm diameter $\times$ 10 cm height). The resin was buffered to pH 4.35 with 0.1 M- NaH_2PO_4 before use and the esters eluted with 0.1 M- NaH_2PO_4 . The graph shows the distribution of biological activity in successive fractions of eluate.

in dry benzene in a sealed tube at 60° for 15 hr. The glassy deliquescent precipitate of acrylylcholine bromide was washed with dry benzene and dried *in vacuo*. It had a molar potency of 30 per cent relative to acetylcholine on the frog rectus and similar chromatographic properties to the natural ester (Fig. 2).

Acrylic acid could be readily separated by vapour phase chromatography from other fatty acids with the exception of propionic acid. The retention volume of the synthetic acrylylcholine and the naturally occurring ester were close to that of propionylcholine which is known to occur in nature.^{14, 15} It is thus difficult to exclude the possibility that some propionylcholine might have been present in the snail extract. However, the presence of the double bond in the molecule and the low relative molar potencies of the ester on the frog rectus and guinea-pig ileum (the corresponding figures for propionylcholine are 160 per cent and 5 per cent) make this unlikely.

Acknowledgements—I am grateful to Miss J. M. Gilson for her skilful and painstaking technical assistance, and to Dr. E. F. Annison for advice on vapour phase chromatography.

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SHORT COMMUNICATIONS

Further biological activities of vincaleukoblastine—an alkaloid isolated from *Vinca rosea* (L.)

OVER a number of years interest in this laboratory has centred on possible biological activity of extracts of the plant *Vinca rosea*. In 1955 the preparation and partial purification of an extract containing a principle causing a fall in circulating leukocytes in the rat was reported.¹ Further studies showed that, while the myelocytes were particularly affected, extensive changes in the bone marrow were induced.^{2, 3} Recently it was reported that a highly active alkaloid, vincaleukoblastine, had been isolated as the crystalline sulphate which in a dose of 0.35 mg/kg administered intraperitoneally caused typical haematological changes in mice, rats or guinea pigs and had little effect on the gut. The blood changes could not be altered by treatment with folic or folinic acid.⁴ Other impure fractions, if given in larger doses, had a similar effect on the haemopoietic system. One fraction was found at a dose level of from 2.5 to 3 mg/kg also to antagonize the effect of oestrogen on the rat uterus, and this effect could be prevented by folinic acid.⁴ In a few studies crude fractions of the extract were found to exhibit some carcinostatic action against a transplanted mammary tumor in DBA mice, and against a transplantable rat sarcoma.⁴ In view of the apparent high degree of activity of extracts of *Vinca rosea* against the blood forming organs, experiments have been extended to include leukemia and other tumours. Only preliminary observations have been made to date in view of the limited quantity of vincaleukoblastine available. It is apparent, however, that this substance can readily inhibit the growth of a number of animal tumours.

It was noted that intraperitoneal administration of an aqueous solution of the alkaloid sulphate gave somewhat variable effects on the blood count. However, reproducible results were obtained if the alkaloid was administered as a solution of the free base in aqueous propylene glycol. The sulphate was therefore neutralized (pH 7.0) with dilute sodium bicarbonate and the resulting suspension of the free base diluted with 2 vols. of propylene glycol. Appropriate amounts of the resulting clear solution were diluted with propylene glycol before administration.

Anti-tumour activity of vincaleukoblastine has been demonstrated against L1210 and P1534 leukaemia transplanted in BDF hybrid mice, AKr leukaemia transplanted in AK mice, and sarcoma 180 in Swiss mice. Limited studies have been made with an acute leukemia in Fischer line 344 rats (leukaemia IRC 741/139B).^{*} Generally, treatment of groups of five mice was instituted 24 hr after inoculation of the animals, and consisted of daily intraperitoneal injections (0.2 cm³) for 10 days. Control animals received daily injections of the same amounts of propylene glycol.

Previous studies had indicated that single doses above 1.5 mg/kg of body weight were toxic for rats and mice. However, from 3 to 4 times this amount was tolerated with little indication of toxicity, if administered over a period of 10 days. The results obtained are summarized below and show that vincaleukoblastine has an inhibitory effect on the development of these tumours. In the case of L1210 leukaemia a comparison has been made with the activity of methotrexate.

Tests with myeloid leukaemia IRC741

This tumour has been maintained by serial transplant in duplicate, and the duplicate animal only has been available for testing. Consequently treatment with Vlb. has not been instituted until the leukaemia was well advanced (7-10 days after inoculation). Many of the animals were acutely ill.

Although survival time has not been significantly increased (15 days for treated vs. 13 days for untreated (three) animals), there has been pronounced alteration of the leukaemic process in peripheral blood. At the time therapy was instituted, the animals showed elevated total white counts (from 30,000 cells/mm³ to 200,000 cells/mm³) with the presence of leukaemic cells (5-24 per cent). Within 3 days of the administration of a single dose of Vlb. (0.2-0.75 mg/g) the count dropped sharply to less than 50 per cent, accompanied by disappearance of the leukaemic cells. In one rat the peripheral

* Kindly supplied by Dr. W. F. DUNNING, University of Miami.

count was maintained at less than 8000 cells/mm³ with no leukaemic cells, for 5 days. This animal also showed a slight reduction in size of the peripheral tumour (from 4.5 cm diameter to 3.0 cm diameter).

Further work on vincalukoblastine is in progress to determine the optimum dosage regimen to affect tumours. A number of other fractions have been obtained from *Vinca rosea* which affect the blood picture and these are being investigated for their possible action on experimental tumours.

TABLE 1. ANTI-TUMOUR EFFECT OF VINCALEUKOBLASTINE (Vlb.)

Tumour	Drug	Dose (mg/kg)	Survival time (days)		Increase (%)
			Treated	Control	
L 1210	Vlb.	0.3 : 9	11.8	7.4	59
		0.4 : 10	12.0	7.7	56
	Methotrexate	0.4 : 10	13.9	10.2	33
		0.6 : 10	13.5	9.8	38
P 1534	Vlb.	0.4 : 10	*	16.5	
		0.6 : 15	*		
AKr	Vlb.	0.4 : 10	3 of 5 living at 80 days†	22	

* One of 5 animals at the lower dose died on day 28. The remaining animals are living and well at 42 days.

† One inadvertently killed on day 4, one died at day 12 showing no evidence of tumour.

TABLE 2. EFFECTS ON TUMOUR S180

Material	Dose (mg/kg)	Take time (days)*		Survival time (days)	
		Treated mean	Control mean	Treated mean	Control mean
Vlb	0.4 × 10	19.5 (2)†	8 (5)	26 (2)†	23.8 (5)

* Time for tumour to reach 1 cm diameter.

† Three failed to develop tumours. There was no evidence of tumour when autopsied at 80 days.

Acknowledgement—We would like to thank Mr. J. Purko, M.Sc., Mr. G. Carpenter and Miss M. Dynes for valuable assistance and the National Research Council, Ottawa and National Cancer Institute of Canada for financial support of this project. Dr. Beer has worked on this problem during the tenure of a Medical Research Associateship of the National Research Council and Dr. Cutts on a Research Fellowship of the National Cancer Institute of Canada.

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Inhibition of an action of adrenaline by D-tartrate*

L-ADRENALINE hydrochloride at a concentration of 4 μg (of base) per ml stimulates the oxygen consumption and amylase secretion of mouse parotid glands *in vitro*.¹ L-Adrenaline D-bitartrate failed to stimulate the oxygen consumption of the parotid glands, although it stimulated secretion of amylase. When sodium D-tartrate (4 μg of tartrate/ml) was added to the medium containing the glands together with the adrenaline hydrochloride (4 μg base/ml) there was again no stimulation by the adrenaline of oxygen consumption, although there was a stimulation of amylase secretion. The inhibitory activities of the four stereoisomers of tartaric acid were compared. The highest inhibitory activity was shown by D-tartrate (82 per cent inhibition); L-tartrate had little inhibitory activity (29 per cent); the inhibitory activity of DL-tartrate was between that of the dextro and laevo forms; meso-tartrate showed no inhibitory activity. Tartrate alone had no effect on the oxygen consumption of the parotid glands. These results are shown in Table 1.

TABLE 1. THE EFFECT OF TARTRATES ON THE STIMULATION BY ADRENALINE OR PILOCARPINE OF OXYGEN CONSUMPTION OF MOUSE PAROTID GLANDS *in vitro*

Conditions	No. of expts.	Oxygen uptake ($\dot{Q}_{O_2}$)		
		Control	Experimental	Stimulation (%)
Adrenaline	4	1.37	1.76	+28
Adrenaline-D-bitartrate	4	1.81	1.86	+2
Adrenaline + D-tartrate	8	1.17	1.23	+5
Adrenaline + DL-tartrate	4	1.53	1.68	+10
Adrenaline + L-tartrate	4	1.47	1.77	+20
Adrenaline + meso-tartrate	4	1.75	2.34	+34
D-Tartrate	4	1.92	1.92	0
Pilocarpine	4	0.99	1.15	+16
Pilocarpine + D-tartrate	2	1.51	1.75	+16

The figures given are in μl of oxygen per mg of tissue (wet wt.) per hr; they are calculated from the oxygen uptake during 60 min incubation. The incubation medium was the calcium-free medium II, type A, of Krebs². The stimulating agent, adrenaline or pilocarpine, was added only to the experimental glands. Tartrate was added to both control and experimental glands except when its own effect on the glands was studied; then it was added only to the experimental glands. Concentrations of compounds used: adrenaline hydrochloride, 4 μg of base per ml; adrenaline bitartrate, 4 μg of base per ml; pilocarpine hydrochloride, 4 μg of base per ml; sodium tartrate, 4 μg of acid per ml.

Since the glands were incubated in a calcium-free medium² (medium II, type A) it was possible that tartrate inhibited the action of adrenaline by removing calcium from the glands. Incubations were therefore carried out in a medium containing calcium² (medium III). In this incubation medium

* The work was supported by U.S. Public Health Service grant number B-1470 (to P. HAGEN).

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D-tartrate had no effect on the stimulation by adrenaline of oxygen consumption; D-tartrate alone had no effect on the oxygen consumption of the glands.

Although the addition of calcium chloride prevented the sodium tartrate from abolishing the stimulation by adrenaline of oxygen consumption, it does not necessarily follow that D-tartrate removed calcium from the tissues. The added calcium might have formed a complex with the tartrate in the medium thus preventing the tartrate from reaching the tissue to exert its inhibitory action. The calcium-precipitating activities of the different stereoisomers of tartaric acid do not correspond to the inhibition of the adrenaline effect obtained. Chio³ studied the calcium-precipitating activities of the different stereoisomers of tartaric acid and placed them in the following order of descending activity: racemic, laevo, dextro, *meso* (Table 2, column II). This order does not correspond to the order of their inhibitory activities in the parotid glands (Table 2, column I).

TABLE 2. THE INHIBITORY ACTIVITIES OF THE DIFFERENT STEREOISOMERS OF TARTARIC ACID IN VARIOUS SYSTEMS

(The most active isomer is given in line (a), and the least active in line (d).)

	I	II	III	IV	V	VI	VII	VIII
(a)	D	DL	L	<i>meso</i>	<i>meso</i>	<i>meso</i>	D	DL
(b)	DL	L	D	—	—	DL	DL	D
(c)	L	D	DL	—	—	L	L	L
(d)	<i>meso</i>	<i>meso</i>	<i>meso</i>	D	D	D	<i>meso</i>	<i>meso</i>

I: Inhibition of the stimulation by adrenaline of the oxygen consumption of mouse parotid glands *in vitro*.

II: Calcium-precipitating activity and toxicity in guinea pigs when injected intraarterially.³

III: Toxicity in guinea pigs when injected intraperitoneally.³

IV: Inhibition of lactic dehydrogenase in *E. coli*.⁴

V: Inhibition of pyruvate oxidation in rat kidney cortex.⁵

VI: Inhibition of the growth of cress seedling roots.⁶

VII: Inhibition of the acid phosphatase of prostate gland.⁷

VIII: Inhibition of the acid phosphatase of mouse parotid gland.

Another action of tartrate is the inhibition of lactic dehydrogenase in *E. coli*;⁴ *meso*-tartrate is considerably more active in inhibiting this enzyme than is D-tartrate (Table 2, column IV). Tartrate also inhibits pyruvate oxidation in rat kidney cortex; *meso*-tartrate is the most active inhibitor and D-tartrate shows little inhibitory activity.⁵

Tartrate inhibits the acid phosphatase of human prostate gland but not that of blood; D-tartrate and DL-tartrate, at a concentration of 0.01 M, caused 92–94 per cent inhibition of this enzyme, while L-tartrate and *meso*-tartrate were not inhibitory.⁷ The acid phosphatase of mouse parotid gland homogenates is also inhibited by tartrate; the inhibitory activity of the four stereoisomers, in descending order, is: racemic, dextro, laevo, *meso* (Table 2, column VIII). Both the racemic and dextro forms are good inhibitors of the parotid gland enzyme, while the laevo and *meso* forms are poor inhibitors. A concentration of 1.5 mg of D-tartrate per ml is required to inhibit the acid phosphatase of mouse parotid gland by 50 per cent; the concentration which inhibits the stimulation of oxygen consumption by adrenaline is only 4 µg/ml.

Thus, the order of inhibitory activity of the four different stereoisomers of tartaric acid on the stimulation by adrenaline of the oxygen consumption of mouse parotid glands *in vitro* does not correspond to the order of activity in any of the known actions of tartrate, with the exception of acid phosphatase inhibition; it is unlikely that inhibition of acid phosphatase could be the mechanism of action of tartrate since the concentration used would not inhibit acid phosphatase in the parotid gland. Therefore, the inhibition by tartrate of this action of adrenaline cannot be explained by any of the other known actions of tartrate.

No other inhibitory effects of tartrate on any other action of adrenaline have been reported. Tartrate does not inhibit the stimulation by pilocarpine of the oxygen consumption of parotid glands (Table 1); thus, this effect of tartrate seems to be specific for the action of adrenaline. Since calcium is present in most biological systems, any possible inhibitory effects of tartrate probably would pass unnoticed, for it would be expected that the effect of the tartrate would be nullified by the calcium in the medium.

However, workers using adrenaline D-bitartrate *in vitro* should bear in mind the possibility of the inhibition of the action of adrenaline by equimolar amounts of D-tartrate.

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A comparison of the distribution of lysosome enzymes and 5-hydroxytryptamine with that of acetylcholine in subcellular fractions of guinea pig brain

(Received 23 December 1958)

THE crude mitochondrial fraction of guinea-pig brain can be resolved into three main subfractions by equilibrium centrifugation in a density gradient: A, particles less dense than 0.8 M sucrose, diameter 2–5 μ ; B, particles intermediate in density between 0.8 and 1.2 M sucrose, diameter 0.02–0.3 μ (median 0.07 μ); C, particles denser than 1.2 M sucrose, mainly mitochondria. Over half the particles of the B layer have diameters within the size range reported for synaptic vesicles (0.02–0.08 μ) (Palay¹), and the bound acetylcholine is concentrated in this layer (Hebb and Whittaker², Whittaker³).

In an attempt to define more closely the degree of homogeneity of this layer, the distribution in the density gradient of other particle-bound activities has been studied. Two lysosome enzymes, acid phosphatase and β -glucuronidase, were included because of the resemblance between the conditions under which acetylcholine is released from the particle-bound state and those which cause the breakdown of lysosomes. 5-Hydroxytryptamine (5-HT) was studied as another substance to which a transmitter role in the central nervous system has been ascribed.

Acid phosphatase and β -glucuronidase were separated by the density gradient technique, acid phosphatase being recovered mainly in the B layer and β -glucuronidase in the C layer. Although lysosome-like particles have been reported in rat brain (Beaufay *et al.*⁴), information as to their sedimentation characteristics was lacking. The present results suggest that the lysosome concept as developed for liver (Duve *et al.*⁵) cannot be applied unmodified to brain.

Bound 5-HT was recovered mainly in the B layer, and could be released from the particles under similar conditions to acetylcholine. Large, dense particles of the type isolated by Baker⁶ from intestinal mucosa were not found in brain. The different distribution of acetylcholine and 5-HT in whole brain makes it unlikely that both substances are located in the same particle. The acetylcholine-containing fraction must thus be recognized as being complex and as containing particles of diverse biochemical and physiological function.

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However, workers using adrenaline D-bitartrate *in vitro* should bear in mind the possibility of the inhibition of the action of adrenaline by equimolar amounts of D-tartrate.

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Inability of newborn mice and guinea pigs to metabolize drugs

INTRODUCTION

TISSUES of the foetus and the newborn of mammals are known to be deficient in a number of enzymes which rapidly increase in activity after the animal is born.^{1, 2} Many drugs easily cross the placenta³ and after giving compounds such as phenobarbitone and morphine to the mother, enough of the drugs may be present in the newborn occasionally to produce anoxia and even death.⁴ These observations suggest that the toxicity of drugs in the newborn may be related to the low activity of enzymes responsible for the metabolism of foreign organic compounds. The present study shows that mice and guinea pigs are born without the enzyme systems which metabolize several common therapeutic agents.

METHODS

Female mice (NIH general purpose), 1, 7, and 21 days old, were given 1 mg of amidopyrine, phenacetin or hexobarbitone by intraperitoneal injection. After 3 hr the animals were killed and then homogenized together with excreta in water using a Waring blender. The homogenates were analyzed for unchanged drugs by previously published methods.^{5, 6, 7} The ability to metabolize monomethyl-4-aminoantipyrine (MMAP), amidopyrine, phenacetin, hexobarbitone and phenolphthalein was determined *in vitro*. Preparation of tissue samples was carried out at 4°C. The livers of female guinea pigs, 1, 7, 21 and 57 days of age, were homogenized in two volumes of 1.15% KCl by means of a Teflon homogenizer. The homogenate was centrifuged at 9000 g for 15 min, and the supernatant fraction was removed and dialyzed overnight against distilled water. Foetuses were removed from parents about 48 hr before birth and their livers were treated similarly. A typical incubation mixture consisted of 2 μ moles of MMAP, amidopyrine, phenacetin or hexobarbitone, 3 ml of dialyzed supernatant, 1 ml of 0.2 M phosphate buffer (pH 7.4), a TPNH-generating system consisting of glucose dehydrogenase and 50 μ moles of glucose, 100 μ moles of nicotinamide, 0.2 μ moles of TPN and 75 μ moles of $MgCl_2$. Phenolphthalein was incubated with 3 ml of dialyzed supernatant, 1 ml of 0.1 M

TABLE 1. METABOLISM OF DRUGS BY GUINEA PIG LIVER
9000 g supernatant fraction was incubated with 2 μ moles of substrate as described under "Methods". In each experiment 30 g of pooled livers were used. Figures represent range of values obtained.

Age (days)	Number of experiments	Amount of substance metabolized (in μ moles)				
		MMAP	Amidopyrine	Phenacetin	Hexobarbitone	Phenolphthalein
-2 (foetus)	(1)	0	—	—	—	0
1	(3)	0	0	0	0	0
7	(3)	0.23-0.35	0.11-0.16	0.16-0.27	0.05-0.07	0.04-0.05
21	(3)	0.37-0.47	0.38-0.55	0.56-0.86	0.26-0.42	0.13-0.18
57	(2)	0.69-0.86	—	—	0.51-0.64	0.33-0.48
Adults (3 months old)	(5)	0.69-0.94	0.62-0.88	0.96-1.08	0.57-0.78	0.44-0.57

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Inability of newborn mice and guinea pigs to metabolize drugs

INTRODUCTION

TISSUES of the foetus and the newborn of mammals are known to be deficient in a number of enzymes which rapidly increase in activity after the animal is born.^{1, 2} Many drugs easily cross the placenta³ and after giving compounds such as phenobarbitone and morphine to the mother, enough of the drugs may be present in the newborn occasionally to produce anoxia and even death.⁴ These observations suggest that the toxicity of drugs in the newborn may be related to the low activity of enzymes responsible for the metabolism of foreign organic compounds. The present study shows that mice and guinea pigs are born without the enzyme systems which metabolize several common therapeutic agents.

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Adults (3 months old)	(5)	0.69-0.94	0.62-0.88	0.96-1.08	0.57-0.78	0.44-0.57

TABLE 2. METABOLISM OF DRUGS BY INTACT MICE
Figures represent range of values obtained

Age (days)	Number of animals	Percentage disappearance in 3 hr		
		Amidopyrine	Phenacetin	Hexobarbitone
1	(6)	0	0	0
7	(6)	17-35	10-14	11-24
21	(6)	51-89	30-61	21-33

TABLE 3. EFFECTS OF HEXOBARBITONE ON MICE OF VARIOUS AGES
Figures in brackets refer to number of animals in each series.

Dose (mg/kg)	Sleeping time (min)			
	1 day old*	7 days old	21 days old	adult
10	> 360 (12)	107 $\pm$ 26 (12)	27 $\pm$ 11 (11)	< 5* (12)
50	Died (12)	243 $\pm$ 30 (10)	64 $\pm$ 17 (10)	17 $\pm$ 5 (12)
100	Died (12)	> 360 (12)	94 $\pm$ 27 (12)	47 $\pm$ 11 (12)

* After administration of drug to 1 day old animals, they lay supine, while control animals placed on their backs attempted to right themselves.

Tris buffer (pH 8.2), 0.5 μ mole of DPN, 100 μ moles of nicotinamide, and 0.5 μ mole of uridine diphosphate glucose. The final volume was adjusted with water to 6.0 ml. Incubations were carried out in a Dubnoff metabolic shaker for 1 hr at 37.5°C in an atmosphere of oxygen. Enzyme activity was defined as μ moles of substrate metabolized per g of liver in 1 hr, except that with MMAP the appearance of 4-aminoantipyrine was measured.⁸ Proof that phenolphthalein disappeared by glucuronide formation was furnished by the method of Talalay *et al.*⁹

RESULTS

The studies *in vitro* showed that newborn guinea pigs lacked the conjugative system that forms phenolphthalein glucuronide¹⁰ and the oxidative systems in liver microsomes that N-demethylate MMAP and amidopyrine, O-dealkylate phenacetin and oxidize the side-chain of O-hexobarbitone¹¹ (Table 1). The enzyme systems were absent 24 hr after birth, appeared during the first week, and increased in activity until the animals were about 8 weeks old.

To confirm that newborn animals are unable to metabolize drugs, studies *in vivo* were carried out on mice of various ages. As shown in Table 2, newborn mice did not metabolize amidopyrine, phenacetin or hexobarbitone. Animals 7 days old were able to metabolize these drugs.

The response to hexobarbitone was determined in mice of various ages. The drug was given intraperitoneally and the duration of action was measured by the time of recovery of righting reflex. The contrast in the response of the various age groups was very striking (Table 3). For example, a dose of 10 mg/kg produced a pronounced hypnosis that lasted more than 360 min in the 1 day old animals and 107 min in the 7 day old animals, but had little effect in adult mice. Larger doses of hexobarbitone were highly toxic to the 1 day old animals, as compared to the effect obtained in older animals. It is probable that the greater effect of hexobarbitone in the newborn results from two factors, the inability of the animals to inactivate the drug and a greater sensitivity of the central nervous system to the drug.

DISCUSSION AND SUMMARY

Newborn mice and guinea pigs lack the enzymatic mechanisms for metabolizing drugs such as monomethyl-4-amino-antipyrine, amidopyrine, phenacetin and hexobarbitone. In addition,⁸ newborn animals are unable to form glucuronides, an observation in agreement with that of Brown and Zuelzer¹². These mechanisms begin to appear in the first week of life and increase in activity until an apparent maximum is reached at about 8 weeks. In addition, the central nervous system of newborn

animals appears to be unusually sensitive to the action of hexobarbitone. These findings are of obvious importance in considering the use of drugs, especially narcotics and nonvolatile anesthetics, in childbirth and for newborn infants.

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The comparison of the effect of an even and an odd unsaturated fluoro fatty acid on kidney particles

(Received 30 December 1958)

THE theory that fluorocompounds with an even number of C atoms are toxic and those with an odd number not so (Saunders^{1, 2}), has received support from recent experiments *inv vivo* for compounds with a longer chain than C₈ (Pattison³; Parker and Walker⁴). The latter also found that accumulation of citrate *in vivo* followed the rule. Confirmation *in vitro* seemed to be desirable.

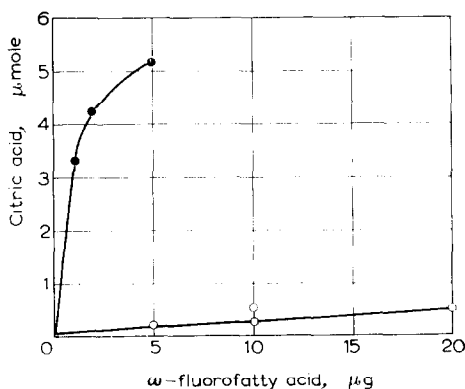


FIG. 1. The effect of fluorofatty acid on accumulation of citric acid with kidney particles from the guinea pig.

Ordinate: μ moles citric acid accumulated in 40 min.

Abscissa: amount in μ g of ω fluorofatty acid added. (The extra point at 10 μ g is from a second experiment).

● 12-fluorododec-2-enoic acid; ○ 13-fluorotridec-2-enoic acid.

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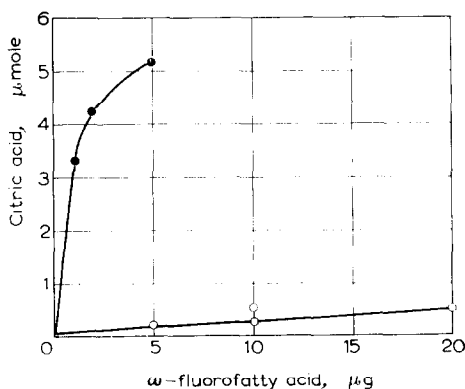


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● 12-fluorododec-2-enoic acid; ○ 13-fluorotridec-2-enoic acid.

The two acids 12-fluorododec-2-enoic acid $F(CH_2)_9CH=CH\ COOH$ and 13-fluorotridec-2-enoic acid $F(CH_2)_{10}CH=CHCOOH$ became available through the kindness of Professor F. L. M. Pattison (London, Ontario). Their effect was tested on our usual preparation of kidney particles from the guinea pig with the results upon citrate accumulation shown in Fig. 1. This was one out of two similar experiments. It is clear that with the long carbon chains the results *in vitro* are similar to those *in vivo* (Parker and Walker⁴), and that the double bond in this position makes no difference. C_{12} is very much more powerful than C_{13} , at least 50 times; as stated elsewhere, the long-chain fluoro-fatty acids with an even number of C atoms are more effective inhibitors than fluoroacetate, so that the system for activation to fluorocitric acid is very active in these kidney particles.

EXPERIMENTAL DETAILS

Kidney mitochondrial particles were prepared as described previously (O'Brien and Peters⁵). The amounts of enzyme used in 1.9 ml were reinforced with $MgCl_2$ (4.0 μ moles), ATP (1.2 μ moles), Na fumarate (20 μ moles), fluoroacid as stated, together with KCl 1.0% to a total volume of 3.0 ml.

The flasks were shaken for 40 min at 37°. Citric acid was estimated by the method of Taylor,⁶ after deproteinization with trichloroacetic acid.

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- TH. J. DE MAN and J. R. ROBORGH: The hypercalcemic activity of dihydrotachysterol₂ and dihydrotachysterol₃, and of the vitamins D₂ and D₃. Comparative experiments in rats.
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