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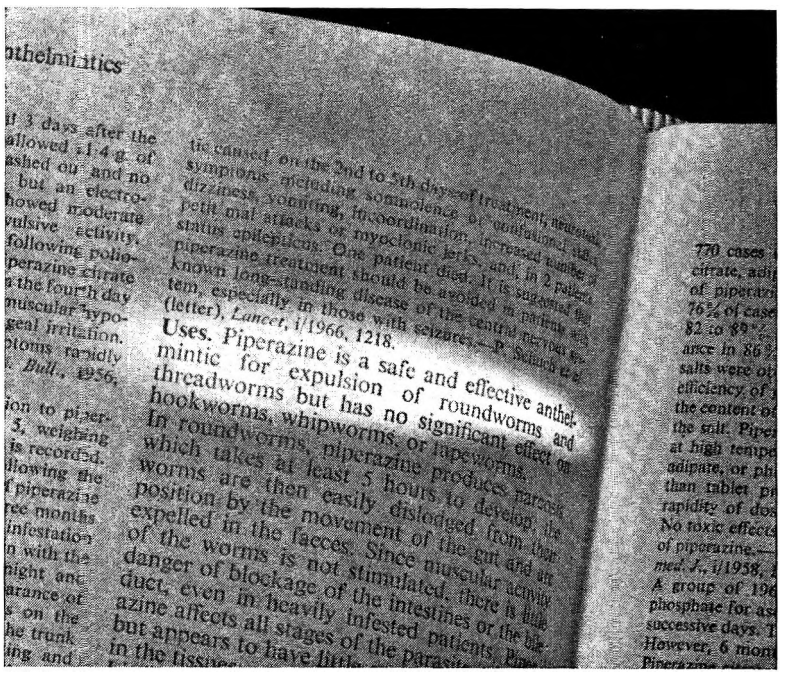

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Kinetics of the hydrolysis of phosphatidylcholine and lysophosphatidylcholine

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An experimental technique capable of estimating as little as 10 nanoequivalents of long-chain carboxylic acids (fatty acids) has been used to study the decomposition of phospholipids. The rate of release of acids varied with the monovalent cation which was associated with the hydroxyl ion in the hydroxide used. Because the concentration of hydroxide was much greater than the concentration of phospholipid, the rate of hydrolysis was first order with respect to the phospholipid. The pseudo-first order reaction velocity constant varied as the first power of the concentration of lithium hydroxide, but as the second power of the concentration of potassium, sodium, tetramethylammonium, or tetraethylammonium, hydroxides. It is postulated that (i) lecithin and lysolecithin, in strong alkaline solution, bind lithium ions much more strongly than potassium, sodium, tetramethylammonium, or tetraethylammonium ions, and (ii) it is only this cation-phospholipid complex which is broken down by hydroxyl ions.

Phosphatidylcholine (lecithin) contains two ester groups per molecule, each of which, in theory, should be hydrolysed both by hydrogen ions and hydroxyl ions.

Many workers, who have analysed hydrolysates of lecithin, have observed a migration of the phosphate group. In explaining the resulting isomerization reaction schemes have been put forward in which it has been assumed that the fatty acids were rapidly removed from the lecithin before any other action could take place, either in acid or alkaline conditions. This is certainly a correct assumption in alkaline solutions which contain a high concentration of hydroxyl ions, but the results with acid solutions and other salt solutions are not so easy to interpret, except under extreme conditions, for example, N HCl at 120° for 7 h (De Koning & McMullan, 1965; Long & Maguire, 1953).

Certain enzymes act specifically on either the 1- or 2-linked fatty acid ester bond only, of lecithin. On the other hand, at 0°, both the 1- and 2-fatty acyl groups are removed by methoxyl ions in chloroform–methanol solution to produce lysolecithins, although there may be "a small preference" for the 1-group (Marinetti, 1962).

Both in the saponification of esters and in acid-catalysed hydrolysis of esters, cleavage is in the $R_1CO–OR_2$ link and not in the $R_1COO–R_2$ link (Polanyi & Szabo, 1934; Roberts & Urey, 1938) and hydroxides break down lecithin and lysolecithin at a much faster rate than H^+ ions, to release the fatty acids.

EXPERIMENTAL

Method

Into a long-necked 100 ml flask, which was immersed (up to an inch below the stopper) in a water bath at a fixed temperature, was added methanol (96%), aqueous hydrolysing agent, and water, to a total volume of 24 ml. The volume of water, kept as low as possible, varied from 1 to 7 ml depending on the hydrolysing agent

used. The quantity of aqueous hydroxide solution was measured in ml, taken from a solution of known strength. To this mixture was added 1 ml of a chloroform solution of phospholipid after the previous mixture had equilibrated in the water bath. 1 ml aliquots of this final mixture were withdrawn at stated time intervals and run into an aqueous solution of 5N HCl, which was above a chloroform layer in a separating funnel. Liberated fatty acids were initially precipitated but, on shaking, they dissolved in the chloroform layer. The lower chloroform layer and washings were run off into a 10 ml beaker and the solvent removed by a vacuum pump, then each sample was titrated separately, as follows.

The acids were dissolved in about 5 ml (a fixed quantity measured by a device delivering a constant volume) of dimethylformamide (DMF), under nitrogen, together with four drops of a methanolic solution of quinaldine red (0·1% w/v), and titrated, under nitrogen, with standardized lithium methoxide which was in a mixed solvent of benzene and methanol. The end point of the titration was a distinct change of indicator colour from pink to colourless. The complete titration was made in an atmosphere free from water and carbon dioxide.

The burette was an Agla micrometer-syringe type readable to 0·0001 ml.

The titrant was standardized using recrystallized benzoic acid as a solid standard, dissolved in DMF as above. A typical series of titrations consisted of two blank titrations against DMF only, followed by two standardizations against accurately weighed samples of benzoic acid. Then another blank titration, followed by the samples in random order, another standardization against benzoic acid and a final blank titration against DMF only.

Materials used

Lecithin was prepared from a sample of purified egg lecithin (Merck A.G.) by chromatography and crystallization.

Lysolecithin was prepared from lecithin by the action of phospholipase A of Russell viper venom and was purified as described by Perrin & Saunders (1960).

RESULTS

Both lecithin and lysolecithin were hydrolysed in the same way by all the hydroxides used. There was no apparent difference in the graphs obtained. The rate of hydrolysis of phospholipid depended both on the concentration of phospholipid and the concentration of hydroxyl ions.

$$-d(L)/dt = k[L]\,[OH^-]^n \quad \ldots\ldots\ldots\ldots \quad (1)$$

L is either lecithin or lysolecithin, k is the reaction velocity constant, n is an integer.

If $[OH^-] \gg [L]$ then equation (1) becomes—

$$-d(L)/dt = k^1[L] \quad \ldots\ldots\ldots\ldots \quad (2)$$

In the experiments reported $[OH^-]$ was at least $100 \times [L]$.

The following quantities are defined: a = the maximum amount of acid released from a 1 ml sample of solution (total volume 25 ml), i.e. it represents the initial concentration of phospholipid in 1 ml of the solution before any hydrolysis occurred. x = the acid present at time t, in a 1 ml sample of solution. $(a - x)$ is, therefore, a measure of the intact phospholipid in the reaction mixture at time t.

Substituting these quantities into equation (2),

$$-d(L)/dt = dx/dt = k^1(a - x)$$

which, on integration, becomes

$$\log (a - x) = \log (a) - k^1t/2{\cdot}303 \quad .. \quad .. \quad (3)$$

Equation (3) represents a first order reaction with respect to phospholipid. Hence on plotting a graph of log (a — x) against t, a straight line (with slope $-k^1/2{\cdot}303$), would indicate such a reaction, which is evident in Fig. 1a and b. The concentration

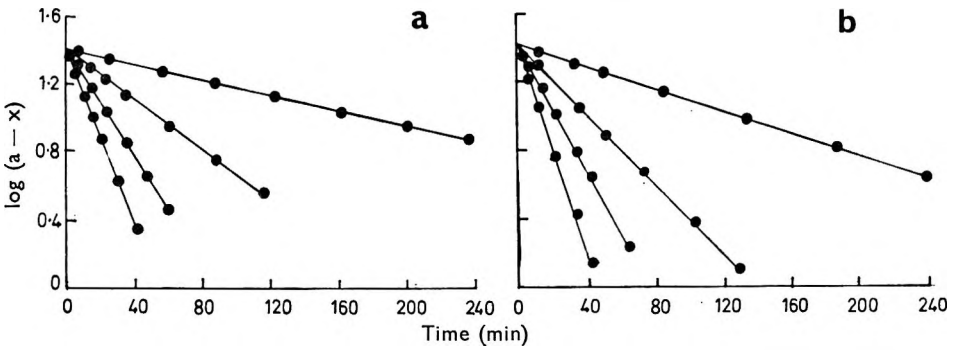

FIG. 1. The breakdown of lecithin (a) and lysolecithin (b) by NaOH under conditions of constant phospholipid concentration, constant temperature and four concentrations of OH^- for each phospholipid.

of hydroxyl ions was constant for each of the straight lines and the slopes of the straight lines depended on the hydroxyl ion concentration. Similar sets of straight lines were obtained for the hydrolysis of either lecithin or lysolecithin using LiOH, NaOH, Me_4NOH or Et_4NOH, at temperatures of 25°, 30° or 35°. Neither sodium borate, sodium bicarbonate, disodium hydrogen phosphate, nor dilute hydrochloric acid released any detectable amounts of carboxylic acids from lecithin in aqueous methanolic solution under the conditions of the experiments. It therefore seems that hydroxyl ions are necessary for the hydrolysis of the phospholipids.

In Fig. 1a it can be seen that the reaction constant, k^1, which is measured as $k^1/2{\cdot}303$ by simply calculating the slope of the straight line, varies with the concentration of hydroxyl ions for a constant lecithin concentration.

Substituting $t = \tau$ and $x = a/2$ into equation (3), we have, on rearranging

$$k^1\tau/2{\cdot}303 = \log 2 \quad .. \quad .. \quad .. \quad .. \quad (4)$$

Equation (4) is seen to hold approximately for the experimental results given in Tables 1 and 2. These results indicate that τ is independent of the initial concentration of phospholipid and confirms that the reaction is first order with respect to the phospholipid.

Assay of the reaction mixtures showed that, ultimately, all the acids were released from lecithin and lysolecithin.

Using the Arrhenius equation in the form

$$k^1 = A \exp(-E/RT) \quad .. \quad .. \quad .. \quad .. \quad (5)$$

the values of E and log A, which were the same for both lecithin and lysolecithin, were calculated (Table 3). When $\log (k^1/2{\cdot}303)$ is plotted against 1/T, the slopes of the lines are $-E/2{\cdot}303$ and the intercept on the $\log (k^1/2{\cdot}303)$ axis is $\log (A/2{\cdot}303)$.

Table 1. *Rate of hydrolysis of lecithin at* 35°

Initial concn of lecithin mM $ml^{-1} \times 10^3$	Cation	[OH^-] g ions $litre^{-1} \times 10^2$	τ min	$k^1/2{\cdot}303 \times 10^3$	$k^1\tau/2{\cdot}303$ (= log 2)
1·119	K	7·6	156	2·04	0·32
0·772			147	2·16	0·32
1·121	K	18·9	25	12·33	0·31
0·768			25	12·63	0·32
1·080	K	23·7	16	17·66	0·28
0·780			18	18·39	0·33
1·077	Na	18·2	44	6·71	0·30
0·785			45	7·93	0·36
1·097	Na	24·2	20	15·05	0·30
0·771			14	22·39	0·31
1·110	Na	30·3	10	27·34	0·27
0·752			10	27·05	0·27
1·078	Li	5·9	46	6·45	0·30
0·749			46	6·39	0·29
1·102	Li	11·8	18	17·27	0·31
0·749			17	17·70	0·30
1·108	Li	17·7	15	21·44	0·32
0·795			17	20·99	0·36

τ is the time for half the total acid to be released from the phospholipid.

Table 2. *Rate of hydrolysis of lysolecithin at* 35°

Initial concn of lysolecithin mM $ml^{-1} \times 10^3$	Cation	[OH^-] g ions $litre^{-1} \times 10^2$	τ min	$k^1/2{\cdot}303 \times 10^3$	$k^1\tau/2{\cdot}303$ (= log 2)
1·799	K	18·9	27	10·61	0·28
1·016			20	15·67	0·31
1·831	K	23·7	13	24·39	0·32
1·014			14	22·81	0·32
1·846	Na	9·7	120	2·65	0·32
1·099			123	2·47	0·30
1·775	Na	18·2	34	9·10	0·31
1·030			36	8·49	0·31
1·753	Na	24·2	17	16·23	0·28
1·123			16	19·62	0·31
1·890	Na	30·3	11	32·29	0·36
1·056			9	38·89	0·35
1·813	Li	5·9	35	8·78	0·31
1·126			36	8·79	0·32
1·856	Li	11·8	14	23·96	0·34
1·078			16	20·30	0·32
1·840	Li	17·7	11	27·79	0·31
1·069			11	23·82	0·26

Table 3. *Arrhenius constants for the hydrolysis reactions*

Hydroxide	E kcal $mole^{-1}$	log A*
LiOH	21·0 ± 2·3	11 to 15
NaOH	18·2 ± 3·6	8 to 14
KOH	14·8 ± 2·3	6 to 9

* Range of log A which varied with hydroxyl ion concentration.

From equations (1) and (2) we have the relation

$$k^1 = k[OH^-]^n \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Taking logarithms of equation 6 we have

$$\log k^1 = \log k + n \log[OH] \quad .. \quad .. \quad .. \quad (7)$$

Table 4. *Effect of hydroxide concentration on rates of hydrolysis*

° C	Cation and phospholipid	$[OH^-]$ g ions litre^{-1} $\times 10^2$	τ min	$[OH^-]^2\tau$
30	K	23·7	27	1·52
	Lecithin	18·9	38	1·40
		14·2	73	1·47
35	K	23·7	16	0·90
	Lecithin	18·9	25	0·89
		14·2	48	0·96
35	Na	30·3	11	1·01
	Lecithin	24·2	19	1·08
		18·2	44	1·46
25	Me_4N	12·2	76	1·13
	Lecithin	8·5	166	1·19
		15·8	44	1·10
25	Et_4N	8·3	105	0·72
	Lecithin	5·0	291	0·73
		11·6	58	0·78
25	K	23·7	45	2·52
	Lysolecithin	18·9	70	2·50
		14·2	123	2·48
30	Na	36·4	16	2·12
	Lysolecithin	30·3	27	2·48
		24·2	38	2·22
35	Na	30·3	11	1·01
	Lysolecithin	24·2	17	1·00
		18·2	34	1·12

Table 5. *Rates of hydrolysis by lithium hydroxide*

° C	Cation and phospholipid	$[OH^-]$ g ions litre^{-1} $\times 10^2$	τ min	$[OH^-]\tau$
30	Li	23·6	18	4·2
	Lecithin	17·7	23	4·1
		5·9	73	4·3
25		23·6	33	7·8
		17·7	48	8·5
		5·9	128	7·6
35		23·6	10	2·4
		17·7	15	2·7
		5·9	44	2·6
30	Li	23·6	16	3·8
	Lysolecithin	7·7	22	3·9
		11·8	42	4·9
25		23·6	23	5·4
		17·7	28	5·0
		5·9	112	6·6
35		17·7	11	2·0
		11·8	16	1·9
		5·9	37	2·2

As before, k^1 is represented as $k^1/2{\cdot}303$ from the slope of the practical curves (Fig. 1a and b), so log ($k^1/2{\cdot}303$) was plotted against log [OH^-] and the slopes of the resulting straight lines are 1 for lithium hydroxide and 2 for all the other hydroxides.

In Tables 4 and 5, $[OH^-]^n\tau$ is constant, within experimental error, with n equal to 1 for lithium, and n equal to 2 for the other hydroxides.

Results obtained for both lecithin and lysolecithin are summarized in Table 6.

Table 6. *Summary*

Symbol	Equation number	Conditions kept constant	Conclusion
k	1	Temperature and hydroxide concentration	$Et_4NOH > Me_4NOH >$ KOH > NaOH
k^1	6	As for k	$Et_4NOH > Me_4NOH >$ LOH > KOH > NaOH
E	5	Hydroxide concn	Has a value between 12·5 and 23·3 kcal KOH < NaOH < LiOH
A	5	Hydroxide concn	KOH < NaOH < LiOH
n	1		Equals 1 for lithium; equals 2 for other hydroxides

DISCUSSION

The values of the activation energy E obtained, are not significantly different from those reported in the literature for ester saponification (Moelwyn-Hughes, 1947). More significance can be attached to the variations in log A.

According to the activated complex theory of reaction rates, the fact that log A (a measure of the frequency factor of the reaction) is smaller for KOH than LiOH, may be due to modifications to the entropy of activation of the reacting molecules (or entropy of formation of the activated complex). Important effects can arise if a reactant, or the complex, has a high dipole moment. The solvent molecules in the neighbourhood of a highly polar activated complex will be acted upon by strong electrostatic forces causing them to have less freedom of motion than they would otherwise have. As a result there is a loss of entropy by the system and an abnormally low frequency factor because the activated complex tends to bind solvent molecules more strongly than the reactant molecules. The entropy of activation for the reaction between KOH and phospholipid is less positive than the entropy of activation for the reaction between LiOH and phospholipid. The KOH-phospholipid interaction can therefore be said to involve processes which are less likely to occur than the processes which occur between LiOH and phospholipid. This suggests that the KOH-phospholipid interaction produces a more polar activated complex than the LiOH-phospholipid interaction.

In the collision theory of reaction rates, the theoretical collision frequency varies but slightly from reaction to reaction, the average value being about $2{\cdot}77 \times 10^{11}$, i.e. for a normal bimolecular reaction log A is about 11·3. The approximate constancy is due to the compensating influences of two factors.

It is to be noted that log A is greater than 11·3 for LiOH, but is less than 11·3 for KOH. The variation of A with hydroxide concentration may be simply due to the relative concentration of OH^- ions. Z, the collision frequency, will vary with the concentration of hydroxyl ions.

The pK for the dissociation of metal hydroxides in aqueous solution ($MOH \rightleftharpoons M^+ + OH^-$) has been calculated as $-0{\cdot}08$ for LiOH (Darken & Meier, 1942), and $-0{\cdot}70$ for NaOH (Bell & Prue, 1949); no evidence for association (into ion pairs) of the K^+ and OH^- ions in KOH could be found (Davies, 1959). Both NaOH and KOH behaved, at low concentrations, as typical strong electrolytes. Lithium hydroxide is incompletely dissociated in such aqueous solutions (Darken & Meier, 1942). This indicates that the presence of OH^- ions in these aqueous solutions decreases in the order KOH > NaOH > LiOH. However, the value of k cannot be compared between LiOH and the other hydroxides because the units of k for LiOH are different from the units of k for the other hydroxides. The pseudo first-order reaction velocity constant, k^1, can be compared though, because the units of k^1 are s^{-1} for all hydroxides studied.

Because both lecithin and lysolecithin are broken down by hydroxides in a similar fashion, the difference between the action of LiOH and the other hydroxides cannot involve the structural difference between lecithin and lysolecithin.

The fact that n is unity for LiOH but 2 for all the other hydroxides can be explained as follows:

(The following symbols are used: p = phospholipid, fp = free phospholipid, tp = total phospholipid, M^+p = cation/phospholipid complex.)

Since the aqueous methanolic solution has a high concentration of OH^- ions the phospholipid will have a net negative charge, due to the phosphate group in the molecule. This net negative charge will tend to repel the OH^- ions, which are needed to breakdown the ester bonds in the phospholipid. If this net negative charge on the phospholipid is neutralized by cations, we have the following equilibrium,

$$M^+ + fp \rightleftharpoons M^+p$$

let
$$K_M = [M^+p]/[M^+]\,[fp] \quad .. \quad .. \quad .. \quad .. \quad (10)$$

and
$$[tp] = [M^+p] + [fp] \quad .. \quad .. \quad .. \quad (11)$$

by experiment, the rate measured was equal to $k^1[tp]$.

If only the neutralized phospholipid molecules are attacked by OH^- ions, then

$$\begin{aligned} k^1[tp] &= k_1[OH^-]\,[M^+p] \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (12) \\ &= k_1[OH^-]\{[tp] - [fp]\}, \text{ from (11)} \\ &= k_1[OH^-]\{[tp] - [M^+p]/K_M[M^+]\}, \text{ from (10)} \end{aligned}$$

Rearranging the last equation

$$k_1[OH^-][M^+p]\,\{1 + 1/K_M[M^+]\} = k_1[OH^-][tp] \quad .. \quad .. \quad (13)$$

From equations (12) and (13) we have

$$k^1[tp] = k_1[OH^-][tp]/\{1 + 1/K_M[M^+]\}$$

Therefore
$$k^1 = k_1[OH^-]K_M[M^+]/\{K_M[M^+] + 1\} \quad .. \quad .. \quad .. \quad (14)$$

From equation (10) we see that

$$K_M[M^+] = [M^+p]/[fp] \quad .. \quad .. \quad .. \quad .. \quad (15)$$

For LiOH, assuming a strong complex is formed between Li^+ ions and phospholipid because Li^+ will be small, $[M^+p]$ will be much greater than [fp], in equation (15); so

$K_M[M^+] \gg 1$. Therefore, from equation (14), $k^1 = k_1[OH^-]$ and so n has a value of 1, for LiOH. For the other hydroxides, a weak complex is formed between cation and phospholipid (the cations are either large themselves, e.g. Et_4N^+, or hydrated); so $[M^+p]$ is not much larger than [fp], which means that equation (14) can be written as,

$$k^1 = (k_1K_M/\{K_M[M^+] + 1\})[M^+][OH^-] \simeq k_2[OH^-]^2$$

($[M^+]$ being taken as proportional to $[OH^-]$) and so n has a value of 2, for all the other hydroxides.

An assumption made in this explanation is that only the cation-phospholipid complex, M^+p, is attacked by OH^- ions. This is reasonable because of the repulsion that is likely to occur between negatively charged phospholipid and OH^- ions. This assumption can be used to explain why the entropy of activation for KOH-phospholipid interaction is less positive than that for LiOH-phospholipid interaction. The KOH-phospholipid activated complex will be more negatively charged than the LiOH-phospholipid activated complex because the lithium ion is smaller than the potassium ion and so would be more strongly held by the phospholipid. This assumes that the lithium ion is dehydrated on complex formation. The dehydration would lead to an increase in entropy, which could explain why the entropy of activation for LiOH-phospholipid interaction is more positive than for KOH-phospholipid interaction. Since the hydrolysis is brought about by OH^- ions the mechanism for KOH/phospholipid interaction involves the combination of two like charges (both negative) whereas the LiOH/phospholipid interaction involves the combination of two unlike charges (either uncharged phospholipid or positively charged phospholipid-cation complex, with OH^- ions).

The difference in charge between LiOH-phospholipid complex and KOH-phospholipid complex can also explain why k^1, for LiOH, is larger than k^1 for the other hydroxides. For the other hydroxides the OH^- ions are repelled by the negatively charged phospholipid-cation complex.

Another assumption is that $K_M[M^+]$, which is equal to $[M^+p]/[fp]$, is much greater than unity for Li^+, but not for any other monovalent cation used. This too is a reasonable assumption because Li^+ ions form the least stable association with solvent molecules, compared with the other cations. Therefore free non-hydrated Li^+ ions will be more strongly held by the negative phospholipid macromolecule than hydrated K^+, Na^+, or quaternary ammonium ions.

This work forms part of a Thesis, accepted for the degree of Ph.D., University of London in 1966.

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Esters of choline and β-trimethylammonio-propionic acid: geometrical isomerism and anti-acetylcholinesterase activity

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The preparation of the choline esters of *cis*- and *trans*-4-t-butylcyclohexanecarboxylic acid is reported. The similar inhibitory potency displayed by these isomers towards the acetylcholinesterase catalysed hydrolysis of acetylcholine is explained on the basis of the binding of a thermodynamically unstable conformation of the *cis*-isomer to the active site. Similar studies employing the β-trimethylammonio-propionate esters of *cis*- and *trans*-4-t-butylcyclohexanol suggest that the "reverse esters" do not bind to the active site in an identical manner to the acylcholines.

Acylcholines have been used in the past to differentiate the cholinesterase enzymes, acetylcholine being a better substrate than butyrylcholine for acetylcholinesterase, whereas the order of activity is reversed for pseudocholinesterase (Glick, 1938, 1941; Nachmansohn & Rothenburg, 1945). The acyl group in such compounds is probably binding to the enzyme surface over and to the side of the esteratic site (see also Ariens & Simonis, 1967) and it thus becomes important to attempt to define the stereochemical requirements for binding to this area. Previous studies using optically asymmetric acylcholines have shown that the enzyme exerts some degree of stereoselectivity when these compounds act as substrates e.g. L-(+)-lactoylcholine is hydrolysed at a rate approximately four times faster than D-(−)-lactoylcholine at their respective optimum substrate concentrations by the enzyme acetylcholinesterase (Auditore & Sastry, 1964), whereas reduced stereoselectivity is shown by pseudocholinesterase (Sastry & White, 1968). There appears however to have been no attempts to employ cyclic compounds devoid of optical asymmetry to study further the geometrical requirements for binding of acylcholines to these enzymes.

It was thus decided to prepare some isomeric acylcholines and to study the effect of geometrical isomerism on inhibitory activity.

The studies of the conformation of 4-t-butylcyclohexane derivatives (for review see Eliel, Allinger & others, 1965) suggest that the *cis*- and *trans*-isomers of 4-t-butylcyclohexanoic acid would provide suitable acyl groups for such studies.

Additionally, Bass, Schueler & others (1950) have shown that the reversed ester of acetylcholine (i.e. methyl β-dimethylaminopropionate methiodide) is a potent cholinomimetic on tissue preparations and is a weak inhibitor of the enzyme acetylcholinesterase. Thus using the *cis*- and *trans*-isomers of 4-t-butylcyclohexanol it is possible additionally to consider the geometrical requirements for inhibitory activity among the reversed esters of acetylcholine.

The compounds shown in Table 1 have therefore been prepared and their anticholinesterase activity determined.

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The preparation of the above esters was accomplished by means of standard synthetic methods, but the stereochemistry or conformational homogeneity of products, or both, was checked whenever possible, by the use of gas-phase chromatography, infrared spectroscopy, and nuclear magnetic resonance (c.f. later section).

Hydrogenation of *p*-t-butylbenzoic acid over platinum oxide at room temperature yields 4-t-butylcyclohexanoic acid enriched in the *cis*-isomer, whereas hydrogenation of the sodium salt over Raney nickel at elevated temperatures and pressure yields a product enriched in the *trans*-isomer (Bekkum, Kleis & others, 1962). The individual geometrical isomers were separated by formation of the thiourea occlusion complex of the *trans*-4-t-butylcyclohexanoic acid (Bekkum, Verkade & Wepster, 1959). The conformational homogeneity of the pure *trans*- and pure *cis*-acids so isolated was checked by gas-phase chromatography of the methyl esters (prepared by means of diazomethane) (Cavell, Chapman & Johnson, 1960), when each sample showed a single peak, with a retention time different from that of its isomer.

The *cis*- and *trans*-4-t-butylcyclohexanoic acids were separately esterified, via the acid chloride, with dimethylaminoethanol. Gas-phase chromatography of the resultant esters showed the "trans" ester contained approximately 5% of the *cis*-isomer and the "cis" ester to be a mixture of the *cis*- and *trans*-isomers in the ratio of approximately 3:1. Eliel & Gerber (1965) have previously suggested that thermal equilibration of 4-t-butylcyclohexanoyl chlorides does take place, presumably via a series of equilibrium reactions as shown in Fig. 1. The results reported here provide further evidence of such an equilibration.

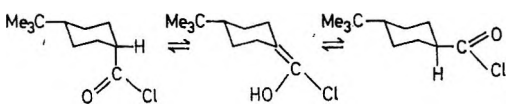


Fig. 1

The retention times on gas-phase chromatography of the *cis*- and *trans*-2-dimethylaminoethyl 4-t-butylcyclohexanoates were sufficiently different to allow a separation on a preparative scale and each purified product was then quaternized with methyl iodide.

The *cis*- and *trans*-isomers of 4-t-butylcyclohexanol were separated by column chromatography (Winstein & Holness, 1955) of a commercial sample of 4-t-butylcyclohexanol (approximately 75% *trans*- and 25% *cis*-isomer). The identity and conformational homogeneity of each product was checked by comparison with published infrared (Eliel & Ro, 1957; Eliel & Rerich, 1960) nmr, (Eliel & Gianni, 1962) and gas-phase chromatographic data (Eliel & Ro, 1957; Roberts 1965). The products were found to be homogeneous by each method.

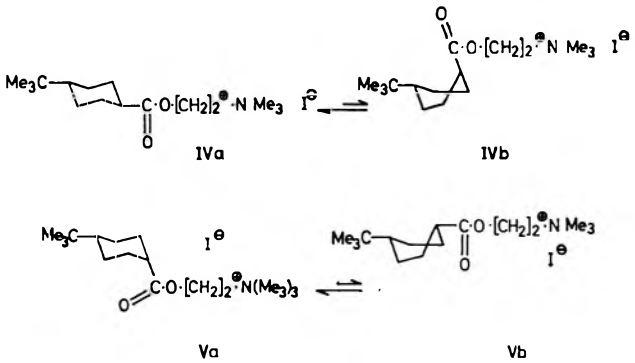
The above alcohols were separately esterified by an ester interchange reaction (Johnson, Paton & Farquharson, 1961) with ethyl β-dimethylaminopropionate. Gas-phase chromatography of the product esters showed the "trans" isomer contained less than 0·5% of "*cis*"-isomer, whereas the "cis" isomer had equilibrated under the reaction conditions and now contained 12% of the "*trans*" isomer. The retention times of the two isomers however were again sufficiently different to allow a separation of the two isomers by means of preparative-scale gas-phase chromatography before they were separately quaternized with methyl iodide.

DISCUSSION

From a comparison of the structures of the acylcholine derivatives with that of acetylcholine, it would be reasonable to assume that the acyl group will interact with an area of the enzyme surface which is close to, but outside, the "active site".

The chemical and physical properties of this area are presently unknown but comparison of the K_I value of compound III with those of compounds IV and V (a lowering of the value of K_I being taken as indicative of stronger binding of the inhibitor to the enzyme) would suggest that this area is hydrophobic in nature, the addition of the t-butyl group having increased the activity by a factor of 10 approximately.

The geometrical requirements for binding to this area are difficult to assess, there being little difference in the activities here reported for compounds IV and V. Although the bulky t-butyl group will act as a "lone handle" to prevent the chair-boat-chair interconversion possible in the parent cyclohexane derivative (III), these compounds in solution could exist in a chair-boat equilibrium conformation.†



Although such a boat conformation is the thermodynamically less stable conformation, this does not preclude such a conformation becoming bound preferentially to the receptor surface if the difference in the free energy of binding of the two conformations Va and Vb exceeds the conformational free energy difference between these two

† The choline moiety could theoretically adopt an infinite number of conformations in solution (for review of possible conformations of acetylcholine c.f. Martin-Smith, Smail & Stenlake, 1967). If the choline moiety in compound V adopts a cyclic conformation (Fig. 2), as suggested by Stenlake for acetylcholine (although no evidence currently exists to substantiate this postulate), the non-bonded interactions due to the bulky axial substituent would cause the equilibrium to be established even more in favour of a boat conformation. However, since the side-chain is common to compounds III, IV, and V, it is possible that this moiety is bound similarly to the "active site" in all the compounds considered, and the problem of the actual conformation of the choline moiety can be neglected in the present discussion.

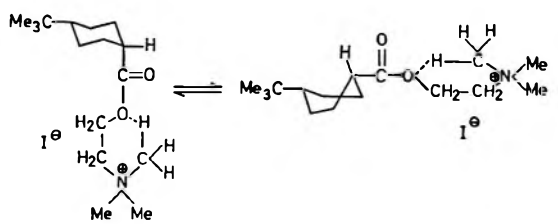


Fig. 2

conformations. Thus a possible explanation of the similar inhibitory potencies of compounds IV and V may lie in a suggestion that the active conformations bound to the receptor area are conformations IVa and Vb respectively. It should be noted here that the "chair" and "boat" conformation are the extremes of an infinite number of conformations of the ring and thus a "twist conformation" of compound V becoming bound to the receptor area is not precluded.

Similar considerations are difficult when applied to the "reversed ester" derivatives. The parent compound (II), although reported to be a potent cholinomimetic (in many tissues equal in potency to acetylcholine), is a weak inhibitor of the enzyme acetylcholinesterase (Bass & others, 1950). It is uncertain at this stage whether the "reversed ester" is binding over the esteratic site of the muscarinic receptor in a manner similar to that of acetylcholine. Schueler, Keasling & Featherstone (1951) have reported that the β-methyl analogue (methyl α-methyl-β-dimethylaminopropionate methobromide) has only about $1/10^4$ of the muscarinic activity of compound II. Comparison of these results with the muscarinic activities reported for acetyl-β-methylcholine (Beckett, Harper & Clitherow, 1963) might suggest that different modes of binding of the drug to this receptor are operative in the cases of acetylcholine and methyl β-dimethylaminopropionate methiodide.

Comparison of the results reported here for the inhibition of acetylcholinesterase by the "reversed esters" and the acylcholine derivatives might also suggest that the two series of compounds are not binding to the enzyme receptor area in identical manners. Although an increase in the lipophilicity of the alkyl-oxygen function produces an increase in the inhibitory potency (compare compounds II, VI, and VII), this increase is not as marked as with acylcholine derivatives (compounds III and IV). A more detailed comparison of the geometrical requirements for binding among these compounds cannot be attempted here, however, since there is a variation in the kinetics of inhibition shown by compound (VIII) at different concentrations.

EXPERIMENTAL

All melting points are uncorrected. Infrared spectra were recorded using a Perkin Elmer Model 237 grating infrared spectrophotometer and nmr spectra were recorded using a Varian A60 spectrometer with tetramethylsilane as internal standard. Gas-phase chromatographic separations were performed by means of a Wilkens Aerograph autoprep Model 705 with flame ionization detector and the following columns *Method A.* 6 ft × ¼ in stainless steel column containing 4% SE30 adsorbed onto Silanized Chromasorb W. *Method B.* 20 ft × ¼ in stainless steel column containing 20% SE30 adsorbed onto Silanized Chromasorb W. *Method C.* 6 ft × ¼ in stainless steel containing Tide detergent (36–60 mesh).

cis-4-*t-Butylcyclohexanoic acid.* Reduction of *p*-t-butylbenzoic acid (12·4 g) dissolved in glacial acetic acid (150 ml) over platinum oxide (1·5 g) at room temperature and atmospheric pressure gave a mixture of isomers (12·67 g) containing predominantly the *cis*-isomer (Bekkum & others, 1962).

The *trans*-isomer was removed as the thiourea occlusion complex (Bekkum & others, 1959) and the *cis*-enriched methanolic filtrate poured into water (300 ml) and extracted with hexane (3 × 200 ml). The combined organic extracts were dried ($MgSO_4$), filtered and evaporated to yield a white solid (8·5 g), m.p. 112–116°. The crude product was purified by sublimation and a material, m.p. 120–121°, was obtained

(2·7 g) (lit. m.p. 116°, Stolow, 1959). Found: C, 72·0; H, 10·5. Calc. for $C_{11}H_{20}O_2$: C, 71·7; H, 10·8%. pK_a = 7·17; 7·25 in 50% ethanol at 25° (lit. pK_a = 6·78; Bekkum & others, 1961).

trans-4-*t-Butylcyclohexanoic acid.* Reduction of *p*-t-butylbenzoic acid (35·6 g) in aqueous sodium hydroxide solution (160 ml, 5% w/v) over Raney nickel at 200° and 100 atmospheres (Bekkum & others, 1962) gave a mixture of isomers containing predominantly the *trans*-isomer. The *trans*-isomer was separated as the thiourea occlusion complex (Bekkum & others, 1959), and the complex decomposed to give *trans*-4-t-butylcyclohexanoic acid (18·0 g), m.p. 173–175° (lit m.p. 176–177°, Stolow, 1959; Bekkum, Verkade & Wepster, 1961). Found: C, 71·3; H, 10·6%. pK_a 6·65; 6·63 in 50% ethanol at 25° (lit. pK_a=6·28; Bekkum & others, 1961).

Methyl cis-4-*t-butylcyclohexanoate. cis*-4-t-Butylcyclohexanoic acid (50 mg) was dissolved in ether (2 ml) and an ethereal solution of diazomethane added until the solution was a persistent yellow colour. After standing overnight at room temperature, the ether was removed and the residue micro-distilled at 10 mm pressure and at a bath temperature of 120° (Cavell & others, 1960).

On gas-phase chromatography (method A, using N_2 as carrier gas at 50 ml/min; injector temperature 220°; column temperature 145°; detector temperature 230°; collector temperature 220°), the product showed a single peak, retention time 2·6 min.

Methyl trans-4-*t-butylcyclohexanoate.* This ester was prepared as for methyl *cis*-4-t-butylcyclohexanoate, but using *trans*-4-t-butylcyclohexanoic acid (100 mg). On gas-phase chromatography (conditions as for methyl *cis*-ester), the material showed only one peak, retention time 3·2 min.

2-*Dimethylaminoethyl* cis-4-*t-butylcyclohexanoate. cis*-4-t-Butylcyclohexanoic acid (1·5 g, m.p. 120–121°) was added to thionyl chloride (10 g) and the mixture allowed to stand at room temperature overnight. The excess of thionyl chloride was removed under reduced pressure at a temperature not exceeding 50° to leave an oily product, which was dissolved in dry benzene (15 ml) and added dropwise during 10 min to 2-dimethylaminoethanol (0·83 g) in benzene (10 ml). The mixture was then heated at a temperature not exceeding 95° for 30 min, cooled and basified with sodium hydroxide solution. The benzene layer was separated and the aqueous phase extracted with ether (3 × 100 ml). The combined organic extracts were dried ($MgSO_4$), filtered and the solvents distilled off. The oily residue was fractionally distilled under reduced pressure, and the fraction, $b.p._{9}$ = 168°, collected (1·68 g); ν_{max} (liquid film) 1740 cm^{-1} (C = O), *picrate* (from ethanol), m.p. 155–157°. Found C, 53·4; H, 6·4. $C_{22}H_{32}N_4O_9$ requires C, 53·2; H, 6·45%.

Gas-phase chromatography of the product ester (method B, using N_2 as carrier gas at 30 ml/min; injector temperature 260°, column temperature 230°; detector temperature 260°; collector temperature 290°), showed the product to contain both *cis*- and *trans*-isomers in the ratio of 3 : 1 (retention times; *cis*-isomer, 15·3 min, *trans*-isomer, 18·3 min). Preparative-scale gas-phase chromatography of the product allowed the isolation of the chromatographically homogeneous *cis*-isomer (0·71 g.).

2-*Dimethylaminoethyl* trans-4-*t-butylcyclohexanoate.* This compound was prepared in a manner similar to that used for the preparation of the *cis*-isomer, but starting with *trans*-4-t-butylcyclohexanoic acid (1·8 g. m.p. 172–175°). The 2-dimethylaminoethyl *trans*-4-t-butylcyclohexanoate was obtained as an oil, $b.p._{11}$ = 166° (1·52 g); ν_{max} (liquid film) 1741 cm^{-1} (C = O), *picrate* (from ethanol), m.p. 158–159°. Found: C, 53·4; H, 6·0%.

Gas-phase chromatography of the product ester (method B, conditions as for *cis*-isomer) showed the product to contain less than 2% of the *cis*-isomer. A small sample was purified by preparative-scale gas-phase chromatography (method B) and converted to the methiodide, m.p. 227° (c.f. preparation of 2-dimethylaminoethyl *trans*-4-t-butylcyclohexanoate methiodide).

2-*Dimethylaminoethyl* cis-4-*t-butylcyclohexanoate methiodide* (V). To 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate (0·70 g, purified by gas-phase chromatography) in ether (20 ml) was added methyl iodide (10 g). The resulting solid product (1·07 g) was collected and recrystallized from absolute ethanol to yield 0·72 g of product as white crystalline plates, m.p. 188°. Found: C, 48·65; H, 8·1. $C_{16}H_{32}INO_2$ requires C, 48·4; H, 8·1%.

2-*Dimethylaminoethyl* trans-4-*t-butylcyclohexanoate methiodide* (IV). This material was made in a manner similar to that used for the preparation of the *cis*-isomer, but using 2-dimethylaminoethyl *trans*-4-t-butylcyclohexanoate (0·8 g, containing approximately 2% of the *cis*-isomer). After recrystallization from absolute ethanol, 1·0 g, of white crystalline plates, m.p. 227°, were obtained. (c.f. preparation of above material using tertiary amino-ester purified by gas-phase chromatography). Found: C, 48·3; H, 8·0%.

2-*Dimethylaminoethyl cyclohexanoate.* This was prepared in a manner similar to that used for the preparation of 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate but using cyclohexanoic acid (4·0 g) and thionyl chloride (10 g). The crude cyclohexanoyl chloride (4·3 g) was reacted, in benzene, with 2-dimethylaminoethanol (3·0 g) to yield 2-dimethylaminoethyl cyclohexanoate (3·4 g), b.p. $_{0\cdot1}$ = 78°; ν_{max} (liquid film) 1740 cm^{-1} (C = O). Equivalent weight, found: 201·1; Calculated for $C_{11}H_{12}NO_2$ = 199.

2-*Dimethylaminoethyl cyclohexanoate methiodide* (III). Prepared in a manner similar to that used for the preparation of 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate methiodide but starting with 2-dimethylaminoethyl cyclohexanoate (2·26 g). The product was recrystallized from ethanol to yield white plate-like crystals, m.p. 173–174°. Found: C, 42·7; H, 7·2. $C_{12}H_{24}INO_2$ requires C, 42·2; H, 7·0%; $C_{12}H_{124}INO_2{\cdot}\frac{1}{2}C_2H_5OH$ requires C, 42·9; H, 7·4%.

Separation of cis- *and* trans-4-*t-butylcyclohexanol.* 4-t-Butylcyclohexanol (29·5 g) (commercial mixture of isomers) was placed on an activated alumina column (1080 g, Peter Spence, Type H, 100/200 mesh.), using pentane. The column was eluted with pentane, followed by pentane containing increasing amounts of ether. Fractions, each of 200 ml, were collected.

cis-4-t-Butylcyclohexanol (5·9 g) was eluted in fractions 217–281 (pentane 60%, ether 40%). Fractions 312 to exhaustion (pentane 60%, ether 40%) gave *trans*-4-t-butylcyclohexanol (17·42 g).

The products were identified by the following properties: m.p. *cis*-isomer 82–83° (lit. m.p. 81–82°, Winstein & Holness, 1955; 82–82·5°, Stork & White 1956; 82·5–83·5° Eliel & Ro, 1957), *trans*-isomer 79–80° (lit m.p. 78–79°, Winstein & Holness, 1955; 81–82°, Eliel & Ro, 1957).

Infrared spectra (CS_2 solution): *cis*-isomer, characteristic peaks at 1010 and 960 cm^{-1}; *trans*-isomer, characteristic peaks at 1060 and 980 cm^{-1} (lit. *cis*-isomer 1010 and 951 cm^{-1} and *trans*-isomer 980 cm^{-1} in CS_2 solution; Eliel & Rerich, 1960; Eliel & Ro, 1957).

Nmr (CCl_4 solution). *cis*-isomer, 6·10 τ (1 proton-multiplet-equatorial C_1 proton) $W\frac{1}{2}$ = 6 Hz *trans*-isomer 6·48–6·80 τ (broad, 1 proton-multiplet-axial C_1 proton) ($W\frac{1}{2}$ = approx. 16 Hz) (lit. *cis*-isomer 6·1 τ, $W\frac{1}{2}$ = 7 Hz, *trans*-isomer 6·63 τ, $W\frac{1}{2}$ = 22 Hz, Lemieux, Kulling & others, 1958; Eliel & Gianni, 1962).

Gas-phase chromatography (method C, N_2 as carrier gas at 30 ml/min; injector temperature 180°; column temperature 160°; detector temperature 185°. Retention time: *cis*-isomer 3·12 min, *trans*-isomer 4·4 min.

Ethyl β-dimethylaminopropionate. Dimethylamine (152·2 ml; 103·5 g; 2·3 mole) and ethyl acrylate (250 ml; 230 g; 2·3 mole) were mixed and the mixture allowed to stand at room temperaure for four days. The reaction mixture was fractionally distilled, the ethyl β-dimethylaminopropionate, b.p.$_{21}$ = 73°, being collected. The product was redistilled (b.p.$_{24}$ = 74°); yield 147 g (lit b.p.$_{12}$ = 56–57°, Adamson, 1949), ν_{max} (liquid film) 1747 cm^{-1} (C = O). Equiv. wt, found: 144·5; 145·1; calculated for $C_7H_{15}NO_2$ = 145.

trans-4-*t-Butylcyclohexyl β-dimethylaminopropionate.* Ethyl β-dimethylaminopropionate (4·83 g) and *trans*-4-t-butylcyclohexanol (5·2 g) were dissolved in xylene (30 ml) and the mixture distilled through a fractionating column until the temperature at which the distillate passed over reached 136°. Sodium (0·1 g) was then added to the residual reaction mixture and the ethanol distilled off. This required about 20 min, by which time the temperature recorded at the head of the fractionating column had again risen to 136°. The reaction mixture was heated for a further hour, cooled, an equal volume of water added and the mixture acidified with dilute HCl and then extracted with ether (3 × 150 ml). The aqueous phase was made alkaline with ammonia solution and extracted with ether (3 × 150 ml). The ether extracts were dried ($MgSO_4$), filtered and the solvent distilled to leave an oil, which was fractionally distilled, the *trans*-4-t-butylcyclohexyl β-dimethylaminopropionate, b.p.$_{0·1}$ = 108–109°, being collected (2·37 g), ν_{max} (liquid film) 1740 cm^{-1} (C = O). Equiv. wt, found: 263; 265; Calculated for $C_{15}H_{29}NO_2$ = 255. *Picrate*, an oil. Gas-phase chromatography (method B) showed the presence of less than 0·5% of *cis*-isomer (retention time; *cis*-isomer, 15·2 min; *trans*-isomer, 18·2 min) and the product was considered to be sufficiently pure for the preparation of the methiodide.

cis-4-*t-Butylcyclohexyl β-dimethylaminopropionate.* This compound was prepared in a manner similar to that used for the preparation of the *trans*-isomer, but using *cis*-4-t-butylcyclohexanol (2·6 g) and ethyl β-dimethylaminopropionate (2·4 g). The product was fractionally distilled, the fraction, b.p.$_{0·1}$ = 104°, being collected; yield 1·04 g. ν_{max} (liquid film) 1745 cm^{-1} (C = O). Gas-phase chromatography (method B) showed the product to contain approximately 12% of the *trans*-isomer. The product was then purified by gas-phase chromatography (preparative scale), whereupon 0·42 g of pure material was obtained. *Picrate* (from ethanol), m.p. 145–147°. Found: C, 52·8; H, 6·35. $C_{22}H_{32}N_4O_9$ requires C, 53·2; H, 6·45%.

Cyclohexyl β-dimethylaminopropionate. This compound was prepared in a manner similar to that used in the preparation of *trans*-4-t-butylcyclohexyl β-dimethylaminopropionate, but using cyclohexanol (3·34 g) and ethyl β-dimethylaminopropionate (4·83 g). The product was fractionally distilled, the fraction, b.p.$_{11}$ = 126°, being collected; yield 3·59 g. Equiv. wt, found: 206·5; calculated for $C_{11}H_{21}NO_2$ = 199.

trans-4-*t-Butylcyclohexyl β-dimethylaminopropionate methiodide* (VII). To *trans*-4-t-butylcyclohexyl β-dimethylaminopropionate (1·71 g) (containing less than 0·5% of the *cis*-isomer) in ether (20 ml) was added methyl iodide (10 g). The resulting solid

was filtered and recrystallized from absolute ethanol to yield white crystals, m.p. 192–196°. Found; C, 48·5; H, 8·2 $C_{16}H_{32}INO_2$ requires C, 48·4; H, 8·1%. Nmr (in deuterated DMSO) 5·58–5·92 τ (broad 1 proton multiplet—axial C_1 proton).

cis-4-*t-Butylcyclohexyl β-dimethylaminopropionate methiodide* (VIII). This was prepared in a manner similar to that used for the preparation of the *trans*-isomer, but using *cis*-4-t-butylcyclohexyl β-dimethylaminopropionate (0·37 g purified by gas-phase chromatography method B). Recrystallization gave a product, m.p. 200–202°, as a white crystalline material. Found: C, 47·6; H, 8·0. $C_{16}H_{32}INO_2$ requires C, 48·4; H, 8·1%; $C_{16}H_{32}INO_2,\frac{1}{2}H_2O$ requires C, 47·3; H, 8·1%. Nmr (in deuterated DMSO) 5·02 τ (1 proton multiplet-equatorial C_1 proton).

Cyclohexyl β-dimethylaminopropionate methiodide (VI). This compound was prepared in a manner similar to that for the *trans*-isomer, but using cyclohexyl β-dimethylaminopropionate (2·16 g). The product was recrystallized from absolute ethanol to give plates, m.p. 146°. Found: C, 42·8; H, 7·4. $C_{12}H_{24}INO_2$ requires C, 42·2; H, 7·0%; $C_{12}H_{24}INO_2,\frac{1}{2}C_2H_5OH$ requires C, 42·85; H, 7·4%.

Methyl β-dimethylaminopropionate methiodide (II). Ethyl β-dimethylaminopropionate (20 g) was dissolved in conc. HCl (30 ml) and refluxed overnight. The solution was evaporated to dryness and the residue dissolved in dry methanol. The solution was saturated with dry HCl gas and then allowed to stand overnight. The reaction mixture was poured into water, the solution made alkaline with solid Na_2CO_3 and extracted with chloroform (3 × 100 ml). The extracts were dried ($MgSO_4$), filtered and the solvent distilled to leave a liquid, which was fractionally distilled, the methyl β-dimethylaminopropionate, b.p. 148–152°, being collected (lit. b.p. 151·5–154°, Halverstadt, Hardie & Williams, 1959). The ester (5 g) was dissolved in methanol (10 ml) and methyl iodide (10 g) added. The precipitated solid was collected and recrystallized from absolute ethanol to yield a product, m.p. 198–200° (lit. m.p. 194–195° Halverstadt & others, 1959). Found: C, 31·1; H, 6·0. $C_7H_{16}NIO_2$ requires C, 30·8; H, 5·9%.

ENZYMIC STUDIES

Bovine erythrocyte acetylcholinesterase (Sigma Chemicals) was used throughout the work.

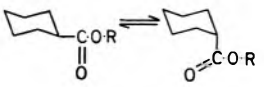
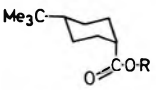
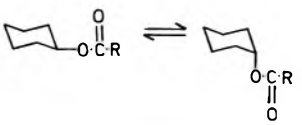
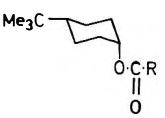
Acetycholine perchlorate (B.D.H.) was used as substrate and the rates of hydrolysis were measured by the pH-stat method, (Alles & Hawes, 1940), using an automatic titrator (type TTTIC), equipped with a recorder (SBR2C) and syringe burette (SBU1A) (Radiometer, Copenhagen).

All incubations were carried out in a total volume of 25 ml of enzyme solution previously made 0·04 M in $MgCl_2$ and 0·05 M in NaCl. The incubations were carried out in jacketted vessels at 25 ± 0·1° and the pH maintained at 7·4 by the addition of 0·02 N NaOH. A CO_2-free nitrogen atmosphere was maintained throughout the experiments.

All inhibitors were pre-incubated with the enzyme for 3 min and the reaction started by the addition of acetylcholine solution. The velocity of the reaction was calculated from the average slope of the recording during the second and third minutes of the incubation. The velocities were all corrected for aqueous hydrolysis.

The K_m value for acetylcholine, under these conditions, was found to be $4·45 \times 10^{-4}$. The K_I values for the inhibitors were calculated from Lineweaver-Burk plots (Lineweaver & Burk, 1934), using a four-fold range of substrate concentration and an

Table 1. *Enzyme-inhibitor dissociation constants* (K_1) *of acylcholines and β-trimethylammoniopropionic acid derivatives*

	Compound	K_i	Nature of inhibition
	$R=[CH_2]^{\oplus}N\,Me_3\ I^{\ominus}$		
I	Me·CO·O·R	$K_m = 4{\cdot}45 \times 10^{-4}$ $V_{max} = 2{\cdot}26 \times 10^{-6}$ mole/min	Substrate
II	Me·O·CO·R	$3{\cdot}95 \times 10^{-3}$	Competitive
III		$1{\cdot}8 \times 10^{-3}$	Competitive
IV		$1{\cdot}5 \times 10^{-4}$	Competitive
V		$1{\cdot}9 \times 10^{-4}$	Competitive
VI		$2{\cdot}3 \times 10^{-3}$	Competitive
VII		$1{\cdot}3 \times 10^{-3}$	Competitive
VIII		$1{\cdot}3 \times 10^{-4}$ (low concentrations $1{\cdot}4$–$2{\cdot}0 \times 10^{-4}$ mole) (At higher concentrations shows mixed inhibition).	Competitive

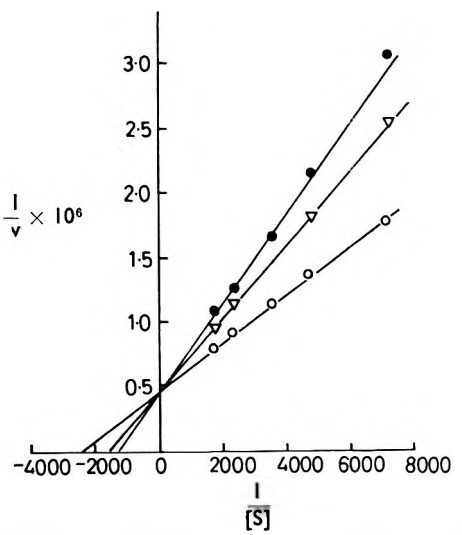


FIG. 3. Competitive Inhibition of acetylcholinesterase by two concentrations of 2-dimethylaminoethyl *cis*-4-t-butylcyclohexanoate methiodide. ●[I] = $1{\cdot}48 \times 10^{-4}$M ▽[I] = $8{\cdot}30 \times 10^{-5}$M. ○[I] = O.

approximately two-fold range of inhibitor concentration. The K_I values were computed from the equation:

$$\text{Gradient} = \frac{K_m\left(1 + \frac{[I]}{Ki}\right)}{V_{max}}.$$

The computed results are shown in Table 1 and a typical Lineweaver-Burk plot obtained for one of the inhibitors in Fig. 3.

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The corticotrophic activity of tetracosactide in the adrenal ascorbic acid depletion test

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Using the subcutaneous adrenal ascorbic acid depletion test in hypophysectomized rats it was found that tetracosactide (synthetic 1–24 corticotrophin) could not be assayed against the International Working Standard (IWS) for natural corticotrophin because of a difference in the slopes of the dose-response curves of the preparations. Valid potency estimates were obtained when tetracosactide was assayed against a standard preparation closely similar to the 2nd International Standard for natural corticotrophin. By comparing this standard with the IWS, the best estimate of the potency of tetracosactide in terms of the IWS was 99 (92–106) units/mg. Five different batches of tetracosactide have yielded identical biological potencies. Since tetracosactide can be adequately standardized by physical and chemical-analytical criteria, labelling and dosage on a mg base instead of in units is recommended.

Peptides with amino-acid sequences corresponding to the N terminal 20 to 24 amino-acids of corticotrophin possess strong corticotrophic activity (Schwyzer, 1964). The member of this group of peptides investigated most extensively is undoubtedly tetracosactide.* In a publication on the pharmacological behaviour of tetracosactide a potency of 100 U/mg has been reported (Schuler, Schär & Desaulles, 1963). This value was established in the adrenal ascorbic acid depletion test, using subcutaneous administration. In these assays a standard preparation of the same type as the International Working Standard was used. No indication of a deviation of parallelism between the log dose response curves was found. However other studies indicate that the shorter peptide shows a pharmacokinetic behaviour different from that of the longer chain of corticotrophin (Rerup, 1966).

This observation is in agreement with data obtained on peptides derived from the natural hormone by pepsin digestion (Hays & White, 1954). Although the connection between ascorbic acid depletion and steroidogenesis is far from being elucidated, the ascorbic acid depletion test is widely employed for assaying biological potencies of corticotrophin. This test provides the basis on which clinical potency of natural corticotrophin preparations can be predicted. Therefore an exact evaluation of tetracosactide in terms of the existing International Working Standard was found desirable. In this paper it will be shown that the slope of the log dose response curve of tetracosactide in the subcutaneous adrenal ascorbic acid depletion test differs from that of the International Working Standard so markedly that in many instances valid potency estimates cannot be obtained. A crude corticotrophin preparation resembling the abandoned 2nd International Standard showed a log dose response curve much more like that of the synthetic tetracosactide, making calculation of relative potencies possible.

* Tetracosactide is the proposed international non-proprietary name for the peptide with the amino-acid sequence of the N terminal 24 amino-acids of corticotrophin. This sequence is the same for all species in which it has been studied, including man.

EXPERIMENTAL

Materials and methods

In this work the following preparations were used.

Tetracosactide. Different production batches of 1-24 corticotrophin were prepared according to Kappeler & Schwyzer (1961). These batches were characterized and standardized *inter alia* by amino-acid analysis, ultraviolet spectrum, specific rotation, electrophoresis, acetic acid, water- and peptide content.

Crude porcine corticotrophin, batch NH 160, having a potency of about 3 IU/mg was prepared from hog pituitaries by the acid-acetone extraction technique of Lyons (1937). Of this preparation a laboratory standard was made, consisting of vials each containing exactly the same amount (about 7 mg) of material which was assayed in the subcutaneous adrenal ascorbic acid depletion test against the 2nd International Standard. Each vial contained 23·4 U with a confidence interval ($P = 0{\cdot}95$) of 20·7 to 26·5.

Carboxymethylcellulose-purified porcine corticotrophin (de Jager, Homan & de Wied, 1963), batch 1094, having a potency of 86 (75–99) U per mg in the subcutaneous test compared to the International Working Standard.

Enzymatically hydrolysed carboxymethylcellulose-purified corticotrophin preparations were made by peptic and carboxypeptidase hydrolysis and purified by column chromatography on carboxymethylcellulose. Preparations with predominantly the 1–31 and 1–26 sequence of porcine corticotrophin had the following potencies in the subcutaneous test using the 2nd International Standard as a reference: Predominantly 1–31 sequence: 106 (89–127) U/mg; 148 (127–172) U/mg. Predominantly 1–26 sequence: 99 (69–177) U/mg; 84 (57–137) U/mg.

Subcutaneous adrenal ascorbic acid depletion tests were made according to the directions given in the U.S. Pharmacopeia XVI (1960) with a few minor modifications. A randomized block design was used and the results were calculated according to the rules given in the USP for such a design. Results of individual assays on the same preparation were tested for homogeneity and combined according to Meier (1953) using the modification of Cochran & Carroll (1953). Usually, the assays were repeated until the fiducial limits of the total estimate, obtained by combination of the individual assays on the preparation, were 87 and 115% of the estimated potency.

RESULTS

Soon after the estimation of corticotrophin activity of tetracosactide, using the subcutaneous ascorbic acid depletion test, was started it became apparent that, when the International Working Standard was used, many results had to be discarded because the tests did not fulfil the criterion of slope parallelism. As it is well known that slope differences exist between crude and carboxymethylcellulose-purified corticotrophin as well as between the latter and hydrolysed purified preparations, the slope of tetracosactide was compared with slopes of various other preparations to classify the behaviour of tetracosactide in this test. In Table 1 the median slopes of different corticotrophin preparations, found in a number of assays, are given. Each line comprises figures obtained in the same assay. The difference between the slopes of the Working Standard and the 2nd International Standard is obvious; so is the fact that the slopes of the latter, and also tetracosactide, crude corticotrophin and the hydrolysed preparations, are similar.

Table 1. *Medians of slopes of different types of corticotrophins in the subcutaneous adrenal ascorbic acid depletion test, using gelatin (16% w/v) as a diluent*

Number of assays	Standard preparation		Tetracosactide	Crude corticotrophin	Hydrolysed corticotrophin*
	IWS	2nd IS			
38	—225		—130		
36	—207			—135	
8	—221				—115
15		—144			—137
17		—181	—124		

IWS = International Working Standard.

2nd IS = Second International Standard.

* Enzymatically hydrolysed carboxymethylcellulose purified corticotrophin preparation, containing polypeptides with amino-acids 1–26 to 1–31.

Table 2. *Slopes of log dose response curves of tetracosactide and NH* 160

Assay number	Slope: Unknown b_u	Slope: Standard b_s	Slope difference $b_s - b_u$	Confidence limits of slope difference ($P = 0.95$)	
1	—192·1	—112·4	79·7	—22·6	182·0
2	—108·0	—104·7	3·3	—95·4	102·0
3	—150·9	—104·7	46·2	—52·5	144·9
4	—132·6	—109·0	23·6	—59·8	107·0
5	—122·1	—153·1	—31·0	—114·5	52·5
6	—159·9	—139·9	20·0	—76·0	116·1
7	—120·1	—129·8	—9·7	—133·7	114·3
8	—88·3	—84·7	3·6	—94·4	101·6
9	—103·5	—84·7	18·8	—79·2	116·8
10	—132·6	—46·2	86·4	—29·7	202·5
11	—114·0	—46·2	67·8	—48·3	183·9
12	—134·0	—41·5	92·5	—4·9	189·9
13	—105·2	—41·5	63·7	—33·7	161·1
14	—119·3	—214·3	—95·0	—200·1	10·1
15	—142·3	—214·3	—72·0	—177·1	33·0
16	—102·4	—114·1	—11·7	—106·6	83·2
17	—71·4	—114·1	—42·7	—137·6	52·2
18	—128·7	—98·6	30·1	—87·6	147·8
19	—134·8	—98·6	36·2	—81·5	153·9
20	—113·0	—147·4	—34·4	—120·2	51·4
21	—101·3	—147·4	—46·1	—113·2	21·0
22	—77·7	—169·8	—92·1	—192·8	8·6
23	—64·8	—105·5	—40·7	—175·0	93·6
24	—16·1	—211·5	—195·4	—405·7	14·9
25	—97·2	—211·5	—114·3	—324·6	96·0
26	—144·0	—157·0	—13·0	—99·0	73·0
27	—118·8	—157·0	—38·2	—124·2	47·8
28	—219·8	—160·3	59·5	—62·9	181·9
29	—137·0	—160·3	—23·3	—145·7	99·1
30	—87·8	—124·0	—36·2	—140·0	67·6
31	—83·3	—124·0	—40·7	—144·5	63·1
32	—140·4	—108·5	31·9	—49·9	113·7
33	—158·1	—91·4	66·7	—67·6	201·0
34	—86·0	—91·4	—5·4	—139·7	128·9
35	—106·5	—85·8	20·7	—91·7	131·1
36	—118·8	—85·8	33·0	—78·4	144·4
37	—194·4	—189·6	4·8	—104·5	114·1
38	—128·7	—189·6	—60·9	—170·2	48·4
39	—85·5	—88·9	—3·4	—103·6	96·8
40	—130·4	—88·9	41·5	—58·7	141·7
41	—117·9	—60·6	57·3	—37·1	151·7
42	—122·1	—60·6	61·5	—32·9	155·9
Mean	—119·—	—111·—	—1·37 ± 1·32		

These findings indicated that tetracosactide should be tested against the 2nd International Standard to obtain valid assays. Because of the limited availability of the latter preparation, a laboratory standard (NH 160) was prepared from hog pituitaries. It had about the same purity as the 2nd International Standard against which it was assayed and found to contain 23·4 (20·7–26·5) U/vial. However, it was essential to know the activity of tetracosactide in terms of the present Working Standard since the 2nd International Standard is no longer in use. Although an exact relation between the activities of these two standards cannot be established by means of the subcutaneous adrenal ascorbic acid depletion test, it should be kept in mind that the WHO used this test as a guide for the assignment of the potency of the Working Standard in terms of the 2nd International Standard (Bangham, Musset & Stack-Dunne, 1962). In the same way an evaluation of the potency of the laboratory standard in terms of the Working Standard was made, applying experience obtained in the past, when the activities of many purified preparations had to be compared to that of the 2nd International Standard then generally in use. According to this line 20 U per vial seemed to be the best estimate of the laboratory standard in terms of the Working Standard.

Using this reference preparation, 42 assays of tetracosactide were made. In Table 2 the slopes of the log dose response curves are given.

None of the assays had to be discarded because of slope difference and the mean value of $b_s - b_u$ is close to zero. In Table 3 potencies of different batches of tetracosactide are given. As they correspond closely, a combined estimate of 99 (92–106) U/mg can be regarded as the best estimate of the potency of tetracosactide in terms of the Working Standard. This value is in close agreement with the value of 106 IU/mg reported by Schuler & others (1963).

Table 3. *Assay of 5 different batches of tetracosactide against NH 160*

Batch of tetracosactide	½ L*	Potency† U per mg
A	0·057	104 (92–119)
B	0·065	96 (83–111)
C	0·063	101 (87–117)
D	0·064	92 (80–106)
E	0·070	86 (73–101)
Combined result of 5 batches		99 (92–106)

* ½L is half the length of the confidence interval in logarithms. 87–115% confidence limits correspond to ½ L of 0·061.

† Confidence interval $P = 0{\cdot}95$ between parentheses.

DISCUSSION

The results presented show that owing to a slope difference, tetracosactide cannot be standardized against the International Working Standard of corticotrophin using the subcutaneous adrenal ascorbic acid depletion test. Hence we have to face the fact that pharmacokinetic behaviour of the two preparations in the rat is sufficiently different to render biological assays of this kind fundamentally invalid. Inspection of Table 1 reveals that enzymatic hydrolysis of purified corticotrophin to a mixture of peptides with chain lengths comparable to that of tetracosactide lowered the slope in the adrenal ascorbic acid depletion test to the same level. Obviously, a relation exists between the slope of the dose response curve and the length of the peptide chain.

This does not, however, explain the existing slope difference between crude and purified corticotrophin.

From the similarity of the slopes of tetracosactide and the 2nd International Standard, or a preparation of a similar nature, it was deduced that, if these preparations were to be standardized against one another, valid results would be obtained. This was proved in 42 assays.

Although this eliminated the problem as to which standard was to be used for tetracosactide it did not solve the problem of evaluating tetracosactide in terms of the Working Standard of corticotrophin, now in general use. This could not simply be done by calculation because of the existing difference in slope between the 2nd International Standard and the Working Standard (Bangham & others, 1962). Therefore, the only possibility remaining was to assess the activity of NH 160 in terms of the Working Standard using an equivalence which had presented itself from many similar comparisons of crude and purified corticotrophin preparations. The best estimate seemed to be 20 Units/ampoule. In fact this figure should be considered as an approximation of an entity not attainable in any exact manner.

Using NH 160 as a standard, it was found that all batches of tetracosactide investigated showed the same biological potency in assays, with a high degree of precision. This result is not unexpected since the synthesis of tetracosactide and its intermediates can be adequately controlled by physical and analytical chemical methods. This constancy of quality of the synthetic product, which has now been confirmed by biological testing, renders regular bioassay of batches superfluous and allows dosage of the preparation on a milligram basis instead of in international units. This can be regarded as a distinct advantage over the natural product, i.e. corticotrophin. The latter contains a mixture of biologically active peptides which cannot be characterized sufficiently by chemical means, so that a biological standardization is essential.

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Buccal absorption and other properties of pharmacokinetic importance of imipramine and its metabolites

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Imipramine and its major unconjugated metabolites have been characterized by their buccal absorption, partition between water and organic solvents—both as a function of pH—and their ionization constants. Furthermore new data are presented on the selective passage of these compounds across the blood brain barrier and on renal excretion as a function of urinary pH in the rat. The shape of the curves of buccal absorption against pH is characteristic for each one of the ten compounds tested and reflects both lipophility of the unionized compound and the pK value. The value of the buccal absorption test for interpreting or predicting pharmacokinetic behaviour of a drug is emphasized.

Overwhelming evidence has been accumulated in past years that absorption, distribution, and renal excretion of most drugs are governed by simple diffusion through lipoid membranes (Brodie & Hogben, 1957; Schanker, 1961, 1962; Vogt, 1967; Parke, 1968). The pH-partition hypothesis for weak electrolyte drugs is based on the observation that only the un-ionized molecules can easily permeate membranes. Diffusion rate therefore is governed by two key properties: degree of ionization (which is dependent on pK of the drug and pH of the media separated by the membrane), and lipid solubility of the un-ionized form of the drug.

Traditionally the partition of a drug between an organic and aqueous phase has been used to obtain gross information on the lipid solubility of a drug at different pH values. Beckett & Triggs (1967) and Beckett, Boyes & Triggs (1968) have used buccal absorption in man to obtain the same information. This test seems to be superior to partition. Its chief advantage is the use of a biological membrane instead of an organic solvent. The buccal absorption test thus has provided us with a better means of characterizing drugs by their behaviour at the membranes of the gastrointestinal tract, renal tubules, blood brain barrier, tissue and cell boundaries.

The metabolism of a drug frequently leads to the formation of a multitude of metabolites. They occur simultaneously and are redistributed, localized and excreted according to their specific diffusion rates. In a recent study (Bickel & Weder, 1968) on the total fate of imipramine in the rat, the formation of 14 metabolites was detected. They cover a wide range of polarity which obviously governs the patterns of their individual distribution, localization and renal excretion.

The present paper is a comparative study of some properties of a family of derivatives occurring together in the body after administration of imipramine. Results are given on buccal absorption, on partition values as a function of pH, and on ionization constants. In addition data on the passage through the blood brain barrier and on the influence of urinary pH upon renal excretion are given. The data are discussed in terms of interpretation and prediction of pharmacokinetic processes.

EXPERIMENTAL

Materials

Imipramine (IP) and its following metabolites were used: Desipramine (DMI), desdimethylimipramine (DDMI), imipramine-*N*-oxide (IPNO), iminodibenzyl (IDB) and the hydroxylated metabolites 2-OH-IP, 2-OH-DMI, 10-OH-IP, 10-OH-DMI, 2-OH-IDB. With the exception of 2-OH-DMI-fumarate, all compounds used were the hydrochloride salts.

Buccal absorption test

General method. In analogy with the indications given by Beckett & Triggs (1967), 0·5 ml drug solution equivalent to 1 mg base was mixed with 24·5 ml buffer solution and agitated in the subject's mouth for 5 min. After expelling the solution and combining with 10 ml of rinsing water (10 s) the solution was adjusted to 50 ml (2-OH-IP, 2-OH-DMI), 100 ml (IDB, 10-OH-IP, 10-OH-DMI) or 200 ml (others) respectively. The following buffer solutions were used (Documenta Geigy, 1960): Sörensen's phosphate buffer pH 5·5, 6·5, 7·4, 8·2, citrate I pH 4·5, borax-phosphate pH 9·0. Three male and three female subjects, aged 17 to 40 were used.

Analytical techniques. 2-OH-IP, 2-OH-DMI: 10 ml aliquots were titrated to pH 10·0 and extracted with three 5 ml portions of peroxide-free diethyl ether. 5 ml of organic phase was extracted with 5 ml HCl (0·1 N). Extinction was measured at 249 nm.

All compounds except the non-fluorescent 2-OH-IP and 2-OH-DMI: 2 ml aliquots were mixed with 0·5 ml NaOH (2 N) (except for 2-OH-IDB). Fluorescence was measured directly with an Aminco-Bowman spectrophotofluorometer at 295/415 nm. With both methods linear standard curves were obtained. Only 2-OH-IDB was pH-dependent. Blank fluorescence due to saliva contamination was almost negligible. Specific fluorescence in relative units per 1 μg/ml was as follows: 2-OH-IDB (3·6–4·7, pH dependent), DMI (4·44), IP (4·14), DDMI (3·78), IPNO (3·72), IDB (2·31), 10-OH-IP (1·35), 10-OH-DMI (1·23). Buccal absorption of IP (pH 8·2) after 1, 2, 5, and 10 min was 46, 56, 64, and 67%. The absence of metabolic conversion of the drugs in the mouth has been proved for all substances used, by thin-layer chromatography according to Bickel & Weder (1968).

Partition experiments

Organic solvents and the following isotonic buffer solutions were used (Documenta Geigy, 1960): Sörensen's phosphate buffer (pH 5–7), borax-phosphate (pH 7–8), glycine II (pH 9–12·5). Previous to partition experiments the organic phases were presaturated by shaking with the same volume of the corresponding aqueous phase for 5–10 min. The substances were dissolved in 5 ml of aqueous phase (40 μg/ml) of a given pH and partitioned by shaking this solution with 5 ml of the organic solvent for 30 min at 20 $\pm$ 2°. Drug concentrations were determined by ultraviolet spectrophotometry of the aqueous phase before partition and of both phases after partition. The extinctions were read at 288 nm for IDB and 2-OH-IDB, and at 249–251 nm for all other compounds. The concentrations were evaluated with linear standard curves. Eight experiments were run per substance and pH value.

Ionization constants

Solutions of the substances (5×10^{-3} M) were prepared with double distilled water. 10 ml of the solutions were titrated with NaOH ($5 \cdot 10^{-2}$ M) in a Metrohm Combititrator 3 D, continuously recording the titration curve. 2–6 experiments were made per

substance and the pK_a values were evaluated from the curves. Controls with dibenzepine, amphetamine, ephedrine, and hydrazine yielded pK_a values in agreement with the literature.

Passage through the blood brain barrier

Male Wistar rats (220–260 g) and guinea-pigs were given high doses of IP or its metabolites by different routes of administration. At different times, brain and extracerebral tissues were homogenized, extracted and submitted to thin-layer chromatography as described by Bickel & Weder (1968). The exclusion from brain of certain metabolites was detected by comparing metabolite patterns of brain and of extracerebral compartments, such as tissues, plasma, excreta.

Renal excretion and urinary pH

Male Wistar rats (about 250 g) were given orally 2·5—5·0 m-equiv NH_4Cl, $NaHCO_3$, or 5 ml H_2O alone and simultaneously 50 mg/kg of IP intraperitoneally. The animals were placed in metabolic cages; urine was collected for 24 h and subjected to periodic pH measurements. Unconjugated and conjugated metabolites in the 24 h urine were determined by means of thin-layer chromatography (Bickel & Weder, 1968).

RESULTS

Buccal absorption

Buccal absorption measurements were made using IP and nine of its unconjugated metabolites. Each substance was tested at pH 5·5, 6·5, 7·4, 8·2 (IP also 4·5 and 9·0) by 3 (IP 4) subjects. The number of tests made per subject and pH value were: 4 (IP), 3 (DMI, DDMI, IPNO, IDB, 2-OH-IP, 2-OH-IDB), 2 (2-OH-DMI), 1 (10-OH-IP, 10-OH-DMI). The mean values of all measurements for a substance for a given pH were calculated.

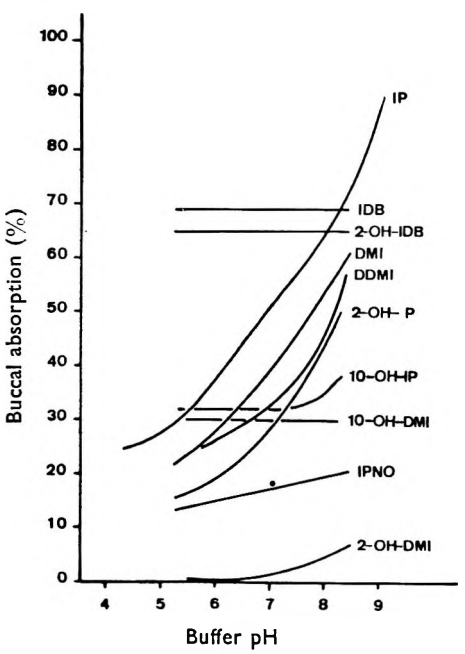

FIG. 1. Buccal absorption of IP and IP metabolites as a function of pH.

The resulting curves of buccal absorption as a function of pH are summarized in Fig. 1. The variations were carefully checked; for IP the mean standard deviation of the inter-subject variation was 8·8% in terms of buccal absorption, and the intra-subject variation was 5·4%. Similar variations were observed for IDB, 2-OH-IDB and 2-OH-IP, whereas the other tested substances showed less variations. Thus the curves are clearly distinct from one another with the reservation of a possible overlap of the pairs IDB and 2-OH-IDB, 10-OH-IP and 10-OH-DMI, and DDMI and 2-OH-IP. Table 1 contains the mean buccal absorption values at pH 7·4 and their total standard deviations.

Table 1. *Partition values of imipramine and imipramine metabolites. % in organic phase after equilibration with isotonic phosphate buffer pH 7·4*

Metabolite	Chloroform	n-Hexane	Diethyl-ether	1,2-Dichloro-ethane	Buccal† absorption
IDB	99·6 ± 1·2	99·2 ± 1·2	93·6 ± 1·2		69 ± 13·1
2-OH-IDB ..	98·0 ± 1·2	44·8 ± 2·0	97·5 ± 1·1		67 ± 7·0
IP	99·8 ± 0·8	99·4 ± 1·4	99·3 ± 1·2	98·5 ± 1·4	57 ± 11·1
DMI	98·5 ± 1·6	65·1 ± 2·1	88·4 ± 1·5	97·8 ± 1·8	45 ± 6·7
DDMI	99·1 ± 1·8	70·5 ± 2·3			36 ± 8·0
2-OH-IP	97·4 ± 1·5	25·2 ± 2·0	84·2 ± 1·7	71·0 ± 1·9	34 ± 13·3
10-OH-IP ..	92·5 ± 2·4				32 ± 7·0
10-OH-DMI ..	77·0 ± 2·8				30 ± 2·9
IPNO	97·2 ± 1·5	10·0 ± 2·2	7·0 ± 2·5	71·0 ± 1·8	18 ± 4·5
2-OH-DMI ..	55·2 ± 2·1	6·3 ± 2·0	55·6 ± 2·6	50·2 ± 3·1	2 ± 2·9

* 8 experiments.
† intra- and inter-subject s.d.

Partition experiments

The partitions of IP and nine unconjugated metabolites between phosphate buffer pH 7·4 and several organic solvents have been measured. The results are summarized in Table 1. Mean standard deviation of the values is ± 1·8%. To compare the partition properties of the solvents with biological partion, this Table also contains the buccal absorption values for pH 7·4. Partition values of the above substances were measured with organic solvents against aqueous buffers at four to seven different pH values in the range of pH 5·0–12·5. The partition-pH plots are given in Figs 2 and 3.

Ionization constants

Since the published pK_a values of IP and DMI show wide variation, we made our own measurements. pK_a values were measured with IP and five of its major unconjugated metabolites. The results are summarized in Table 2 which also includes values

Table 2. *pK_a values of imipramine and its metabolites*

	(1)	(2)	(3)	(4)
IP	8·0	7·94	8·6	9·5
DMI	9·4	9·22		10·2
DDMI	9·4	9·32		
IPNO	4·7			
2-OH-IP	8·0	7·93		
2-OH-DMI	9·3	9·10		

(1) This study.
(2) Geigy Inc., Basel, (methyl cellosolve), (unpublished).
(3) Haefliger (1959).
(4) Green (1967).

obtained by other authors. The dissociation of the cyclic amine group is too weak to be measured (IDB). The dissociation of the phenolic groups begins several pH units above the pK_a of the side-chain amine group (cf. Fig. 3).

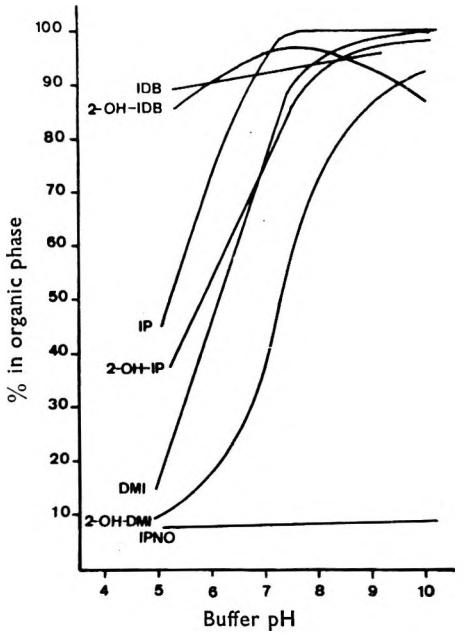

FIG. 2. Partition of IP and IP metabolites between aqueous buffer solutions and diethyl ether.

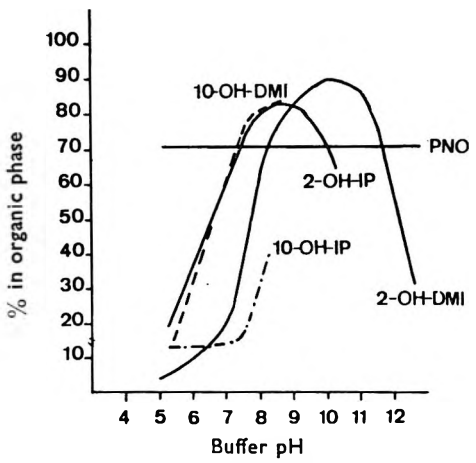

FIG. 3. Partition of IP metabolites between aqueous buffer solutions and 1,2-dichloroethane (———), chloroform (- - - - -), and n-hexane (-·-·-).

Passage through the blood brain barrier

Metabolite patterns in brain and extracerebral compartments were compared after administration of IP and its metabolites at different dosage and time schedules. The results are summarized in Table 3. Some experiments were aimed specifically at the study of blood brain barrier passage whilst others were part of pharmacokinetic studies (Bickel, Weder & Baggiolini, 1966; Bickel & Weder, 1968). One to 28 animals

were used for each type of experiment in Table 3. In most cases hydroxylated and conjugated metabolites could be detected in extracerebral compartments (plasma, excreta, liver, other organs) but not in brain. Glucuronides and minor metabolites (DDMI, 2-OH-IDB, 10-OH-metabolites) were not administered.

Table 3. *Appearance of imipramine and its metabolites in brain after administration to rats*

Species	Drug	Dose (mg/kg)	Route	Time	Detected in brain*	
					IP	DMI
Rat	IP	10	i.v.	5 min	+	—
	IP	50	s.c.	1; 6 h	+	+
	IP	50	i.m.	6 h	+	+
	IP	50	oral	1; 6 h	—	+
	IP	50	i.p.	15–120 min	+	+
	IP	chron.	i.p.	30 min	+	+
	IP	chron.	i.p.	6 h	—	+
	IDB†	50	i.p.	15 min	—	—
	DMI	50	i.p.	15 min	—	+
	IPNO	50	i.p.	1 h	—	+
	IPNO	50	i.p.	2 h	—	+
	IPNO	15	i.v.	5 min	—	—
	2-OH-IP‡	20	i.v.	5 min	—	—
	2-OH-DMI	40	i.p.	15 min	—	—
Guinea-pig	IP	50	i.p.	1 h	+	+
	DMI	50	i.p.	1 h	—	+
	IPNO	50	i.p.	1 h	+	—
	2-OH-DMI	50	oral	1 h	—	—

* Hydroxylated metabolites present in extracerebral compartments

† IDB detected only.

‡ 2-OH-IP detected only.

No IPNO or 2-OH-DMI were detected after imipramine and the other metabolites.

Renal excretion and urinary pH

IP and its metabolites were determined in 24 h urine specimens from normal, aciduric and alkaluric rats dosed with IP. The molecular species determined were summarized as three groups: low, medium, and high polarity metabolites. The results of these experiments are listed in Table 4.

Table 4. *Influence of urinary pH on the excretion of metabolites after administration of imipramine to rats* (50 mg/kg i.p.)*

Pretreatment	ml urine mean	pH ± s.d.	Metabolites		
			Low polarity (1)	medium polarity (2)	high polarity (3)
(a) 24 h excretion (% of dose):					
NH_4Cl	9·7	5·8 ± 0·6	1·7 — 2·6	0·8 — 1·5	8·5 — 34·5
H_2O	9·0	6·3 ± 0·3	0·3 — 1·5	0·1 — 4·7	8·3 — 25·3
$NaHCO_3$ ..	9·5	8·5 ± 0·4	0·03 — 0·2	0·2 — 1·1	7·6 — 13·2
(b) Metabolite patterns (%, mean):					
NH_4Cl			17·7	10·0	72·3
H_2O			11·2	31·3	57·5
$NaHCO_3$ ..			1·0	5·1	93·9

* 3 rats per group.

(1) IP, DMI. (2) IPNO, 2-OH-IP, 2-OH-DMI. (3) Glucuronides.

DISCUSSION

Our testing of the buccal absorption test fully confirms the findings of Beckett & Triggs (1967) and Beckett & others (1968a) about reliability, inter- and intra-patient variations, kinetics and other parameters. All buccal absorption-pH curves of IP and its metabolites depicted in Fig. 1 have a distinct and characteristic position. Buccal absorption of IP, DMI, DDMI, and 2-OH-IP strongly increases in the tested pH range 5–9. A second group containing IDB, 2-OH-IDB, 10-OH-IP, 10-OH-DMI, IPNO and 2-OH-DMI shows little or no pH dependence of buccal absorption. The members of the first group are medium strength bases with pK_a values between 7 and 10 (Table 2) and thus are partly ionized at the tested pH range. Therefore with increasing pH there is a decrease in ionization and an increase in diffusion through the (buccal) membrane. On the other hand the members of the second group, IDB, 2-OH-IDB, and IPNO are weak bases with pK_a values far below neutral (IPNO = 4·7). The basicity is decreased by the partial sharing of the terminal-N free electron pair by oxygen in the case of IPNO and of the cyclic-N free electron pair by resonance mesomers in the case of IDB. These substances are practically un-ionized over the tested pH range, and their lipid solubilities and diffusion rates are therefore not pH dependent. With 2-OH-DMI it must be assumed that even the un-ionized base (pK_a 9·3, equalling DMI) is very hydrophilic because of the amino- and phenolic-groups and therefore does not easily penetrate the buccal membranes. The lipid solubility of all compounds at a given pH value is reflected by the (vertical) order in the pH buccal absorption plot. For reasons discussed below it must be inferred that the 10-OH metabolites are relatively hydrophilic medium strength bases.

The partition of a substance between a polar and a non-polar solvent is dependent on the lipid solubility of the substance, the polarity (dielectric constant) of the organic solvent, the pH of the polar solvent and the temperature. Furthermore the solubility of the substance in the non-polar solvent can limit partition. The partition values for a constant pH of 7·4 (Table 1) are in accordance with these considerations. The differences of partition values of one substance in the four solvents must be ascribed to differences of dielectric constants (chloroform 5·2, n-hexane 1·9) which also determine the discrimination of the substances tested. Except for a few inversions, the (vertical) order of lipid solubility of the substances is similar with all solvents. The substances can be divided into a group of low polarity metabolites (IDB, 2-OH-IDB, IP, DMI, DDMI) and a group of medium polarity metabolites (phenolic, alcoholic and *N*-oxide metabolites), although there seems to be no absolute division between the members at the boundary. The conjugates, not included in this study, could be labelled as high polarity metabolites. Preliminary partition experiments with the glucuronides of 2-OH-IP and 2-OH-DMI did not show detectable amounts in the chloroform phase. Inversed phase paper chromatography (Herrmann, 1963) leads to an Rf sequence of unconjugated IP metabolites comparable to the lipid solubility order in Table 1.

The pH dependence of the partition values (Figs 2 and 3) are obvious with IP, DMI, 2-OH-IP, and 2-OH-DMI (pK_a 8·0–9·4). 10-OH-IP and 10-OH-DMI are also pH-dependent suggesting that their pK_a values are in a similar range. Partition values of the 2-OH-metabolites decrease again in the strongly alkaline range due to dissociation of the phenolic group. The slope of the pH-partition curve is comparable for all these metabolites. On the other hand the metabolites with pK_a values far below neutral show practically identical partition values over the tested pH range 5–12·5 (IDB, 2-OH-IDB, IPNO).

It is of interest to compare the partition values with the buccal absorption data. At pH 7·4 the gross order of buccal absorption corresponds to the order of lipid solubility obtained with all solvents. Again, the same groups of low polarity and medium polarity metabolites can be distinguished with the reservation of a possible overlap of the members at the boundary. Comparison of discrimination by the solvents shows that the highly discriminating and most lipophilic of these, n-hexane, displays partition properties closer to buccal absorption than do the less lipophilic ether, dichloroethane, and chloroform. However, the search for a solvent that truly imitates (buccal) absorption remains as an important goal of further research. Both partition in different solvents and buccal absorption distinguish identical groups of pH-dependent and pH-independent metabolites. The relative similarity of n-hexane and the buccal membrane is also reflected in the pH dependence of 10-OH-IP (Figs 1 and 3). The poor buccal absorption of 2-OH-DMI—sharing the same pK_a value with DMI—can be explained by the fact that the highly lipophilic membrane strongly discriminates against this metabolite unless it is highly un-ionized which would only occur at unphysiological pH values (compare partition behaviour of 2-OH-DMI and the 10-OH-metabolites in Fig. 3).

For the purpose of predicting the behaviour of drugs at biological membranes the buccal absorption test is no doubt superior to the partition test using organic solvents. However, pH-partition values will keep their importance as a basis for selective extraction procedures. In the case of IP metabolites an example of this has been described by Weder & Bickel (1968).

The experiments aimed at a detection of selective passage through the blood brain barrier demonstrate a physiological application of the above findings. The results summarized in Table 3 show that only IP, DMI, and IDB easily cross from plasma into brain. Crossing of the more polar 2-OH-IP was detected only once under extreme conditions. 2-OH-DMI and IPNO did not cross the blood brain barrier, and other phenolic, alcoholic, and conjugated metabolites were formed from precursors and detected in extracerebral but not cerebral tissue (see also Bickel & Weder, 1968). Further information supporting these results is given by Quinn, Marano & Greengard (1964) who found 2-OH-IP in the brain but no 2-OH-DMI, the major plasma metabolite. In a human suicide case with IP, Faragó (1965) found no phenolic metabolites except 2-OH-IDB, and in a similar DMI case (Bickel, Brochon & others, 1967) the major metabolite, 2-OH-DMI, was present in body fluids and tissues except brain. The only indication of 2-OH-DMI in brain was reported after an extreme overdosage of IP (4500 mg) (Herrmann, 1963). Despite the crude experimental approach which does not take into account the complex interrelation between blood, brain and cerebrospinal fluid (Davson & Oldendorf, 1967) the results are in full agreement with the general observations that the blood brain barrier behaves like a pure lipid membrane (Vogt, 1967). The fact that several medium polarity metabolites, which do not enter the brain, can appear in liver and other cellular tissues (Bickel & Weder, 1968) agrees with the suggestion of a greater discrimination in favour of more lipid soluble substances by the blood brain barrier than by the (liver) cell barrier (Kurz, 1964). All imipramine metabolites which easily cross the blood brain barrier belong to the low polarity group emerging from buccal absorption and partition experiments at pH 7·4.

The studies of urinary excretion of IP and its metabolites are another physiological application of buccal absorption and partition studies. In urine of normal IP-treated rats the ratio low : medium : high polarity metabolites is 1 : 3 : 6, and the corresponding

ratio in plasma is 5 : 1 : 4 (Bickel & Weder, 1968). The reason for the small amounts of low polarity metabolites (IP, DMI) in urine is found in their high degree of tubular reabsorption by non-ionic diffusion. Due to the pK_a values of these substances, pH changes of the tubular fluid markedly influence the degree of reabsorption and hence of excretion, which significantly decreases in the order of urinary pH 5·8, 6·3, 8·5. On the other hand, the excretion of phenolic and conjugated metabolites does not significantly change as a function of pH. Since the low polarity metabolites are only a small fraction of the urinary metabolites, total excretion is not significantly influenced by urinary pH (Table 4). Unaffected total drug excretion of IP-treated alkalinuric rats was also reported by Crammer, Scott & others (1968). Urinary IP and DMI determinations in patients (Demiaux, Motin & others, 1966; Gaultier, Fournier & others, 1967) point in the same direction and explain the failure of drug-induced aciduria, dialysis, or forced diuresis in the treatment of IP poisoning.

Again the behaviour of IP metabolites at the tubular membrane is in agreement with the characterization of these substances by the buccal absorption test. Relations between buccal absorption and pH-dependent tubular reabsorption have been extensively studied by Beckett & others (1968a, b) using amphetamine.

Acknowledgements

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Metabolism and excretion of promazine by the horse

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The urinary excretion of promazine and its metabolites has been examined in five horses, by ultraviolet spectrometry and thin-layer chromatography. In each horse, excretion continued for at least 96 h although the amount excreted was low, being about 11% of the dose. No correlation of excretion and pH or urinary volume was observed. Glucuronic acid conjugates predominated, their ratio to unconjugated metabolites being about 5:1. Promazine was found only in small amounts and traces of unconjugated sulphide metabolites were found in only one horse. At least nine metabolites of promazine were detected, the major one being the glucuronide of 3-hydroxypromazine.

The metabolism and excretion of the phenothiazine tranquillizers* is complex and shows marked variations from species to species (Goldenberg & Fishman, 1961; Goldenberg, Fishman & others, 1964).

Five common metabolic pathways of these drugs occur; namely, oxidation at the sulphur atom (Salzman & Brodie, 1956; Fishman & Goldenberg, 1960), oxidation at the terminal side-chain nitrogen atom (Fishman, Heaton & Goldenberg, 1962), demethylation at the terminal dimethyl amino-group (Ross, Young & Maass, 1958; Walkenstein & Seifter, 1959), and hydroxylation of the phenothiazine nucleus followed by conjugation with glucuronic acid (Lin, Reynolds & others, 1959; Huang, Sands & Kurland, 1961; Fishman & Goldenberg, 1963). A large number of metabolites are thus possible. With promazine some 30 metabolites have been detected in human urine (Goldenberg & others, 1964). Studies have also shown extensive localization of such compounds in the body (Salzman & Brodie, 1956; Fyodorov, 1958; Walkenstein & Seifter, 1959), low percentage urinary excretion (Fyodorov & Shnol, 1956; Nadeau & Sobolewski, 1959; Goldenberg & others, 1964), and prolonged excretion, sometimes lasting several months (Forrest, Forrest & Mason, 1961; Huang & others, 1961). (See review by Emmerson & Miya, 1963.)

These drugs have proved most useful in veterinary practice, both for the restraint of temperamental animals, and for pre-anaesthetic medication, particularly in the fields of equine medicine and surgery (Raker & English, 1959; Raker & Sayers, 1959).

Little information is available about their metabolism and excretion in the horse. Carey & Sanford (1963) detected the presence of at least seven metabolites of promazine in horse urine, and found that polar metabolites predominated and were excreted for a much longer period. Schubert (1967) suggested that urinary excretion in the horse continues for several days after a single intravenous injection of chlorpromazine, and that a small percentage of the dose is excreted. He detected

* Those phenothiazine derivatives possessing an amino-alkyl side-chain attached to the ring nitrogen atom.

four metabolites, the predominating one being chlorpromazine sulphoxide. The unchanged drug was found in only very small amounts.

It was decided to make a detailed analysis, both of metabolism and excretion, on one of this group of drugs. Promazine was chosen as a representative of the group because it may be given safely in a relatively large dose.

EXPERIMENTAL

Urine samples

The horses used were light hunters (3 geldings and 2 mares). Each horse was brought in from grass three days before the experiment and was fed the same diet of hay and concentrates before and during the experiment. A single intramuscular dose of promazine hydrochloride (5 mg/kg as a 5% solution), was administered at about 11 a.m. This dose invariably caused deep sedation lasting 12–18 h, but the horses remained standing. Urine was collected over successive periods of 8 h for 96 h after injection and the pH of each sample was measured. For quantitative analysis, 100 ml of each sample was used, and 300 ml for obtaining a qualitative excretion pattern. The residual urine was pooled for further analysis.

Urine collections were made from mares by passing a catheter at 2 hourly intervals. In geldings, collections were made by strapping a polythene container to the animal. All samples were stored in sterile bottles at $-20°$ because in preliminary experiments storage at 4° allowed a build-up of impurities which tended to mask the phenothiazine peaks on the ultraviolet spectra of extracts.

Extraction procedures

The procedure for quantitative analysis was similar to that of Bolt (1965). However, owing to the large amounts of contaminants found in horse urine, and its tendency to form emulsions with organic solvents, several extra purification steps were included. Emulsions were usually broken at any stage by centrifuging at approximately 2,500 rev/min. If centrifugation failed, the addition of a little anhydrous ammonium sulphate succeeded.

Unconjugated metabolites (*Procedure* 1). Each 100 ml aliquot of urine was adjusted to pH 9·5–10 using N ammonia solution. The white precipitate which formed was removed by centrifuging, and decanting the supernatant urine. This precipitate contained no phenothiazines as shown by reaction with 50% sulphuric acid. The urine was then extracted with three 10 ml portions of 1,2-dichloroethane (previously washed with half its volume of 0·1N ammonia solution, 0·1N hydrochloric acid, and distilled water successively), and the organic extracts were pooled. The residual urine was stored for analysis of conjugated metabolites.

After removal of any emulsion, the organic extract was washed with 0·1N ammonia solution (2 × 50 ml), followed by distilled water (2 × 50 ml), and was finally extracted with 10 ml 0·1N hydrochloric acid. This acid extract was assayed for phenothiazine content by ultraviolet spectrometry.

Glucuronide conjugated metabolites (*Procedure* 2). As analysis of residual urine from Procedure 1 for glucuronide conjugates involved a prolonged incubation step to hydrolyse the glucuronides to their aglycones, this urine was heated for 10 min on a boiling water bath at three successive 24 h intervals before incubation, and was used thereafter under sterile conditions to avoid the possibility of formation of contaminants by microbial action at 37°.

The urine was adjusted to pH 4·5 using 5N hydrochloric acid and 5 ml β-glucuronidase (Ketodase 5,000 units/ml) were added. The sample was then incubated for 24 h at 37°. After incubation the pH was adjusted to 9·5–10 using ammonia solution, and the extraction carried out as for Procedure 1.

The concentration of sulphide, and of sulphoxide metabolites in each group was calculated using the background cancellation method of Flanagan, Lin & others (1959), as modified by Bolt (1965) for promazine assay.

The rates of excretion for four groups of metabolites—unconjugated sulphides, unconjugated sulphoxides, glucuronide conjugated sulphides, and glucuronide conjugated sulphoxides—were calculated for successive pooled 8 h samples. The methylated or hydroxylated derivatives and promazine *N*-oxide, which are common metabolites of promazine in other species, produce maxima on the ultraviolet spectrum at the same wavelength as the parent drug, and are included in the group of unconjugated sulphide derivatives.

The same procedures were used for qualitative analysis, using larger volumes of solvent. However, the extracts were not taken into acid solution, but dried over anhydrous ammonium sulphate, then evaporated under reduced pressure to approximately 1/300 of their original volume. The resultant solutions were used for thin-layer chromatography. In addition, the heating step was omitted from Procedure 2, as the interfering compounds gave no abnormal spots when chromatographed.

Chromatography

Activated silica thin-layer plates, after spotting, were developed to a height of 15 cm. For unconjugated metabolites the solvent system used was methanol–acetic acid–water (5:3:2), and for glucuronide conjugated metabolites the system was acetone–isopropanol–1% ammonia (9:7:4) (Goldenberg & others, 1964). Spots were visualized by spraying with a 50% sulphuric acid solution, and were identified by comparison with known standards.

RESULTS

Ninety-six h excretion patterns were plotted for six experiments. On examining the combined rates of excretion of the four groups of metabolites for each horse, it was noted that the patterns fell into two groups.

In the first group (A) a maximum rate of excretion was reached within 16 h after injection, but in the second group (B) this was not achieved until 24–32 h after injection. Each pattern of excretion was shown in 3 out of the 6 experiments and the average rates are shown in Fig. 1.

For each horse, excretion patterns of individual groups of metabolites proved to be varied and irregular. Excretion was also prolonged, continuing, in at least one of the four metabolic groups, up to 96 h. Typical examples from each group of horses are shown in Fig. 2A,B. Excretion of unconjugated sulphoxide metabolites did not exceed a rate of 2·5 mg/h for any horse, and the average rate of excretion remained at around 0·5 mg/h for the duration of the experiment. Unconjugated sulphide metabolites appeared in small quantity only in one case (Horse 1), from approximately 32 h after injection until the end of the experiment.

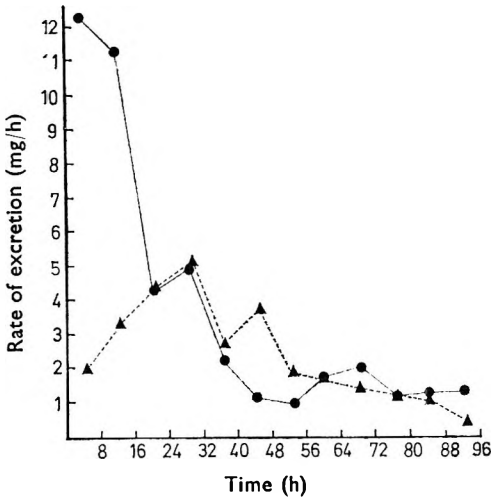

FIG. 1. Urinary excretion of total promazine metabolites by the horse. Rates of excretion were obtained from the total concentration of metabolites in pooled 8 h samples. Each point represents the average of three experiments. In excretion pattern (A), ——●——, the maximum rate was reached within 16 h, and in pattern (B), – – – ▲ – – –, within 24–32 h.

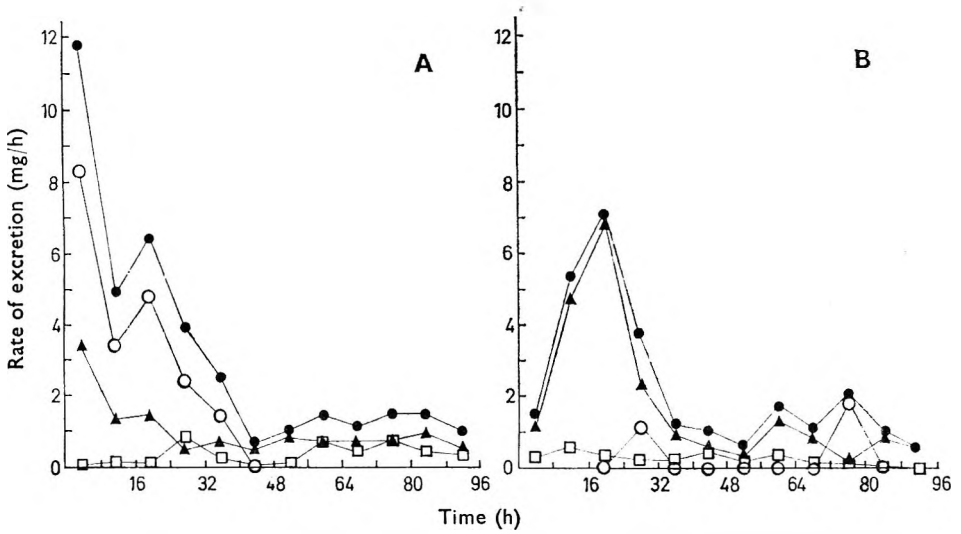

FIG. 2. Excretion patterns of individual groups of metabolities from (A) one horse representative of those shown in Fig. 1A and (B) one horse representative of those shown in Fig. 1B. □ Unconjugated sulphoxide metabolites. ▲ Glucuronide conjugated sulphoxide metabolites. ○ Glucuronide conjugated sulphide metabolites. ● Total metabolites.

Excretion of glucuronide-conjugated sulphoxide metabolites continued throughout the 96 h period in five horses. The remaining horse (animal No. 2) showed no trace of this type of metabolite. With one exception (No. 5b), the rate of excretion was less than 3·5 mg/h, and the average rate of excretion was less than 2 mg/h for the duration of the experiments.

Glucuronide-conjugated sulphide metabolites were in great evidence in four of the horses. The fifth horse, which was dosed on two occasions, showed no trace of this type of metabolite the first time, and, on the second occasion, it was only detected in

trace amounts in two samples. In the other four horses, excretion was very varied throughout each series of samples, but had ceased by 80 h after injection.

The pH of the urine samples varied between 7·0 and 8·5. No correlation was seen between differences in pH and the rate of excretion of metabolites, neither was this related to urinary volume.

Table 1. *The urinary excretion as % of dose after intramuscular administration of promazine hydrochloride to the horse*

Distribution	Animal No. 1	2	3	4	5a	5b	Average % of dose
% Unconjugated	3·6	1·8	0·2	1·3	3·2	1·0	1·8
% Glucuronides	18·1	7·8	7·5	9·6	5·9	6·1	9·2
Total	21·7	9·6	7·6	10·9	9·1	7·1	11·0
Glucuronides : unconjugated ..	~5 : 1	~4 : 1	~63 : 1	~7 : 1	~2 : 1	~6 : 1	~5 : 1

Table 1 shows the percentage of the dose excreted in the urine during each experiment, the average ratio of glucuronides to unconjugated metabolites being 5 : 1. The percentage of the dose excreted over the 96 h was low, the average value being approximately 11%.

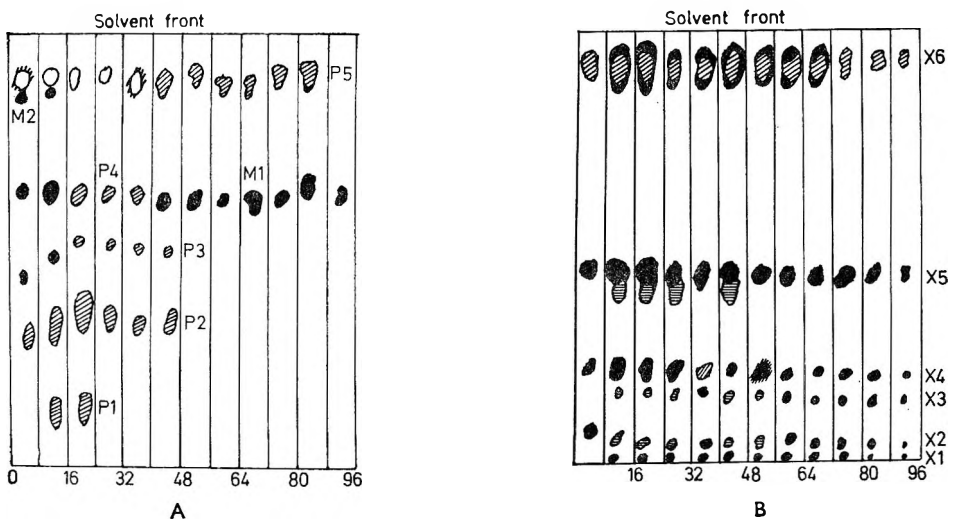

FIG. 3. Typical chromatograms of urinary extracts of (A) unconjugated and (B) glucuronide conjugated metabolites of promazine over 96 h. Each division represents the chromatogram of an 8 h pooled urine sample. The unconjugated metabolites were developed in the system methanol–acetic acid–water (5 : 3 : 2) and the conjugated metabolites were developed in the system acetone–isopropanol–1% ammonia (9 : 7 : 4). Chromatograms were visualized with a 50% sulphuric acid solution. Spot P2 was identified as promazine sulphoxide; P4 was identified as the parent drug and both M1 and X5 were identified as 3-hydroxypromazine. P1, P3, P5, M2, X1–4 and X6 were not identified. Colours: Black = purple, diagonal hatch = pink, cross hatch = bluish purple, white = yellow.

Fig. 3 shows typical chromatograms following the excretion of individual metabolites over 96 h. At least four unconjugated metabolites were detected in addition to the unchanged drug, which was also present in low concentration (P4). By comparison with standard metabolites two of these were tentatively identified as promazine sulphoxide (P2) and 3-hydroxypromazine (M1).

At least five glucuronide-conjugated metabolites were detected, the major one being identified as the glucuronide of 3-hydroxypromazine (X5), by comparison with the standard metabolite. The spot was also eluted from the plate using methanol, and identified conclusively by examination of its ultraviolet spectrum after reaction with 50% sulphuric acid as described by Beckett & Curry (1963).

DISCUSSION

The four groups of metabolites investigated in this series of experiments constitute the major metabolites known to be excreted in other species. The other common pathway of sulphate conjugation was investigated but only trace amounts were found.

Collection of urine over a 96 h period was chosen since work on other species had suggested prolonged excretion of these compounds (Forrest & others, 1961; Huang & others, 1961). Indeed, the present results indicate the need for more lengthy excretion studies on phenothiazine derivatives in the horse.

Masking of the ultraviolet spectra of urinary extracts of phenothiazine derivatives has previously been reported by Schubert (1967). The formation of further interfering compounds, by bacterial action, occurs at 4°, and is greatly increased by prolonged incubation at 37°. In view of this, thorough washing and a heating procedure to reduce growth of micro-organisms should be adhered to in the analysis of metabolites in horse urine.

So far we have eluted, and identified by ultraviolet spectroscopy, the major metabolite of promazine found in horse urine. It was not possible to elute the minor metabolites. The difficulty is thought to be due to the small concentrations of such metabolites found on thin-layer plates.

The varied and irregular excretion pattern noted in this series of experiments is in accordance with findings in other species. Eiduson, Geller & Wallace (1963) reported variable urinary excretion patterns of thioridazine after oral administration to man, and suggested a relation between the amount of drug excreted and urinary volume. No such relation was seen for promazine in the present experiments. Neither was there any obvious explanation for the two sets of metabolic patterns experienced in the present series of experiments. Carr (1962), reviewing the metabolism of this type of drug, mentioned "their unusual metabolic patterns and their individual excretion differences that vary with the different drugs and different patients".

The low percentage of dose recovered also agrees with research carried out in other species (Fyodorov & Shnol, 1956; Nadeau & Sobolewski, 1959), although reports varied considerably (Emmerson & Miya, 1963). Beckett, Beaven & Robinson (1963) recovered 7% of an oral dose of chlorpromazine from human urine, and attributed the low recovery to poor reabsorption of conjugated metabolites excreted in the bile, suggesting the possibility of a high concentration of metabolites in the faeces. Indeed, high levels of phenothiazine metabolites have been reported in the faeces of various species (Fyodorov & Shnol, 1956; Emmerson & Miya, 1963; Eiduson & Geller, 1963).

On the other hand, extensive localization of these drugs in various organs has been reported (Salzman & Brodie, 1956; Fyodorov, 1958; Walkenstein & Seifter, 1959). Slow release from sites of localization may explain the delayed excretion, and could also account for the low recoveries of metabolites.

The predominance of glucuronide-conjugated metabolites agrees with findings in other species. Beckett & others (1963) reported the ratio of glucuronides to

unconjugated to sulphate-conjugated metabolites recovered after an oral dose of chlorpromazine to man as approximately 12 : 5 : 4. Only trace amounts of sulphate-conjugated metabolites were found in our investigation, which may explain the larger ratio of glucuronides to unconjugated metabolites obtained.

In conclusion, the present series of experiments has borne out the varied and irregular excretion patterns experienced in other species, and the large number of metabolites associated with this group of drugs. Since at least one of the four groups of metabolites can be detected over a period of 96 h, this method of analysis should prove of value in cases of suspected doping by phenothiazine derivatives.

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Effect of *Coprinus atramentarius* on the metabolism of ethanol in mice

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The ethanol-soluble material extracted from *Coprinus atramentarius* was more toxic to mice, as measured by the LD50 and potentiation of the ethanol-induced sleeping time, than the whole mushroom or the residue remaining after ethanol extraction. Also, the ethanol-soluble fraction when fed to mice 4 h before administration of ethanol markedly increased the blood acetaldehyde and ethanol levels.

Previously Genest, Coldwell & Hughes (1968) reported that the fungus, *Coprinus atramentarius* (inky cap), potentiated the toxic effects of ethanol in mice but that *Coprinus comatus* (shaggy cap, lawyer's wig) did not. The toxic and ethanol-potentiating effects in mice of *Coprinus atramentarius* has now been investigated and also its ethanol-acetic acid soluble and ethanol insoluble fractions.

EXPERIMENTAL

Coprinus atramentarius (Bull. ex Fr.) Fr. (20 lb) was collected on Ottawa lawns during the autumn of 1967 and dried as described by Genest & others (1968). The dried powdered fungus (IC/A) was percolated in 200 g batches with 3 litres of 96% ethanol at a rate of 2–4 ml/min. After adding 0·72 ml of 50% sulphuric acid to ensure retention of volatile bases (List & Reith, 1960), the percolate was evaporated at 30° *in vacuo* in a rotary evaporator to 200–400 ml. The marc was treated twice with 200 ml each of 10% acetic acid on a water bath at 80°. After filtration, the marc was dried at 60° (IC/B). The ethanol and acetic acid extracts were combined, the ethanol completely expelled in a rotary evaporator and the remaining water removed by freeze-drying. The residue (IC/C) represented 43% of the whole mushroom. The pH of aqueous suspensions of samples of IC/A, IC/B and IC/C were 6·6, 4·7 and 4·8, respectively. Freshly prepared suspensions of either one of fractions IC/A, B or C (1 g/10 ml) in 0·25% aqueous gum tragacanth were fed to mice (2·5 ml/30 g body weight) by stomach tube.

Non-fasted male albino mice of Connaught strain weighing 27 ± 4 g were assigned randomly to the experimental groups. They were housed in all-metal cages, 10 or 12 per cage with free access to Master Fox Chow and water in a room at 72° ± 2° F. They were acclimatized to the environment of the animal quarters at least 10 days before beginning the experiments.

Solutions of 30% w/v ethanol in water were administered by stomach tube. A non-lethal dose of 6 g of ethanol in solution per kg was given; this was sufficient to cause loss of the righting reflex in about 20% of the animals.

RESULTS

Induction and sleeping times

These were measured as described by Genest & others (1968), in 270 mice in groups of 10, and treated as follows.

IC/A 8 g/kg, 4 h later water 0·5 ml/30 g (10 mice); IC/B 4·6 g/kg, water 0·5 ml/30 g (10 mice); IC/C 3·4 g/kg, water 0·5 ml/30 g (10 mice); 0·25% gum tragacanth, 2·5 ml/30 g, 4 h later ethanol 6 g/kg (60 mice); IC/A 8 g/kg, ethanol 6 g/kg (60 mice); IC/B 4·6 g/kg, ethanol 6 g/kg (60 mice); IC/C 3·4 g/kg, ethanol 6 g/kg (60 mice). Both IC/B and IC/C were given at doses equivalent to the amount of each present (% w/w) in IC/A (8 g/kg). The results are summarized in Table 1.

Table 1. *Induction time (I.T.), sleeping time (S.T.) and incidence of sleeping after administration of ethanol alone and with* C. atramentarius *and its ethanol-soluble and insoluble fractions to mice*

	Treatments			
Measurement	Ethanol (a)	IC/B + ethanol (b)	IC/A + ethanol (c)	IC/C + ethanol (d)
Number of mice sleeping ..	12/58	28/59	47/59	54/59
% of mice sleeping	21	47	80	92
Probability of χ^2_Y	<0·001 (a, b)	<0·001 (b, c)	<0·07 (c, d)	
I.T.—median and range (min) ..	40 (16–97)	35 (5–109)	33 (9–187)	30 (2–230)
S.T.—median and range (min) ..	163 (15–258)	236 (98–544)	438 (31–945)	639 (22–1092)
Probability† ..	<0·02 (a, b)	<0·05 (b, c)	<0·01 (c, d)	

† Calculated by the Wilcoxon rank sum test (Wilcoxon, 1945).

The incidence of sleeping among the mushroom + ethanol-treated mice was increased significantly over that observed in mice treated with ethanol alone confirming our earlier observation (Genest & others, 1968). Also, the incidence of sleeping was highest after treatment with IC/C + ethanol and higher in the IC/A + ethanol group than in the IC/B + ethanol treated animals. IC/A, B, or C administered without ethanol did not cause loss of the righting reflex. The induction times exhibited no significant differences between treatments. However, the sleeping times were increased significantly when IC/A, B or C were given 4 h before administration of the ethanol, the greatest effect being caused by IC/C, followed by IC/A and IC/B. The results on incidence of sleeping and on sleeping time strongly indicate that the toxic component(s) of *C. atramentarius* are at least partially extracted with ethanol-acetic acid.

LD50 of IC/C

The ethanol-soluble fraction of *C. atramentarius* (IC/C) was administered orally at 0, 6·5, 8·1, 10·2, 12·7 and 15·9 g/kg. The numbers of deaths in 24 h were respectively 0/10, 0/12, 2/12, 4/12, 9/12, 9/12. The LD50 with 95% confidence limits as estimated by the method of Litchfield & Wilcoxon (1949) was 11·2 (9·4–13·3) g/kg. The animals exhibited clonic convulsions immediately before death. The LD50 of IC/A and IC/B could not be estimated because the thickness of the suspensions prevented administration of more than 10 and 16 g/kg, respectively, and these quantities caused no deaths. Similar amounts of IC/C killed 38 and 60% of the animals, respectively.

Effect of IC/C on ethanol metabolism

The ethanol-acetic acid soluble material extracted from *C. atramentarius* (IC/C) was administered orally, at the estimated LD0 dose of 3·5 g/kg, to groups of mice housed

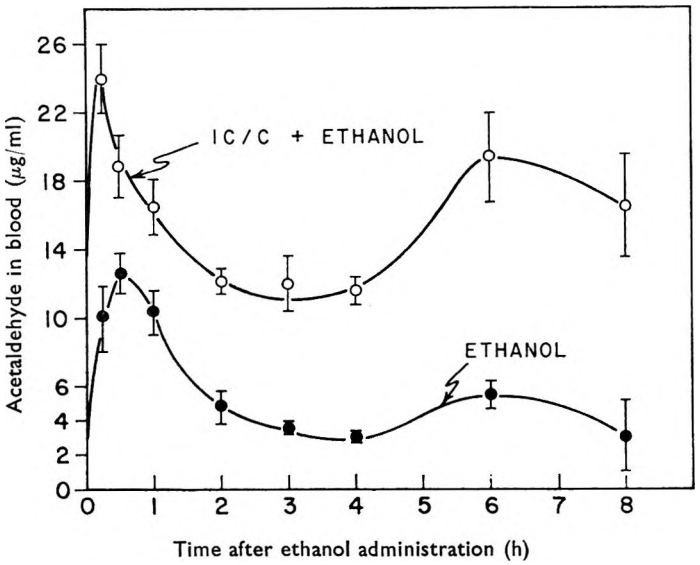

FIG. 1. Acetaldehyde levels in the whole blood of mice administered ethanol alone (—●—●—) and with the ethanol-soluble fraction (IC/C) of *C. atramentarius* (—○—○—). Vertical bars indicate ± one standard error.

5 per cage. Ethanol (6 g/kg) was administered orally 4 h later. Control groups were treated as follows: no treatment; aqueous tragacanth vehicle (2·5 ml/30 g) first, then ethanol 4 h later; IC/C (3·5 g/kg) first, followed by distilled water (0·6 ml/30 g) 4 h later. The animals were decapitated at various times (5 to 10 mice at each time interval) after administration of ethanol or water (controls) and the blood collected in small tubes containing oxalate. The tubes were stoppered and refrigerated until analysed (within 48 h) for acetaldehyde and ethanol by the gas chromatographic procedure of Duritz & Truitt (1964). The results are summarized in Fig. 1 and Table 2.

Table 2. *Blood ethanol concentrations (mg % ± s.e.) in mice treated orally with ethanol, alone and in combination with the ethanol-soluble fraction of* Coprinus atramentarius

Time after ethanol admin. (min)	Treatment		Probability
	Ethanol	IC/C + Ethanol*	
15	356 ± 45 (5)	379 ± 35 (5)	N.S.
30	398 ± 55 (5)	420 ± 93 (5)	N.S.
60	475 ± 81 (10)	443 ± 52 (10)	N.S.
120	547 ± 28 (10)	372 ± 26 (10)	<0·001
180	324 ± 40 (10)	411 ± 32 (8)	N.S.
240	328 ± 68 (5)	550 ± 61 (5)	<0·05
360	179 ± 41 (5)	393 ± 41 (5)	<0·01
480	64 ± 42 (5)	293 ± 67 (5)	<0·02

* IC/C designates the alcohol-soluble fraction of dried, powdered, *Coprinus atramentarius*.

( ) indicate number of animals.

Administration of IC/C resulted in a highly significant increase in the acetaldehyde concentration which was evident 15 min after ethanol was given and persisted throughout the 8 h observation period (Fig. 1). There was no significant difference in the blood ethanol levels in the two groups until at 2 h when the mean concentration in the animals receiving ethanol alone was elevated significantly above the concentration in the animals that were given both IC/C and ethanol. Thereafter, the ethanol concentration in the latter group was higher than in the former at each collection period (Table 2). No acetaldehyde or ethanol was detected in blood from untreated mice given IC/A, IC/B or IC/C only.

DISCUSSION

The present work confirms our earlier observation that *C. atramentarius* potentiates the action of ethanol in mice. We have now shown that the toxicity and the potentiating effect of the ethanol-acetic acid soluble fraction of this mushroom significantly exceeds that of either the whole plant or the ethanol-insoluble residue. The acetaldehyde blood level curves in Fig. 1 suggest that the ethanol-acetic acid soluble fraction inhibits the conversion of acetaldehyde to acetate. The disulfiram-like action of IC/C might be ascribed to the presence of disulfiram in *C. atramentarius*, as claimed by Simandl & Franc (1956), but not confirmed by List & Reith (1960) or by Wier & Taylor (1960). It is possible that other substances are present which inhibit the oxidation of acetaldehyde.

The lower blood ethanol levels at 2 h and the higher levels at 4 to 8 h after ethanol administration in the IC/C + ethanol-treated animals, compared to the levels a these periods in blood from mice given ethanol alone, probably are due to an effect of IC/C on the rate of absorption of ethanol from the gastrointestinal tract, since the rates of disappearance of ethanol from the blood in the two groups of animals (64 and 66 mg %/h, respectively) are essentially similar.

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Anticholinergic effects and passage through the intestinal wall of *N*-butylhyoscine bromide

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The effects of *N*-butylhyoscine bromide (Buscopan) were examined on responses of guinea-pig isolated intestine to transmural stimulation and to peristalsis induced by raising the intraluminal pressure. The drug acted rapidly and in low concentration to abolish peristaltic activity and responses to transmural stimulation when applied to the serosal surface of the ileum, but from 100 to 1000 times the concentration was required when it was applied to the mucosal surface. The effects were more persistent after mucosal than after serosal application. *N*-Butylhyoscine bromide was bound to mucus and mucosal material, the ratio of free to bound drug being 2:1. The drug passed through portions of the intestinal wall containing Peyer's patches more rapidly than through portions containing no macroscopically visible lymphoid tissue. The rate of passage through Peyer's patches, but not through other portions, increased with increasing hydrostatic pressure. These findings help to explain why enterally administered *N*-butylhyoscine bromide exerts anticholinergic effects on the gut without producing systemic actions.

Parenteral administration of *N*-butylhyoscine bromide is effective in the treatment of spasm of the smooth muscle of the alimentary tract (Wick, 1951). It also affects other cholinergically innervated structures (Brownlee, Wilson & Birmingham, 1965; Herxheimer & Haefeli, 1966). However, Herxheimer & Haefeli (1966) reported that oral-*N*-butylhyoscine bromide in volunteers had no effect on heart rate, salivary secretion or accommodation, suggesting that it was not absorbed from the gut. It has been said that orally the drug is inactive, even on the gut, in the doses usually administered (20 mg, 4 to 5 times daily) (Herxheimer & Capel, 1963, 1965). On the other hand, Wick (1967) and Pennefather, McCulloch & Rand (1963) produced evidence from animal experiments for the absorption of the drug after enteral administration, and Pennefather & others (1968) showed that it abolished cholinergically-induced responses of the gut.

This paper deals with the effects of *N*-butylhyoscine bromide in blocking cholinergic responses in isolated gut after application to mucosal or serosal surfaces. The passage of the drug through the intestinal wall was also examined. The findings go some way towards reconciling the apparently conflicting observations previously reported.

EXPERIMENTAL

Method

Observations were made on portions of guinea-pig isolated ileum suspended in an organ bath in McEwen solution (McEwen, 1956) bubbled with 5% CO_2 in oxygen, and maintained at 35·5°.

Peristaltic activity of segments of ileum was studied by Trendelenburg's method (see Burn, 1952), intraluminal pressure being measured with a water manometer and longitudinal movements with a frontal lever exerting 1 g tension and giving a 6-fold magnification. Care was taken to ensure that no leakage of fluid occurred from the lumen of the segment of ileum to the McEwen solution surrounding it in the organ bath. The resting intraluminal pressure was raised to 2 to 6 cm of water pressure for periods of 1·5 to 3 min to induce peristaltic activity. The preparation was then allowed to rest for 3 min.

Twitches of the ileum were produced as described by Paton (1957) by transmural stimulation with 50 V pulses of 1 msec duration at a frequency of 0·1 s for periods of 1·5 to 2·5 min. After such a period of stimulation, the preparation was allowed to rest for 2 min. In some preparations, peristaltic activity and responses to transmural stimulation were recorded in alternate periods.

The amounts of *N*-butylhyoscine bromide (Buscopan) referred to in the text are in terms of the salt. The drug was applied to the serosal surface by placing it in the fluid surrounding the preparation. It was applied to the mucosal surface, that is, into the lumen of the intestinal segment, by passing a fine polythene tube through the Trendelenburg cannula so that the tip was within the intestinal sac. *N*-Butylhyoscine in McEwen solution was injected through the polythene tube in sufficient volume to flush out the fluid already present through a T-piece in the tubing connecting the cannula with the reservoir: care was taken to avoid increases in pressure during this procedure. In some experiments, everted segments of intestine were used, in which case the drug was applied to the serosal surface by giving it into the lumen.

Some observations were made on the passage of the drug through circumscribed regions of the wall of portions of intestine. A segment of intestine was slipped over a glass tube of 5 mm external diameter and a selected region of the intestine was located over an oval hole (5 mm × 7·5 mm) in the side of the tube. The segment was then tied in place using double ties to ensure there was no leakage. Drug solutions were passed through this tube and the rate of flow and the hydrostatic pressure could be varied. The segment of intestine tied over the tube was immersed in an organ bath. In some experiments, this organ bath also contained another segment of ileum arranged for recording of responses to transmural stimulation, and this served to detect the drug passing through the selected region of the wall of the perfused segment. In other experiments, samples were taken from the bath surrounding the perfused segments. At the conclusion of each experiment, azovan blue or Congo red solution were perfused through the tube to ensure that there had been no leakage.

The potential difference across the wall of the ileum was measured in segments that were converted to flat sheets by a longitudinal division of the wall. The sheets were rinsed in McEwen solution and laid flat under paraffin. A platinum electrode was applied to the lower surface and a platinum probe was applied to selected portions of the upper surface. The potential was measured on a Tetronix 502A oscilloscope.

Mucus and mucosa were collected by opening a long segment of ileum and gently scraping the mucosal surface with a scalpel blade.

RESULTS

Relative anticholinergic activity of N-*butylhyoscine applied to serosal and mucosal surfaces of the ileum*

The drug added to the fluid bathing the preparation, that is, applied to the serosal surface, abolished responses to transmural stimulation in concentrations of 0·1 to 0·6 μg/ml. Concentrations of 4 to 6 μg/ml were required to abolish peristaltic responses to increased intraluminal pressure. The longitudinal contractions associated with peristaltic activity were abolished earlier and with slightly lower doses of the drug than were the pressure waves due to propagated contractions of circular muscle. The blockade of responses to transmural stimulation and of peristaltic activity by the drug, in the appropriate concentrations, was fully established in 30 to 60 s. Fig. 1 illustrates its effects on circular and longitudinal muscles involved in peristaltic activity.

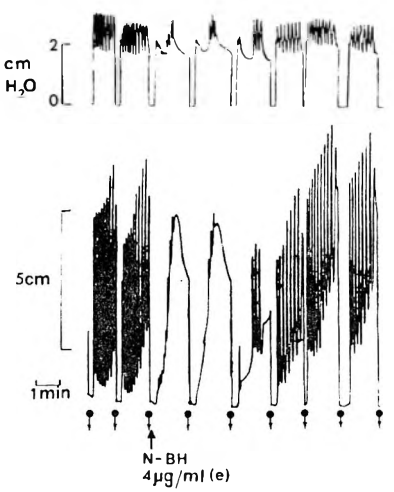

FIG. 1. Blockade of peristaltic activity by *N*-butylhyoscine (N–BH) applied to the external (e), serosal surface of guinea-pig ileum. Upper trace: intraluminal pressure. Lower trace: longitudinal movements. The drum was stopped and the bath washed out after each period of peristaltic activity as indicated by the symbol ⏚.

With doses of the drug that reduced but did not abolish responses to transmural stimulation (0·03 to 0·1 μg/ml), the twitches were reduced in height within 30 s, but then recovered somewhat and reached a constant depressed level within 5 min (Fig. 2A). The effect of the drug was easily removed by one exchange of the bath fluid with fresh McEwen solution.

When *N*-butylhyoscine was applied into the lumen of the ileal sac, that is, to the mucosal surface, much larger concentrations (600 to 800 μg/ml) were required to abolish peristaltic activity and responses to transmural stimulation within 5 min. There was no apparent difference in the concentrations affecting the two types of response (Fig. 3).

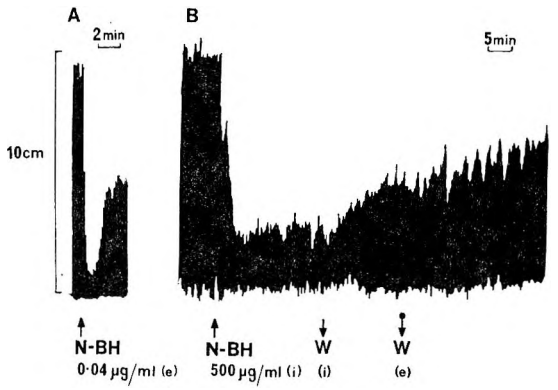

FIG. 2. Effects of *N*-butylhyoscine on transmurally stimulated segments of guinea-pig ileum, in A, applied to the external (e), serosal surface, and in B, applied intraluminally (i) to the mucosal surface. The preparation was washed at W by exchanging the intraluminal fluid (i) or external bath fluid (e) with fresh McEwen solution.

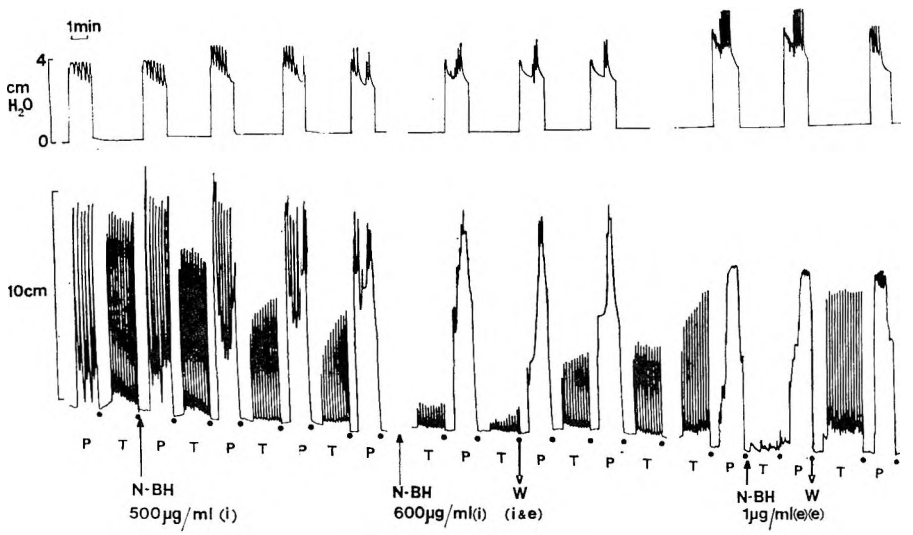

FIG. 3. Effects of *N*-butylhyoscine (N–BH) applied to the internal mucosal surface (i), and to the external serosal surface (e), on responses of guinea-pig ileum to transmural stimulation (T) and to increases in intraluminal pressure (P). The drum was stopped for 2 to 3 min after each period of stimulation as indicated by a dot. The drug-containing solution was washed out at W. *N*-Butylhyoscine caused approximately 50% reduction in responses in a concentration of 500 μg/ml and about 90% reduction on increasing this to 600 μg/ml. The responses gradually recovered after washing out the lumen of the gut (i) and the organ bath (e). Then, 1 μg/ml applied to the external surface abolished responses to transmural stimulation but had no effect on peristaltic activity. Washing out the external fluid resulted in an immediate restoration of responses.

Replacing the drug solution in the lumen with one exchange of fresh McEwen solution either resulted in a slight restoration of responses or failed to cause any restoration. An exchange of the external fluid usually resulted in a slight increase in responses, suggesting that traces of the drug had passed through the wall of the ileal segment (Fig. 2B). With further exchanges of intraluminal fluid, responses to transmural stimulation were restored to a greater extent and more rapidly than was peristaltic activity. Two exchanges of intraluminal fluid with fresh McEwen solution usually resulted in restoration of responses to transmural stimulation to the control

level, but 5 to 6 exchanges of the intraluminal fluid were required before peristaltic activity was restored.

Experiments were made with everted segments of intestine to establish that the difference in anticholinergic potency of *N*-butylhyoscine was in fact due to differences in its penetration when applied to serosal or mucosal surfaces. With everted segments, responses to transmural stimulation and peristaltic activity were obtained. These were abolished by low concentrations of the drug applied intraluminally, that is, to the serosal surface, whereas high concentrations were required to abolish responses when the drug was added to the fluid bathing the preparation, that is, applied to the mucosal surfaces. The effective concentrations for application at each surface of everted segments were of the same order as those found with normal segments.

With doses of the drug applied intraluminally that reduced but did not abolish responses (400–600 μg/ml), both types of responses were equally depressed. In about half of the preparations, the effects developed fully during the course of 5 min and there was no further depression in the following 30 min. However, in the remaining preparations, there was a slow continuing decline in responses throughout the ensuing period of observation (up to 2·5 h). Records from the preparations showing these two types of behaviour are illustrated in Fig. 4. It was noticed that preparations in which there was a slow continuing decline in responses contained at least one Peyer's patch (lymph node) in the wall of the segment. The rate of decline of responses was sometimes increased by raising the intraluminal pressure in inducing peristaltic activity.

Some of the preceding observations led to experiments designed to test the influence of hydrostatic pressure on the passage of the drug through the intestinal wall after intraluminal application to the mucosal surface. In these it was also possible to

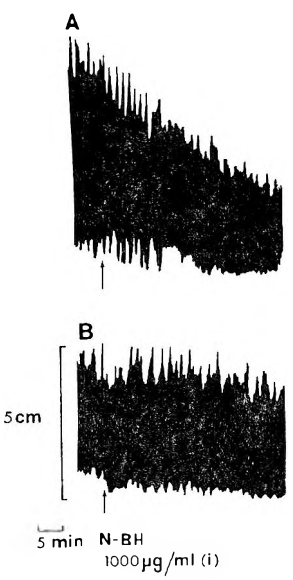

FIG. 4. Effects of *N*-butylhyoscine perfused through segments of ileum in a concentration of 1 mg/ml on transmurally stimulated detector segments in the same bath. In A, the perfused segment contained a Peyer's patch and in B, there was no macroscopically visible lymphoid tissue in the segment.

test whether the drug passed through the wall more rapidly when a Peyer's patch was present. Segments of intestine were slipped over a glass tube and selected portions of the intestinal wall were orientated over an oval hole in the wall of the tube. Various concentrations were circulated through the glass tube. Drug passing through the portion of wall covering the hole in the tube was detected by observing its effect in depressing responses to transmural stimulation in another piece of ileum. When *N*-butylhyoscine was perfused at zero hydrostatic pressure, responses of the detector segment to transmural stimulation were depressed about equally whether or not a Peyer's patch was present. However, when the hydrostatic pressure was increased there was a much greater and more rapid rate of onset of depression of responses in the detector segment when a Peyer's patch was present in the perfused segment, but this did not occur in segments without a Peyer's patch. Fig. 5 illustrates typical results with an internal of concentration of 400 μg/ml. Similar results were obtained with 800 μg and 1 mg/ml.

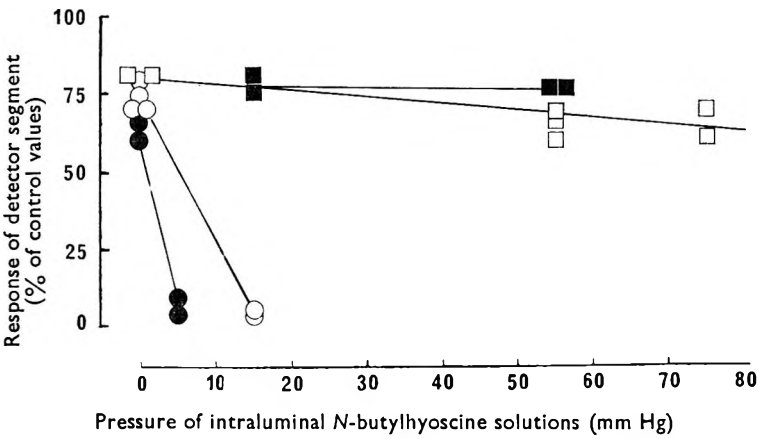

FIG. 5. Effect of *N*-butylhyoscine passing through the wall of perfused segments of guinea-pig gut on responses of transmurally stimulated detector segments. *N*-butylhyoscine in a concentration of 400 μg/ml was applied to the mucosal surface. The perfused segments were in a 10 ml bath. The bath fluid was removed at 5 min intervals and assayed. Results from segments of gut containing a Peyer's patch are indicated by ● and ○, those from segments without macroscopically visible lymphoid tissue are indicated by ■ and □. The open symbols are of segments taken from the caecal end of the gut, and the solid symbols are from the duodenal end. The sensitivity of detector segments was as follows: 80–95% depression, 80–90 ng/ml; 20–40% depression, 20–40 ng/ml.

On careful inspection of the mucosal surface of ileum, it was noticed that there was less mucus over a region containing a Peyer's patch. It had also been noticed that some segments used in the earlier experiments had large amounts of mucus present and these were unusually resistant to the anticholinergic action of the drug applied to the mucosal surface. Therefore, experiments were made in which 0·4 g of mucus and mucosal surface was suspended in 3 ml of solutions containing the drug. The mixture was centrifuged and the supernatant was assayed for anticholinergic activity on transmurally stimulated ileal segments. They were found to have activities corresponding to 60 to 70% of the concentration of *N*-butylhyoscine orginally present, this relation holding good over a concentration range of 10 to 800μg/ml. Therefore, 30–40% of the drug was removed, presumably by binding.

A potential difference across the intestinal wall, the serosal surface being positive with respect to the mucosal surface, would tend to prevent the *N*-butylhyoscine cation, when applied to the mucosal surface, from passing through the wall. An endogenous potential difference of about 2 mV across the wall of 32 day avian ileum was reported by Hudson & Levin (1968). Measurement of transmural potential difference in portions of guinea-pig ileum gave values of 6 to 8 mV. There was no difference between the potential over a Peyer's patch and that over a region macroscopically free of lymphoid tissue. There was no difference between segments taken from jejunal and caecal ends of the ileum.

DISCUSSION

N-Butylhyoscine bromide acts rapidly and in low concentrations in abolishing peristaltic activity and responses to transmural stimulation when applied to the serosal surface of the ileum, but from 100 to 1000 times the concentration is required when it is applied to the mucosal surface. This suggests that there is a substantial barrier to its penetration from the mucosal surface to cholinergic receptors on the smooth muscle in the wall. The drug, being a quaternary nitrogen compound will be virtually insoluble in the lipoid component of the mucosal cell membranes, and this probably represents the most important barrier to the drug. Furthermore, the drug is bound to mucus and mucosal tissue, and this binding may provide a further barrier to passage of the drug into and through the wall. An additional factor providing a barrier to penetration is that *N*-butylhyoscine, being a cation, would be moving against the electrical gradient of the transmural potential. Nevertheless, it can exert anticholinergic effects on the ileum after application to the mucosa providing it is present in a sufficiently high concentration.

However, passage of the drug through the wall, rather than action within the wall is poor, even with high concentrations. It is generally recognized that the absorption of ionized drugs from the intestine is poor and erratic. This applies particularly to drugs such as *N*-butylhyoscine bromide which contain a quaternary group (Levine & Pelikan, 1964). It appears from our studies with guinea-pig ileum that the passage of *N*-butylhyoscine through the wall will depend on the numbers and distribution of Peyer's patches, on the amount and distribution of mucus, on the intraluminal pressure and on the concentration of the drug. The finding that *N*-butylhyoscine passed more readily through the wall of the ileum containing a Peyer's patch than through other regions suggests that the drug may be carried into the general circulation via the lymphatics rather than via the portal system. The increased passage of *N*-butylhyoscine through portions of wall containing a Peyer's patch may be due to the fact that there is less mucus on the surface in these regions, but we have no direct evidence on this point. The binding of the cation *N*-butylhyoscine to mucus may be due to the polyanionic nature of mucus.

Herxheimer & Haefeli (1966) produced evidence that there was insignificant absorption of *N*-butylhyoscine from the gut even after oral administration of 600 mg which is six times the recommended dose. They suggested that it was without action on the gut after oral administration. However, absence of systemic effects does not preclude a local action. In fact, it has now been shown that high concentrations of the drug applied to the mucosal surface of the guinea-pig ileum abolished peristaltic activity and responses to transmural stimulation of cholinergic nerves, but that only small amounts actually passed through the wall of the ileum.

Wick's (1967) evidence that *N*-butylhyoscine is absorbed was based on the findings that LD50 was lower and death occurred much earlier after intraduodenal than subcutaneous administration. Wick also noted that the LD50 after instillation of *N*-butylhyoscine into the stomach was higher than with instillation into the duodenum. He suggested that this was due to decreased gastric peristalsis caused by the drug, and hence a slower passage to sites of absorption in the small intestine. The present observations that a high concentration of the drug decreased peristaltic movements supports Wick's suggestion.

The present findings extend the observations made by Pennefather & others (1968). They demonstrated that *N*-butylhyoscine was absorbed from the gastrointestinal tract as shown by the blockade of cardiovascular responses to vagal stimulation. However, their main finding was that *N*-butylhyoscine, administered into the duodenum, had a much greater antagonistic effect on cholinergically induced responses of the gut than on responses of other effectors. In the light of the present findings, this may be interpreted as a local effect on the gut with relatively little absorption.

The binding of *N*-butylhyoscine to mucus and mucosal tissue may explain the slow return of responses after washing out the drug-containing fluid from the lumen of isolated intestinal segments and the necessity for repeated washing to obtain full recovery of responsiveness, the bound *N*-butylhyoscine being slowly released. Furthermore, it may explain the persistence of action of enterally administered *N*-butylhyoscine as compared with intravenous injections in the experiments of Pennefather & others (1968).

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Competitive antagonism of isoprenaline-induced cardiac necroses by β-adrenoreceptor blocking agents

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Histological methods show that low doses of (±)-isoprenaline may produce cardiac necroses in the rat. The percentage of animals with focal necroses was related to the dose within the range 0·005 to 0·5 mg/kg administered subcutaneously. Therefore the heart-damaging effect of isoprenaline as well as the antagonistic effect of β-adrenoreceptor blocking agents could be evaluated quantitatively. Pre-treatment of animals with dichloroisoprenaline, pronethalol and propranolol produced a parallel displacement to the right of the dose-response line for isoprenaline. However, when lesions were already in progress, these agents were without protecting effect.

Isoprenaline and other sympathomimetic drugs produce myocardial necroses in the heart of the rat. Isoprenaline-induced necroses are reproducible although the severity of lesions depends on many factors, such as weight, sex, age, and strain of animals.

The criteria used to assess the extent of cardiac necroses, and also the doses used, have differed among authors. According to Rona (1967) high and repeated doses of isoprenaline are needed to obtain consistent lesions.

We show that a single low dose of isoprenaline produces dose-dependent lesions which allow for an easy quantitative evaluation of β-adrenergic blocking activity.

EXPERIMENTAL

Methods

Groups of male Sprague-Dawley rats, 200–220 g, were injected subcutaneously with increasing doses of (±)-isoprenaline hydrochloride in a volume of 0·2 ml/100 g of body weight. At least 10 animals were used for each dose. The antagonist drugs were injected subcutaneously either 15 or 60 min before isoprenaline or 4 h after.

The animals were decapitated 24 h after isoprenaline administration. Hearts, fixed for 24 h in 6·25% phosphate buffered (pH 7·2) glutaraldehyde solution, were embedded in jelly. Cryostatic frontal sections (10 μm) including the ventricles and the septum were stained with sudan-black.

Two contiguous sections of each heart were examined by two different observers and the hearts were considered lesioned when at least one focus of necrosis was found by both observers. No attempt was otherwise made to grade the severity of the lesions.

The percentage of animals with lesions was plotted against the dose on probit-log paper. Statistical analysis of results was according to the method of probits analysis of Finney (1952).

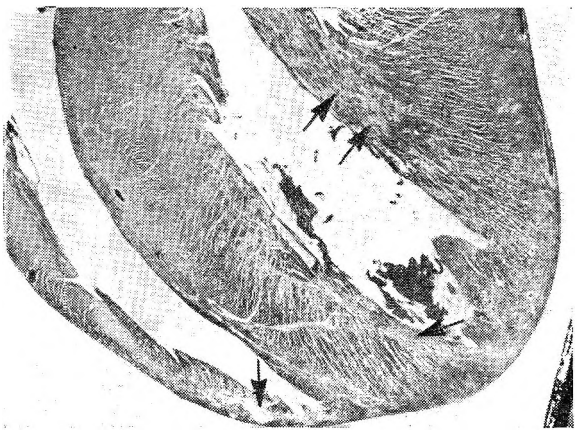

FIG. 1. Frontal section of the heart of the rat. Several cardiac necroses, indicated by arrows, after isoprenaline (0·180 mg/kg s.c.) (×4).

RESULTS

Administration of a single dose of isoprenaline to rats caused necrotic lesions in the cardiac ventricles (Fig. 1). The morphological picture 24 h after isoprenaline injection was typical of focal necrosis. Loss of cell structure and a decrease of sudanophilic droplets at the muscle fibre were associated with infiltration of leucocytes (sometimes rich in lipidic material) in the affected areas (Fig. 2).

The dose of isoprenaline active in 10% of animals was as low as 0·005 mg/kg subcutaneously, while at 0·5 mg/kg, 100% of animals showed numerous focal lesions in each histological section.

The percentage of animals with lesions was related to the dose of isoprenaline, as illustrated in Fig. 3, where satisfactory dose-response curves for isoprenaline, alone and in the presence of different doses of dichloroisoprenaline, pronethalol, and propranolol, are shown. The dose-response lines for animals treated with different doses of β-adrenoreceptor blocking agents were parallel and displaced to the right.

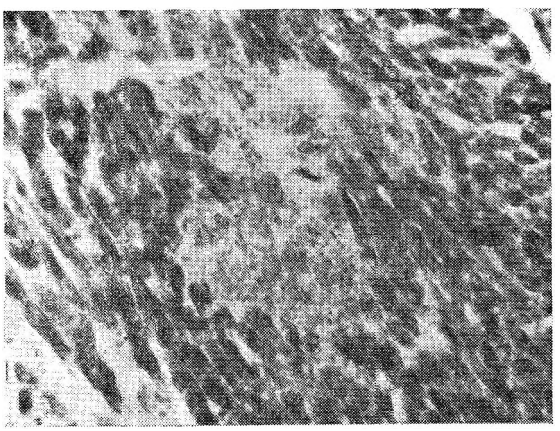

FIG. 2. A focus of cardiac necrosis. Hearts showing at least one of this kind of focus were considered to have lesions. (×250).

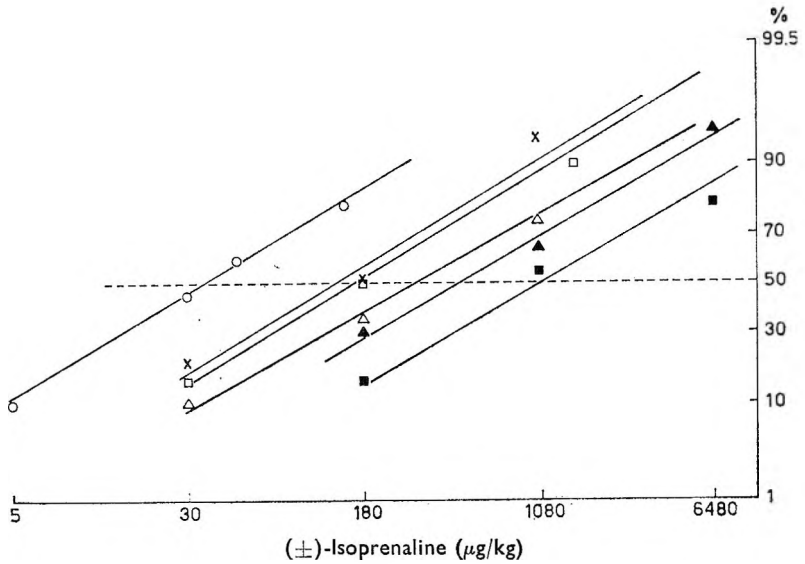

FIG. 3. Dose response curves of isoprenaline (○), isoprenaline after dichloroisoprenaline 5 mg/kg (×), isoprenaline after pronethalol 5 mg/kg (□), isoprenaline after propranolol 2 mg/kg (△), isoprenaline after propranolol 5 mg/kg (▲), and isoprenaline after pronethalol 25 mg/kg (■).

Table 1. *Dose of isoprenaline producing cardiac lesions in* 50% *of the animals* (*ED*50) *in the presence of β-adrenergic blocking agents administered* 15 *min before the agonist*

Antagonist and dose	Isoprenaline ED50 (μg/kg)	Straight line equation
	33·7 (21·7–63·5)*	y = 2·358 + 1·681 x
Dichloroisoprenaline 5 mg/kg ..	143 (72·5–284)*	y = 1·454 + 1·645 x
Propranolol 2 mg/kg	311 (151–638)*	y = 1·376 + 1·454 x
,, 5 mg/kg	487 (250–974)*	y = 0·817 + 1·557 x
Pronethalol 5 mg/kg	166 (83·5–333)*	y = 1·381 + 1·629 x
,, 25 mg/kg	1140 (485–2704)*	y = 0·983 + 1·313 x

* Confidence limits for $P = 95\%$.

Potencies of antagonistic drugs are shown in Table 1.

The pretreatment of the rats with high doses of dihydroergocryptine (20 or 40 mg/kg, s.c., 15 min before 0·5 mg/kg, s.c., of isoprenaline) or of phenoxybenzamine (5 or 25 mg/kg, s.c., 15 and 60 min before isoprenaline) did not reduce the necrotic effects of isoprenaline.

In the presence of β-adrenoreceptor blocking agents, the ED50 for isoprenaline was raised from 33·7 to 1140 μg/kg, for instance, in animals pretreated with 25 mg/kg of pronethalol.

As can be seen from the Table, propranolol was the most active antagonist. On the other hand, α-adrenoreceptor blocking agents were without effect.

Groups of animals treated 4 h after isoprenaline with 5 mg/kg of propranolol, or with 25 mg/kg of pronethalol, showed focal necroses of the type described above 20 h later.

DISCUSSION

From the present study it is evident that myocardial necroses may be produced in the rat by a single administration of isoprenaline and by dosages smaller than those used previously (Leszokovszky, Gal & Tardos, 1967). Hence, isoprenaline may induce severe myocardial lesions even in the range of doses of pharmacological and therapeutic importance.

The percentage of animals with lesions was directly related to the dose of isoprenaline. While the antagonistic effect of β-adrenoreceptor blocking agents could be confirmed by our results (Méhes, Raykovits & Papp, 1966) we showed, in addition, that dichloroisoprenaline, pronethalol, and propranolol caused a parallel displacement of dose response lines for isoprenaline in proportion to the potencies of the blocking agent. Hence, β-blockers seem to act as competitive antagonists not only to the pharmacological but also to the morphological effects elicited by the β-adrenoreceptor stimulant agent, isoprenaline. However this type of antagonism was evident solely in animals pretreated with β-blockers and not when lesions were already in progress (Ferrans, Hibbs & others, 1964), i.e. when the blocking drugs were injected 4 h after isoprenaline administration.

The antagonistic effects of phenoxybenzamine and dihydroergocryptine were also investigated. Neither prevented the development of isoprenaline-induced lesions, although according to Méhes & others (1967), lesions produced by α-receptor stimulants (adrenaline, noradrenaline, phenylephrine, and methoxamine) are inhibited by dibenamine.

In conclusion our results have shown that isoprenaline-induced cardiac necroses may be put on a quantitative basis by plotting numbers of animals with at least one histological lesion against doses of isoprenaline.

Since known β-adrenoreceptor blocking agents were shown to possess specific and dose-related antagonistic properties, the procedure, as modified by us, could be used to study the potency and the type of antagonism induced by other β-blockers.

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Detection of drugs with peripheral vascular effects similar to those of nicotinic acid

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Guinea-pigs anaesthetized with urethane respond with a marked rise in skin temperature due to dilation of the cutaneous vessels after the administration of nicotinic acid. Recording the ear temperature of these animals can therefore be used as a simple and sensitive method for the detection of drugs with peripheral vascular effects similar to those produced by nicotinic acid.

Flushing of the skin due to dilation of the cutaneous vessels (Bean & Spies, 1940) especially in the brachiocephalic region is frequently experienced in man after the administration of nicotinic acid. This phenomenon is associated with a rise in skin temperature and is difficult to reproduce in animals. It has not been observed in mice, rats, rabbits, ground squirrels, sheep and dogs, even after large doses (Altschul, 1964a). However, it has been reported that nicotinic acid (Chevillard, Giono & Laury, 1958) and β-pyridylcarbinol (Fromherz & Spiegelberg, 1948) elicit flushing of the guinea-pig ear. This led us to develop a method for detecting drugs with vascular effects similar to those of nicotinic acid.

EXPERIMENTAL

Male guinea-pigs weighing 250–300 g were anaesthetized by the intraperitoneal injection of 1500 mg/kg of urethane (6 ml/kg of a 25% w/v solution).

A chromium-nickel-constantan thermo-couple (type RM6, Ellab instruments, Copenhagen, Denmark) was placed with its thermo-junction on the skin of the ear. The temperature was recorded on a six channel strip chart recorder (type Z8, Ellab instruments, Copenhagen, Denmark). The accuracy of the recordings was ±0·1°.

The compounds were injected intraperitoneally (8 ml/kg). Substances, sparingly soluble in water, were suspended in 0·9% saline containing 2% methylcellulose. The pH and tonicity of the solutions were adjusted to physiological levels by adding NaCl, NaOH or HCl. The experiments were made at a room temperature of 22–23°.

RESULTS

The ear temperature of 100 unanaesthetized guinea-pigs was 33·2 ± 1·5° (mean ± s.e.) and was markedly affected by the handling of the animals. However, the temperature of guinea-pigs anaesthetized with urethane did not vary more than ±0·1° during a period of 10 min shortly after the onset of anaesthesia. The temperature during the 10 min pre-drug period as obtained on 100 animals was 28·8 ± 1·0° (mean ± s.e.). Under similar conditions anaesthesia induced by sodium pentobarbitone (35 mg/kg, i.p.) gave variations of several degrees.

In 15 control animals the intraperitoneal administration of 8·0 ml/kg of a 0·9% NaCl solution gave a small and gradual decrease in ear temperature in all animals which amounted to 0·3 ± 0·2° (mean ± s.e.) 60 min after the injection. The

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corresponding value after the injection of a solution containing 2% methylcellulose was 0·3 ± 0·3° (mean ± s.e., n = 15). An increase in ear temperature of at least 0·3° above the pre-drug value was therefore defined as a significant effect.

Nicotinic acid in doses between 5–150 mg/kg gave a significant increase in ear temperature (Table 1). In most cases the temperature began to rise within 2 min after the injection and reached its peak value after 3 to 8 min, returning to pre-drug values usually within 10–20 min after the onset of the rise. No significant correlation of the maximum rise in skin temperature and the dose given was observed.

Table 1. *The effect of a single intraperitoneal injection of nicotinic acid on the ear skin temperature of guinea-pigs anaesthetized with urethane. An increase in temperature of* 0·3° *was considered significant*

Nicotinic acid dose in mg/kg	Number of responders / Number of animals injected	Maximum rise in ear temperature in ° C (mean ± s.e.)
1·5	2/10	0·4 ± 1·2
5	6/10	1·4 ± 1·1
15	7/15	1·7 ± 0·8
30	13/15	1·3 ± 0·8
50	12/16	2·2 ± 1·2
100	14/15	2·2 ± 1·1
150	21/29	1·8 ± 1·0

DISCUSSION

The reason for the different responses of various peripheral vascular beds (Altschul, 1964) in man to nicotinic acid is not clear. The large differences in the vascular reactivity towards this drug between various species is also unexplained. The unresponsiveness of laboratory animals has prevented the development of simple methods for detecting drugs with vascular effects similar to those of nicotinic acid.

Measurements of fore-paw temperature in conscious mice is used as a screening method for detecting drugs with peripheral vasodilatory properties (Richter, 1964; Campbell & Richter, 1967). In our hands this method fails to detect nicotinic acid in doses up to 500 mg/kg intraperitoneally. The present method seems to fulfil the requirement of a simple, sensitive screening procedure for this purpose. The number of animals which reacted increased with dose (Table 1). However, no correlation was found between the magnitude of the rise in ear skin temperature and the dose (Table 1). This is similar to the situation in man where no clear relation has been found between the vascular effects and the dose of nicotinic acid (Altschul, 1964b).

Acknowledgement

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LETTERS TO THE EDITOR

Inhibition by phenobarbitone of oestrogen-stimulated increases in uterine enzymes

Some barbiturates, carcinogens and insecticides enhance the activities of drug-metabolizing enzymes in hepatic microsomes (Conney, 1967). Pretreatment of immature rats with phenobarbitone modifies the responses of uterine tissue to oestrogenic hormones. Levin, Welch & Conney (1967) found phenobarbitone to inhibit the increases in uterine wet weights induced by oestradiol as well as the incorporation of [2-^{14}C]glycine into uterine protein. Subsequently these workers (Levin, Welch & Conney, 1968) demonstrated that phenobarbitone, and several unrelated inducers of liver microsomal enzymes, blocked not only the uterotrophic action of oestradiol and oestrone but also decreased the amount of tritiated oestrogen in the uterus after an injection of the labelled hormone.

We interpreted the rapid increases in the activity of uterine phosphofructokinase induced by oestradiol-17β to represent enzyme synthesis *de novo* (Singhal & Valadares, 1967; Singhal, Valadares & Ling, 1967a), but whether drug-induced stimulation of hepatic microsomal enzyme activities could modify the effects of oestradiol-17β on uterine enzyme biosynthesis remained unknown. We now report the ability of phenobarbitone to alter the oestrogen-induced increases in several glycolytic and hexosemonophosphate shunt enzymes in the uterus of the ovariectomized rat.

Female Wistar rats, 180–200 g were ovariectomized, and 2 weeks later were divided into three groups: control rats injected with physiological saline; animals treated intramuscularly with oestradiol-17β (0·1 μg/100 g); and rats treated by intraperitoneal injections of phenobarbitone (3·7 mg/100 g) twice daily for 3 days before giving the oestrogen. Animals were killed 16 h after injection of the hormone. Uteri were rapidly excised, carefully trimmed of extraneous tissue and homogenates and supernatant fluids were prepared (Singhal & others, 1967a, b). The activities of uterine hexokinase (Valadares, Singhal & Parulekar, 1968), aldolase (Warburg & Christian, 1943), as well as glucose 6-phosphate and 6-phosphogluconate dehydrogenases (Glock & McLean, 1953) were assayed in the supernatant. Uterine pyruvate kinase activity was also measured in the supernatant fluid (Weber, Stamm & Fisher, 1965). All enzyme activities were assayed under strictly linear kinetic conditions and calculated as μmol of substrate metabolized per g of tissue per h at 37° × the weight of the uterus.

Table 1 summarizes the effects of phenobarbitone pretreatment on the oestradiol-induced alterations in the activities of uterine hexokinase, aldolase, pyruvate kinase and the two NADP-specific dehydrogenases of the hexose monophosphate shunt pathway. Sixteen h after a single injection of oestradiol-17β, uterine hexokinase increased to 229%, aldolase to 331% and pyruvate kinase to 370% of the values of control animals. Likewise, the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases were raised respectively, to 268 and 264% of control values in uteri of oestrogenized rats. Chronic treatment with phenobarbitone inhibited completely (98%) the increases in uterine hexokinase activity induced by oestradiol-17β. Phenobarbitone also reduced markedly the oestrogen-stimulated increases in uterine aldolase (138%) and pyruvate kinase (179%). Additionally, the steroid-induced rise in the activities of the two NADP-dependent dehydrogenases was inhibited significantly

Table 1. *Influence of phenobarbitone administration on oestradiol-stimulated increases in several uterine enzymes*

Treatment	Hexokinase	Aldolase	Pyruvate kinase	Glucose 6-phosphate dehydrogenase	6-Phospho-gluconate dehydrogenase
Control	4·8±0·2 (100)	10·2±0·3 (100)	108±4·0 (100)	9·6±0·2 (100)	2·48±0·02 (100)
Oestradiol-17β ..	11·0±0·3 (229)*	33·8±0·3 (331)*	399·5±13·3 (370)*	25·7±0·4 (268)*	6·6±0·3 (264)*
Oestradiol-17β + phenobarbitone	4·7±0·1 (98)†	14·1±0·7 (138)*†	193±10·1 (179)*†	16·0±0·4 (167)*†	3·8±0·2 (152)*†

Each value represents the mean ± s.e. based on 3 assays of enzyme activity in uteri pooled from 2–3 rats. Rats were treated with phenobarbitone (3·7 mg/100 g), twice daily for 3 days. Oestradiol-17β (0·1 μg/100 g) was administered intramuscularly 16 h before death. Enzyme activities are expressed as μmol of substrate metabolized per g of tissue per h at 37° × weight of the uterus. The results are also given in percentages (in parentheses) taking the control values as 100%.

* Statistically significant difference as compared to the values of control rats ($P = <0·05$).

† Statistically significant difference as compared to the values of oestradiol-injected rats without pretreatment with phenobarbitone ($P = <0·05$).

by phenobarbitone and in these animals, uterine glucose 6-phosphate dehydrogenase increased to only 167% and 6-phosphogluconate dehydrogenase to 152% of the values of saline-treated controls. Chronic phenobarbitone treatment did not influence the basal levels of uterine phosphofructokinase although this barbiturate produced an almost complete blockade of the enzyme response elicited by a 2·5 μg/100 g dose of oestradiol-17β (Singhal & others, 1967b).

It has been suggested that phenobarbitone accelerates the metabolism of oestradiol in the body by inducing the synthesis of drug-metabolizing enzymes (Conney, 1967). The observed inhibition by phenobarbitone of the oestradiol-stimulated increases in the activities of several uterine carbohydrate-metabolizing enzymes may be the result of a decrease in the amount of oestradiol-17β available to the uterus.

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The effect of diet in West Africa on the pH of human urine

It is well known that the pH of urine can have pronounced effects on the excretion of partially ionized drugs (Beckett & Tucker, 1967). In the Western world, in subjects receiving a normal balanced diet, urinary pH varies between subjects as well as within subjects throughout the day but the average pH is about 6·0 (Yarbro, 1956; Elliot, Sharp & Lewis, 1959; Beckett, unpublished observations).

In Ghana, 12 men aged 20–30 years were selected at random from each of two classes of persons: (i) students in Halls of Residence at the University who were receiving their usual balanced diet, the carbohydrates consisting of bread, cakes, yams, cassava, rice and maize; (ii) laboratory assistants living at home on their usual low-protein diet, their carbohydrate intake consisting of rice, gari, maize, yam, plantain and cassava. Urine was collected from the subjects at 2 h intervals from 7 a.m. to 1 p.m. on two successive days and the pH of each collection measured immediately. Also all urine samples were collected for a period of 36 h starting from 7 a.m. on one day and the pH of the pooled urine of each subject over this period was measured.

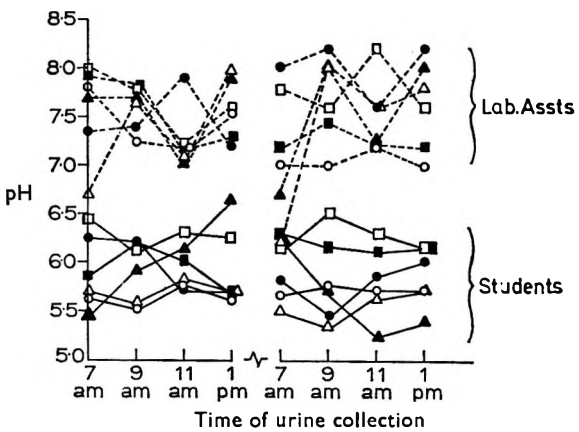

FIG. 1. Urinary pH in subjects on their normal different diets. ———, students on balanced protein diet. – – – –, laboratory assistants on low protein diet.

Typical results for the pH of separate samples of urine of students and laboratory assistants are given in Fig. 1. There is virtually no overlap in the pH profile in the two classes of subjects. Very few laboratory assistants had urine collections more acidic than pH 7 whereas none of the students had urine collections more alkaline than pH 7.

For the pooled urines, the pH values were: students, 5·9 $\pm$ 0·56 (s.e., n = 12), range, 5·45–6·3; laboratory assistants, 7·5 $\pm$ 0·25 (s.e., n = 12), range, 7·3–7·7.

The two classes of subjects must inevitably excrete partially ionized drugs to a different extent, e.g. basic drugs would be expected to be excreted much less rapidly by laboratory assistants than by students. Preliminary investigations with amphetamine support this conclusion.

Since the pH of the urine can alter the excretion of partially ionized drugs and the ratio of metabolites to parent drugs in the body, the present results indicate that it may be unwise to extrapolate from the observed effects and side-effects of drugs in clinical trials in developed countries to predict the effect of these drugs in communities with different dietary customs. Also the effect of diet should not be overlooked

when clinical trials of partially ionized drugs are being considered for different countries.

I thank the subjects for their co-operation in this study and Prof. A. H. Beckett, University of London, for suggesting the project.

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Effect of actinomycin D on the recovery of cardiac noradrenaline after depletion with guanethidine

It has been suggested that the recovery of tissue noradrenaline levels after reserpine depletion is dependent upon the synthesis of new storage vesicles in the cell body and their subsequent transport down the axons to the nerve terminals (Dahlström, 1965; Dahlström & Haggendal, 1966). Evidence for such a suggestion comes from studies showing that fresh vesicles begin to reach the nerve terminals within 24 h after reserpine administration (Dahlström, 1967). Prolongation of the recovery of tissue noradrenaline in reserpine-treated animals by agents which inhibit protein synthesis, such as actinomycin D or SKF-525A (β-diethylaminoethyldiphenylpropyl acetate), is consistent with this hypothesis (Mueller & Shideman, 1968). Guanethidine in addition to producing pharmacological effects different from those of reserpine, has been shown to produce depletion of noradrenaline stores by a mechanism that appears to be, at least in part, similar to the one mediated by reserpine, i.e. by blocking of the granular pump (Shore & Giachetti, 1966). It was therefore of interest to determine the effect of actinomycin D on the recovery of cardiac noradrenaline after depletion with guanethidine.

Male Sprague-Dawley rats weighing 125 to 150 g were injected i.p. with 20 mg/kg of guanethidine, a dose which has been shown to produce over 90% depletion of cardiac noradrenaline (Westfall & Osada, 1968), and 48 h later half of these rats were treated with actinomycin D (100 μg/kg,i.p.). The animals not receiving actinomycin D were injected with a comparable volume of saline. Since actinomycin D has been shown to reduce food consumption, a paired feeding technique similar to that of Mueller & Shideman (1968) was used. The quantity of food eaten by each experimental animal on one day was given to its control on the following day. Animals were killed by decapitation at 6 h, 3 days, 4 days and 6 days after guanethidine. The hearts were removed and analysed for endogenous noradrenaline according to the trihydroxyindole procedure of Euler & Lishajko (1961).

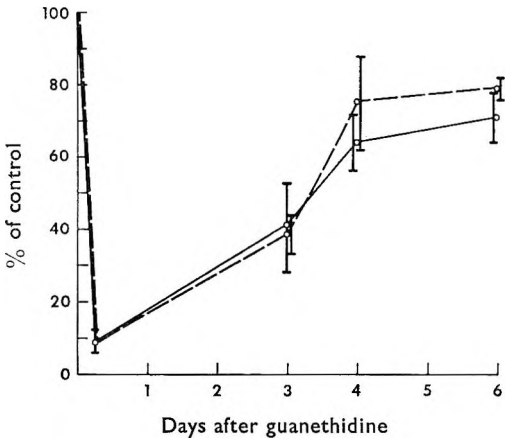

FIG. 1. The influence of actinomycin D on the recovery of myocardial noradrenaline levels after guanethidine. Guanethidine was administered in a dose of 20 mg/kg i.p. and 2 days later half of the rats were injected with actinomycin D at a dose of 100 μg/kg i.p. Data are plotted as % of control level ± standard error of the mean *vs* time in days. Each point represents the mean from at least 4 animals. ——; guanethidine alone. – – – –; guanethidine and actinomycin D.

In these experiments, no difference was found in the recovery of heart noradrenaline in animals receiving the antibiotic and the controls (Fig. 1). These experiments, therefore, fail to provide evidence that resynthesis of storage vesicles is a necessary requirement for repletion of noradrenaline stores following depletion with guanethidine. Thus, either the mechanism by which guanethidine causes depletion of noradrenaline differs, in some way, from the depletion brought about by resperpine, even though both drugs appear to inhibit transport across the granular membrane, or a reversal of guanethidine-induced inhibition of noradrenaline transport by storage granules is produced before new storage vesicles can be synthesized and transported to the nerve terminals.

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The presence of dopamine in cat spleen and blood

We have measured the resting concentrations of noradrenaline and dopamine in the spleen and blood of cats under chloralose anaesthesia. Using a modification of the column separation method of Bertler, Carlsson & Rosengren (1958), and the very sensitive assay methods of Vendsalu (1960) for noradrenaline and adrenaline and of Laverty & Sharman (1965) for dopamine, and expressing the catecholamine content of the spleen in terms of its tissue mass as estimated by its content of deoxyribonucleic acid phosphorus (Dearnaley & Geffen, 1966), we have made the following observations.

From 22 cats the mean resting concentration of dopamine in the spleen was found to be 4·82 ng/μmol DNA-P (s.e. $\pm$ 1·16) which was approximately 10% of the noradrenaline content (mean 50·20 $\pm$ 5·71 ng/μmol DNA-P, n = 16). In 9 other cats, however, the dopamine content was estimated to be some 4 to 20 times higher than this (mean 33·77 $\pm$ 8·55 ng/μmol DNA-P), and a few spleens therefore contained more dopamine than noradrenaline. Why these spleens contained so much more dopamine than the others we do not know (there appeared to be no obvious correlation between factors such as age, sex or diet and the high dopamine levels) but we feel sure that it was dopamine for a number of reasons.

We have been able to show, for example, that dopamine is present in plasma from the blood of some cats at a resting concentration of 0·69 to 1·52 ng/ml, but in the plasma from other cats we found levels much in excess of this (3·85 to 14·46 ng/ml). These levels do not take into account the low recovery of dopamine, 25%, which was obtainable from plasma by the techniques adopted. In those animals where both blood and spleen concentrations of dopamine were measured they were both in either the low group or the high group. Similarly, in some of our experiments where cat spleens have been divided (Dearnaley & Geffen, 1966), when one portion was shown to contain high levels of dopamine, perfusion of the other portion with McEwen solution (Thoenen, Hürlimann & Haefely, 1963) resulted in the appearance of large amounts of dopamine in the effluent (1·49 to 4·26 ng/ml after 30 min perfusion). This was in contrast to the situation in spleens containing low levels of dopamine (less than 0·08 ng/ml). Finally we have shown that the fluorescence characteristics of the amine from spleens apparently containing high levels of dopamine corresponds to that of authentic dopamine, and that the fluorescence intensity and hence the dopamine content of samples was essentially the same before and after subjecting the samples to chromatography and elution at the dopamine Rf value (Laverty & Sharman, 1965).

The experiments in which the spleens were perfused, also allow us to speculate on the nature of the storage of this dopamine in the tissue. When the wash-out of dopamine from spleens with a high dopamine content was determined at various times during the perfusion, the rate of output declined in an exponential manner (Fig. 1) similar to the decline observed by Iversen (1965) after the accumulation of adrenaline and noradrenaline by the Uptake$_2$ process in the rat isolated heart. We can demonstrate an identical wash-out of accumulated dopamine after its infusion into spleens containing dopamine at the lower level and suggest therefore that the high levels of dopamine found in some of our experiments had accumulated in Uptake$_2$ tissue storage sites. Whether the high spleen levels precede the high blood levels or vice versa we do not know, nor is the original source of this dopamine apparent. We hestitate to comment on the possible functional significance of our findings but it would appear that endogenous amines can occur in Uptake$_2$ storage sites.

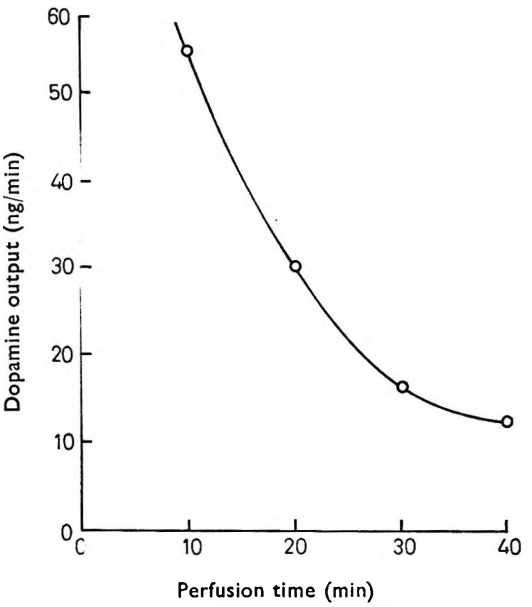

FIG. 1. The relation between the rate of output of dopamine (ng/min) and the time of perfusion of a cat isolated spleen containing a large amount of dopamine.

Table 1. *The influence of pargyline hydrochloride on catecholamine levels in cat spleen and plasma*

		Spleen content (ng/μmole DNA-P)		Plasma concentration (ng/ml)	
		Untreated (n = 16)	Pargyline pretreated (n = 15)	Untreated (n = 6)	Pargyline pretreated (n = 7)
Noradrenaline	Mean	50·20	30·09	1·15	1·34
	s.e.	±5·71	±4·88 (P <0·01)	±0·17	±0·24
Adrenaline	Mean	14·68	7·72	1·66	0·60
	s.e.	±2·10	±1·92 (P <0·001)	±0·44	±0·15 (P <0·05)
Dopamine	Mean	7·66	19·16	4·75	4·40
	s.e.	±2·16	±4·38	±2·15	±1·27

Pargyline (50 mg/kg) was administered subcutaneously 16 h before the experiment.

Pretreatment of cats with the monoamine oxidase inhibitor pargyline, 50 mg/kg injected subcutaneously 16 h before the experiment, was found to significantly lower the noradrenaline and adrenaline contents of spleens and the adrenaline content of plasma. The dopamine content of both spleen and plasma was not significantly altered by pargyline (Table 1).

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Removal of plasma metyrapone in rats submitted to previous pharmacological treatment

Metyrapone [SU 4885; Metopirone; 2-methyl-1,2-di(3-pyridyl)propan-1-one] a relatively specific 11-β-hydroxylase inhibitor, is currently used for testing the pituitary ACTH reserve (Liddle, Estep & others, 1959). Several authors have reported that the metyrapone test is not reliable in patients under therapy with other drugs like for instance phenobarbitone and diphenylhydantoin (Krieger, 1962; Rinne, 1966, 1967; Werk, Thrasher & others, 1967). Metyrapone is metabolized by the liver to form a reduced compound [SU 5236; 2-methyl-1,2-di(3-pyridyl)propanol] (Kraulis, Traikov & others, 1968), a process which is blocked *in vivo* by the administration of an inhibitor of liver microsomal enzymes such as SKF 525 A (S. Szeberenyi, unpublished). It may be possible therefore that the level and the disappearance of metyrapone from plasma are affected by treatment with various drugs known to influence the activity of liver microsomal enzymes. This note summarizes preliminary data obtained by measuring the half-life of metyrapone in plasma of rats pretreated with several drugs known to increase the rate of metabolism (induction) of foreign compounds. Female Sprague–Dawley rats (140 g) were treated with various drugs twice a day (9.00 a.m. and 9.00 p.m.) for 5 days as reported in Table 1. 36 h after the last treatment, metyrapone hydrochloride was injected intraperitoneally at the dose of 66 mg/kg. 5, 15 and 30 min after metyrapone administration, animals were killed and metyrapone was determined in plasma according to the method of Szeberenyi, Szalay & Tacconi (1968). At least 12 animals per drug were used.

Table 1. *Half-life* (t $\frac{1}{2}$) *of metyrapone in plasma of rats pretreated with several drugs*

Drug	mg/kg (twice daily for 5 days)	$t\frac{1}{2}$ (min) of metyrapone in plasma
Saline	(0·5 ml)	17 ± 0·6
Niketamide	100 orally	14
Phenylbutazone	62·5 i.p.	13·5
Diazepam	50 i.p.	13
Meprobamate	100 i.p.	12
Diphenylhydantoin	37·5 i.p.	12
Pentaerythritol tetranitrate	25 i.p.	11
Hydrocortisone	40 s.c.	10·5
Phenobarbitone	37·5 i.p.	9

From Table 1 it is evident that all drugs tested are able to shorten the half-life of metyrapone. The effect was maximum for phenobarbitone and then in decreasing order for hydrocortisone, pentaerythritol tetranitrate, diphenylhydantoin, meprobamate, diazepam, diphenylbutazone and nikethamide. All these drugs are known to induce microsomal enzymes (Conney, 1967).

Although it is impossible to extrapolate these data to humans, it may be that previous therapeutic treatment could affect the metabolism of metyrapone and therefore influence the functional significance of this test.

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Corticosteroid modification of guinea-pig anaphylaxis

It is well established that a wide variety of experimental allergic reactions may be modified by prior administration of cortisone or related compounds (Rose, 1954, 1959). One notable exception is the anaphylactic reaction in the guinea-pig, for which a mass of contradictory evidence has been published.

The most inconsistent evidence has resulted from experiments on anaphylaxis induced by intravenous administration of antigen; the severity of the reaction being evaluated by subjective scoring, or mortality methods. A protective influence of corticosteroids was observed by Hajos (1926), Wolfram & Zwemer (1935), Simonsen (1950), Humphrey (1951), Zelenka, Zitka & Jirasek (1957) and Jaques (1961). Using similar methods no significant protective effects were observed in experiments described by Stoerck (1950), Dworetsky, Code & others (1950), Friedlander & Friedlander (1950), Malkiel (1951), Dews & Code (1951), Arbesman, Neter & Bertram (1951), Landau, Nelson & Gay (1951), Germuth, Ottinger & Oyama (1952), Criep, Weigler & Meyer (1952), Marcus, Carlquist & others (1952), Bertola (1958) and Csaba & Kassay (1966). Literature concerning the protective effects of corticotrophin against guinea-pig anaphylaxis may similarly be divided into two conflicting groups.

In contrast, much more consistent evidence has been produced, employing antigen-aerosol induced reactions accompanied by evaluation of anaphylactic dyspnoea. Certain corticosteroids were shown to depress the severity of the reactions, particularly when administered at an optimal 18 h before induction of anaphylaxis and in conjunction with antihistamine treatment (Herxheimer & Rosa, 1952; Feinberg & Malkiel, 1952; Feinberg, Malkiel & McIntire, 1953; Winter & Flataker, 1955; Mendes, 1957; Goadby & Smith, 1964; Hicks, 1968). Even with these experiments significant protective effects were established only for the more potent and water-soluble corticosteroids.

In view of the widespread use of guinea-pig anaphylaxis as a model for evaluation of anti-asthmatic compounds, it is of considerable importance that the effectiveness of corticosteroids should be clearly established. This survey indicates that the adequacy of the earlier experimental methods must be questioned, but suggests also that the sensitivity of the guinea-pig to anti-anaphylactic steroids is low. This insensitivity may be due to the very high resting blood concentrations of corticosteroids in this species (Done, Ely & others, 1952).

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Proteolytic activity, its inhibitors, and the blastic reaction in graft rejection

The role of proteolytic enzymes in eliciting reactions to transplantation have been concerned with changes in serum enzymatic activity after making allografts (Patkowski, 1968; Patkowski, Halawa & Giełdanowski, 1969), or with prolongation of graft survival under the influence of the proteolysis inhibitor ϵ-aminocaproic acid (EACA) and its acetamide (Bertelli & Frontino, 1963; Gillette, Findley & Conway, 1963; Bertelli, Bisiani & others, 1964; Cramer, Hinshaw & Spar, 1964; Zukoski, Sachatello & Tinsley, 1965). Because of the discussion on the mechanisms of the action of EACA in suppressing the transplantation barrier, we have investigated the relation between enzyme activities in the serum and lymph nodes and morphologic changes in the regional lymph node with respect to the graft in animals treated chronically with the natural inhibitor trasylol (Bayer) (a trypsin and kallikrein inhibitor from animal glands) and synthetic ϵ-aminocaproic acid (Polfa). The same parameters were observed in animals not treated with the drugs.

Experiments were made with 52 young, randomly bred, rabbits weighing 2–3 kg each. The drugs were injected twice daily, intravenously, beginning one day before the transplantation and to the end of the period of graft survival. EACA was injected in doses of 0·2 and 0·5 g/kg body weight, and trasylol in doses of 5000 u/kg. Allogenic skin grafts were made in typical manner on the dorsal side of the auricula of the rabbits. Enzyme activities in the blood serum and lymph nodes were measured (Heuson, 1959) and blastic changes in the draining lymph nodes were also evaluated (Woolf, 1950).

Results. Skin allografts evoked a biphasic increase in serum proteolytic activity, about 3–4 and 10–14 days after the operation. Early changes in enzymatic activity after transplantation were observed also after autografts and seem to be immunologically non-specific. The rise of activity after 10–14 days, on the other hand, occurred only in allografts, indicating a specific immunologic reaction (Fig. 1).

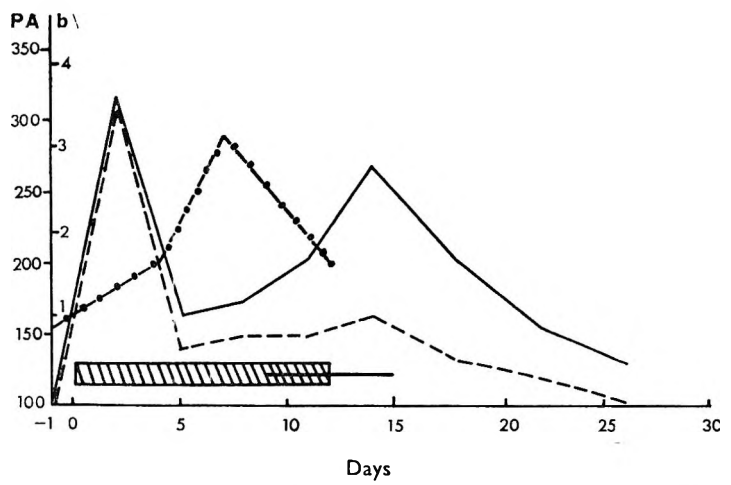

FIG. 1. Proteolytic activity and blastic activity in rabbits after transplantation. Ordinate axis: PA = proteolytic activity, as percentage. b = blastic cells, as percentage. Abscissa axis: time in days/transplantation on "O" day. ——— allograft. – – – – autograft. —●—●—●— blast cells. Hatched area = mean survival time of skin allografts with rule showing the extreme range of results.

Changes in proteolytic activity in the regional lymph node were erratic and permitted no conclusions to be reached.

The blastic reaction in the draining lymph nodes reached a peak after about 7 days. At that time the number of blasts was 3·18%; the number of plasma cells was not affected.

Administration of the proteolysis inhibitors trasylol and EACA in the lower dose gave similar effects. The survival of the skin allografts increased from 10·5 to 13–14 days, and the number of blasts in the lymph node stimulated by the graft decreased slightly, from 2·59 to 2·28%. Suppression of the blastic reaction in the strictest sense, was therefore unlikely; only the time after which the changes appeared was prolonged. Serum proteolytic activity behaved similarly. The rise in enzymatic activity after transplantation was less pronounced, but was not suppressed (Table 1).

Table 1. *Survival of skin allografts and recorded transformative changes in the regional lymph node*

Compound/dose		Survival time of skin allografts	Blastic reaction	
			Blast cells	Plasma cells
Trasylol	5·000 u/kg	13·2 ± 0·5	2·29 ± 0·53	0·48 ± 0·13
EACA	200 mg/kg	13·9 ± 2·1	—	—
EACA	500 mg/kg	13·5 ± 1·9	2·28 ± 0·30	0·48 ± 0·13
Control		10·5 ± 1·4	2·59 ± 0·45	0·58 ± 0·24

After the higher doses of EACA, survival of the allografts was not proportionally prolonged, and the blastic reaction was not significantly weakened. Proteolytic activity, however, was suppressed more strongly, persisting at the level of the initial changes (Table 2).

Table 2. *Serum proteolytic activity in animals treated after transplantation with trasylol or* EACA (*mg of digested casein/ml*)

Compound/dose		Initial	Days after allografting		
			5	10	15
Trasylol	5·000 u/kg	0·056 ± 0·013	0·077 ± 0·008	0·068 ± 0	0·053 ± 0·008
EACA	200 mg/kg	0·066 ± 0·007	0·076 ± 0·022	0·094 ± 0·015	0·076 ± 0·011
EACA	500 mg/kg	0·056 ± 0·010	0·055 ± 0·015	0·056 ± 0·019	0·062 ± 0·033
Control		0·081 ± 0·018	0·133 ± 0·046	0·157 ± 0·037	0·205 ± 0·028

In the control animals without grafts, both drugs caused 60–65% reduction of the physiologic enzymatic activity.

Cramer (1965) has suggested that EACA acts by competing for lysine, and Taylor & Fudenberg (1964) that it suppresses complement activity, which plays an important role in the transplantation reaction (Hager, Du Pay & Wallach, 1964; Cramer, 1965). On the basis of our observations on the enzymatic and blastic reactions, it must be concluded that the absence of any correlation of the degree of inhibition of proteolysis and the graft reaction arises from the manifold points of attack of EACA.

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BOOK REVIEWS

POWDERED VEGETABLE DRUGS. By Betty P. Jackson and Derek W. Snowdon. Pp. viii — 203 (including Index). J. & A. Churchill Ltd., London, 1968. 65s.

Sixty-four years separate a standard reference work from its obvious heir but the new Jackson and Snowdon is a worthy successor to the old Greenish and Collin; it will be welcomed by all concerned with the analytical microscopy of powdered drugs of vegetable origin.

If the need to authenticate such materials is somewhat less frequent than formerly, it is nonetheless necessary for some analysts to be able to determine the nature and quality of the still-large number of botanical drugs in current use. Indeed, in view of the new Medicines Legislation, it is desirable to exercise quality control not only on products containing some of the ninety-three crude drugs described but also on the range of herbal remedies used in less conventional medicine.

The book is an atlas in which diagnostic microscopical characters of each powdered drug are described on the page facing annotated drawings of these characters. The itemized descriptions are sufficiently full to enable those already familiar with plant histology to appreciate the detail in the drawings which, with a few exceptions, are of such meticulous accuracy that they will enable even the less expert to recognize what is there to be seen under the microscope. The arrangement is according to morphological grouping and information includes biological sources and drug synonyms, which also appear in the comprehensive index. Useful footnotes are given on comparative data for closely similar drugs and only in such instances are sizes recorded.

Any criticism of such painstaking work must be of a minor nature but the representation of cuticular striations on Belladonna Leaf is an example of the occasional lapse; the absence of sieve areas on the side walls of sieve tubes or of simple round pits in the phloem parenchyma of Cascara point the occasional omission; and the inference on relative prominence of tuberosities on starch granules of Potato and Maranta suggest the occasional query. Although most drawings are of features observed in cleared preparations and the authors state that certain cell contents were intentionally omitted, there are some drugs for which resultant illustration seems not quite complete. The aleurone grains (and often more calcium oxalate than shown) in Umbelliferous fruits and the fixed oil in Linseed are as necessary for completeness as is the protein illustrated in Capsicum.

Spices, unless also used in medicine, are deliberately excluded but perhaps the authors might reconsider this decision for future editions and give thought to further expanding the scope (and title) to include culinary herbs. Materials such as powdered grass or lucerne, as permitted diluents of Prepared Digitalis, and a small section on microscopy applied to unorganized drugs are further suggestions for expanding fractionally an already excellent book. It will be invaluable for the practising analyst and a constant source of information, perhaps inspiration, to both teachers and students of pharmacognosy. It is well that the book is of good paper, strongly bound, for copies will be much used.

This is a book which should meet the success it so clearly merits.

FRANK FISH

PRACTICAL PHARMACEUTICAL CHEMISTRY. Second Edition. Part I. By A. H. Beckett and J. B. Stenlake. Pp. x + 316 (including index). The Athlone Press, London, 1968. 55s.

During the six years which have elapsed since the first edition of this book, it has become established in most schools of pharmacy in this country as the standard work on pharmaceutical analysis. This period has seen a continuation of the trend, already well underway in the early 1960's, towards instrumental methods in both the quantitative and qualitative analysis of pharmaceutical chemicals. It is perhaps not surprising, therefore, to find that this second edition has been divided into two parts. Part One, reviewed here, deals with general methods of volumetric and gravimetric analysis, while Part Two will deal with physical instrumental methods. This is a sensible move and well suited to the structure of most pharmacy degree courses. The only disadvantage is the inevitable price increase of the complete work; these days, this will not be a new experience to the majority of readers!

In twelve chapters and 291 pages, the book covers essentially the same ground as was covered in eleven chapters and 238 pages of the first edition. The chapter headings remain the same and the general approach and much of the subject matter remains unaltered.

The first chapter on chemical purity and control has been considerably expanded and the contents organized in a more rational manner; this gives the reader a better appreciation of the need for the various tests included in a pharmacopoeial monograph. The sources of impurity in pharmaceutical chemicals are particularly well covered and a valuable addition is the section dealing with the standardization of pharmaceutical chemicals and formulated products. Chapter three on the technique of quantitative analysis deals more fully than its predecessor with the balance and includes a valuable table of comparative data on three types of modern balance. No mention is made of the use of glass fibre mats in the preparation of Gooch crucibles. Although not applicable where the contents have to be heated above about 200°C, these are so eminently superior to the use of asbestos for most assays, being both cheap and reliable and giving consistently good results in the hands of relatively inexperienced students. Filter papers are briefly mentioned in appropriate sections, but a valuable addition would be a comprehensive table of the characteristics of the various types of filter paper available and their special uses.

The new chapter on ion exchange and gel filtration gives a descriptive account of the two processes and the various types of material available on the market. Some practical exercises in ion exchange are given, but there are no exercises for gel filtration, although a brief mention is made of some practical applications.

The remaining chapters on the theoretical basis of quantitative analysis, acidimetry and alkalimetry, non-aqueous titrations, oxidation-reduction titrations, argentimetric titrations, complexometric analysis, gravimetric and alkaloidal type analysis and miscellaneous methods remain substantially as in the earlier edition.

Textual mistakes are remarkably few; the only important one noted by the reviewer being on page 87 where a definition for constant weight is too high by a factor of ten.

This book is a must for every new pharmacy student; existing owners of a first edition and potential purchasers of second-hand copies may not feel that the changes are sufficiently great to warrant the purchase of a new copy.

J. N. T. GILBERT

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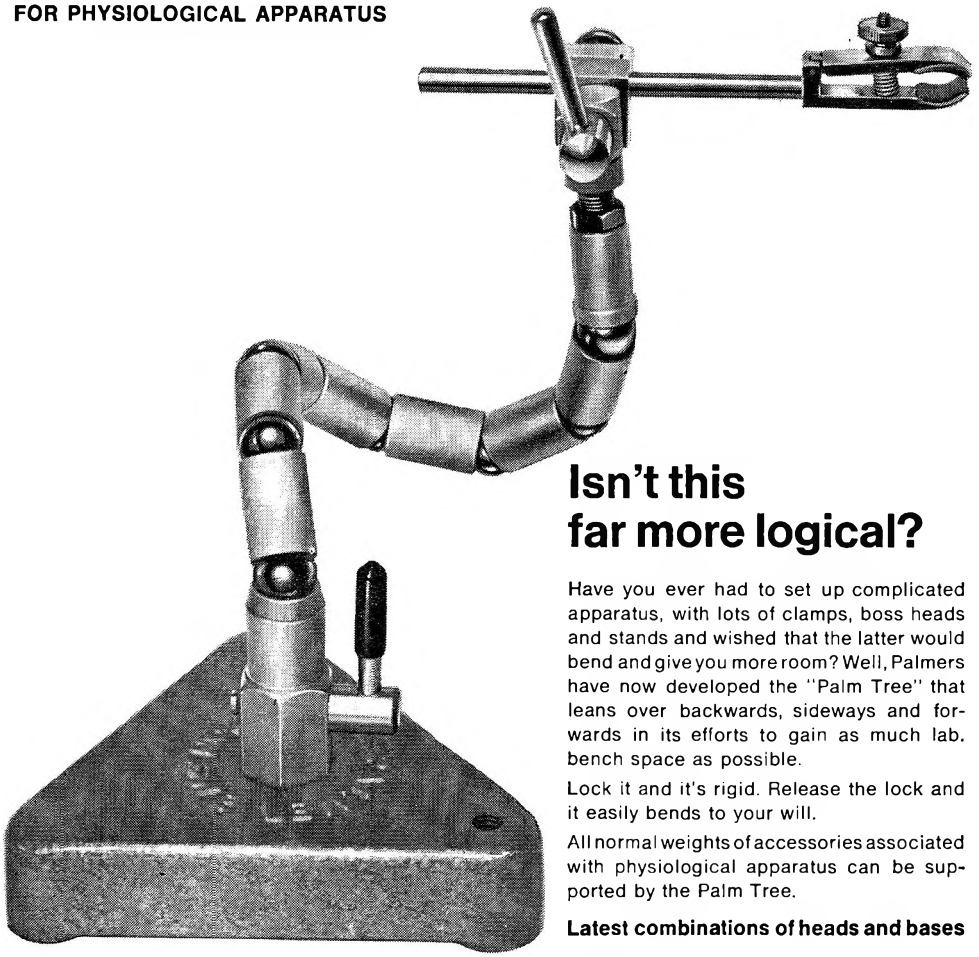

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