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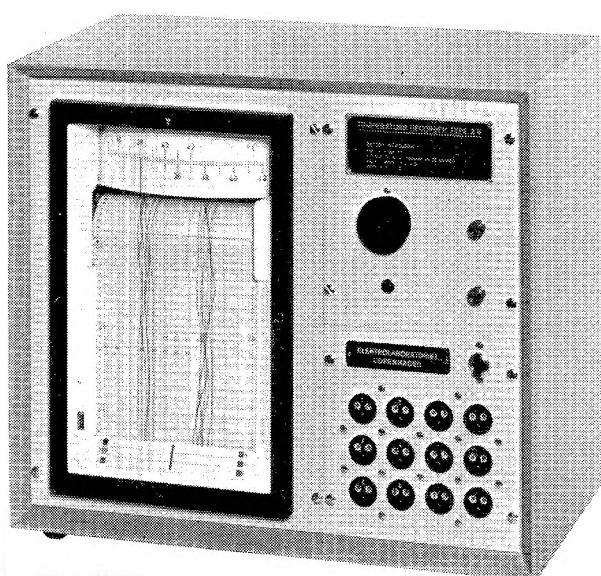

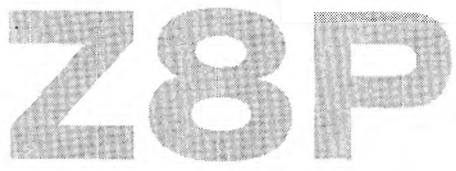

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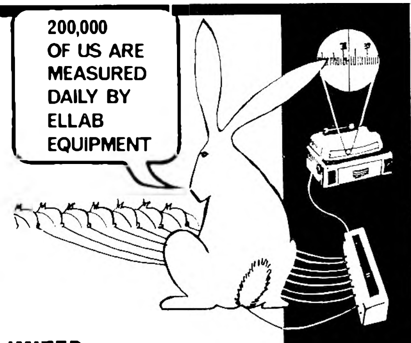

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The metabolism of ethionamide and its sulphoxide

J. P. JOHNSTON, P. O. KANE,* AND M. R. KIBBY†

The fate of orally administered ethionamide (2-ethyl-4-thiocarbamoylpyridine) and ethionamide sulphoxide in man, rats, mice and dogs has been examined. Though spontaneous interconversion between the two antituberculosis drugs does not take place, there was extensive interconversion *in vivo*, both appearing in the blood within 15 min of dosing irrespective of which compound was administered. Species differences were evident in both the rate of metabolism and in the ethionamide: ethionamide sulphoxide ratio. Apart from this interconversion, ten probable urinary metabolites were detected. These consisted of six fluorescent and four non-fluorescent metabolites, three of the latter being identified as 2-ethylisonicotinamide, 2-ethylisonicotinic acid and inorganic sulphate. A scheme is proposed to cover part of the common metabolic route of the two drugs.

ETHIONAMIDE (2-ethyl-4-thiocarbamoylpyridine or 2-ethylisothio-nicotinamide) is an active antituberculosis drug (Rist, Grumbach, Libermann, Moyeux, Cals & Clavel, 1958; Brouet, Marche, Rist, Chevallier & LeMeur, 1959). Recently the sulphoxide of ethionamide has been shown to behave similarly (Kreis, B., unpublished).

The structure of ethionamide shows similarities to other antituberculosis compounds, notably the thiosemicarbazones and isonicotinic hydrazide. Unlike these drugs the metabolism of thioamides has not been investigated although the behaviour of the $-CSNH_2$ group in thioureas and thiosemicarbazones is to some extent understood (Arita, 1956; Shibata, 1958; Scheline, Smith & Williams, 1961).

Thiorea and thiosemicarbazide (released from thiosemicarbazones) appear to be stable. Mono-*N*-substituted thioureas are highly toxic to rats (Dieke, Allen & Richter, 1947), probably due to release of hydrogen sulphide (Smith & Williams, 1961), whereas *NN'*-diarylthioureas are less so. On the basis of toxicity thioamides would be labile, being converted into the corresponding amide with the associated production of hydrogen sulphide.

Preliminary clinical trials of ethionamide indicated a very low urinary recovery of unchanged drug; blood concentrations, although not high, indicated that appreciable absorption of the compound had occurred (Hughes, Smith & Kane, 1962). Since then other workers have demonstrated extensive metabolism of ethionamide in man. Bieder & Mazeau (1962) have detected 2-ethylisonicotinamide and unchanged ethionamide in human urine by chromatography; they have also detected and obtained data on fluorescent metabolites. Kane (1962) demonstrated convincingly that a product found by polarography in the serum of patients receiving ethionamide was ethionamide sulphoxide; ethionamide was also detected. Bieder, Brunel & Mazeau (1963) have further shown the conversion of ethionamide sulphoxide into ethionamide in man. Yamamoto (1962) claimed to have identified a fluorescent product found by chromatography

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as 1-methyl-2-ethyl-4-thiocarbamoyl-6-pyridone, and has recently described the isolation and identification of the amide and acid from the urine of rabbits receiving ethionamide only. This is the first time that the biochemical conversion of $-CSNH_2$ into $-C\overset{\overset{O}{\uparrow}}{S}NH_2$ has been described, but the chemical conversion has been demonstrated (Walter & Curts, 1960).

We have attempted to extend and integrate existing knowledge of the metabolism of ethionamide and its sulphoxide in man and experimental animals.

Experimental

MATERIALS

Ethionamide was used as a finely milled yellow powder m.p. 165°. Ethionamide sulphoxide was supplied by Rhône-Poulenc as a bright yellow finely crystalline solid m.p. 141–142°. The remaining reference compounds were synthesized in the Research Laboratories of May & Baker Ltd. These were 2-ethyl-isonicotinamide–white prisms m.p. 131–132°; 2-ethyl-isonicotinic acid–white solid m.p. 237–239°; 2-ethyl-4-pyridoyl-glycine–white prisms m.p. 186–187°. (Found C, 57·5; H, 5·8; N, 13·7; $C_{10}H_{13}N_2O_3$ requires C, 57·7; H, 5·8; N, 13·5%); 2-ethyl-4-carboxy-6-pyridone–off-white solid m.p. 308° (decomp.); 2-ethyl-4-carboxamide-6-pyridone–white solid m.p. 303–304° (decomp.). (Found, C, 58·1; H, 6·55; N, 16·6; $C_8H_{10}N_2O_2$ requires C, 57·8; H, 6·05; N, 16·85%). The 1-methyl derivatives of these last two pyridones were obtained only partially purified.

DOSAGE AND SAMPLE COLLECTION

Ethionamide and ethionamide sulphoxide were given in 0·1N hydrochloric acid to rats, 150–200 g, and mice, 15–25 g, by stomach tube. Dogs received the compounds as aqueous suspensions. A few experiments using 10 mg/kg (the approximate dose in man) showed identical metabolic patterns to those obtained with 100 mg/kg which was used in most experiments. Blood samples were obtained by cardiac puncture from lightly ether-anaesthetized rats and mice. Dogs were bled by inserting a wide-bore hypodermic needle into the cephalic vein of the foreleg. Serum was obtained by collecting the blood in well-oiled syringes and transferring it to a centrifuge tube where it was allowed to clot. Plasma was obtained by collecting the blood into citrated syringes and subsequently spinning off the cellular fraction. With mice, three animals were required to provide sufficient material for one analysis; for other species one animal was used.

Urine was obtained from rats and mice housed in metabolism cages. The animals were allowed access to food for ½ hr each day; water was given *ad libitum*. Dogs were fed normally. Samples of human urine were also obtained from volunteers who took single doses of 0·5–1 g of ethionamide in the form of uncoated 250 mg tablets of Trescatyl.

CHROMATOGRAPHY

Urine was prepared for descending chromatography on Whatman No. 1 paper by concentration to a small volume under reduced pressure at 45°, extraction into ethyl acetate and concentration of the extract. Unless otherwise indicated, the running time was 16 hr. The Dragendorff spray reagent was prepared according to Vagùjfàlvi (1960).

ESTIMATION METHODS

Plasma and serum were analysed polarographically (Kane, 1959). In addition to the waves due to ethionamide and its sulphoxide (Kane, 1962), a third wave with $E_{\frac{1}{2}} = -1{\cdot}15$ V was recorded.

Urinary inorganic sulphate excretion was determined gravimetrically by precipitation of barium sulphate from urine.

Results

EXAMINATION OF BLOOD

The blood concentrations (Table 1) show that both ethionamide and its sulphoxide occur in the blood of mouse, rat and dog irrespective of which is administered. The concentrations of the two compounds parallel each other, except in the rat where detectable sulphoxide concentrations are transient. The peak ethionamide level occurs earliest in the mouse and latest in the dog. A third compound found in these animals forms the major component detectable in the later blood samples.

EXAMINATION IN URINES

Urines were collected and chromatographed after similar dosage with either compound. The metabolites found are listed in Table 2, which also contains data from man after the administration of ethionamide. Eleven different probable metabolites were found by chromatography, ten of which occur in more than one species. These included six unidentified fluorescent compounds. Three of the remainder (M_3, M_9 and M_{10}) have been further investigated. M_{10} has been identified as ethionamide from its Rf, ultraviolet quenching, colour reaction with Dragendorff reagent and polarographic half-wave potential after elution. The Rf values of M_9, using several solvent systems, showed no significant difference from those of 2-ethylisonicotinamide. Polarography of eluates of this spot confirmed this identification by revealing a wave with the characteristic half-wave potential of the amide. The polarographic records further show a second wave characteristic of ethionamide sulphoxide, demonstrating that the spot is a binary mixture of 2-ethylisonicotinamide and ethionamide sulphoxide. (In all the solvent systems so far tested it has proved impossible to separate the sulphoxide from this amide by chromatography). On the basis of its chromatographic behaviour (Table 3), ultraviolet and polarographic properties and colour reactions M_3 was indistinguishable from 2-ethylisonicotinic acid. A fraction obtained by passing the urine of human volunteers, who had taken ethionamide,

TABLE 1. MEAN SERUM OR PLASMA CONCENTRATIONS (μG/ML) OF ETHIONAMIDE, ETHIONAMIDE SULPHOXIDE AND THE THIRD METABOLITE IN THREE SPECIES FOLLOWING ORAL DOSAGE (100 MG/KG) WITH EITHER ETHIONAMIDE OR ITS SULPHOXIDE

		Mouse†						Rat*						Dog†					
		Ethionamide dosed			Sulphoxide dosed			Ethionamide dosed			Sulphoxide dosed			Ethionamide dosed			Sulphoxide dosed		
Time (hr)		S	E	T	S	E	T	S	E	T	S	E	T	S	E	T	S	E	T
0·25	Mean	19·0	41	15·7	26	23	20	2·4	18·4	5·6	7·5	19·1	16·9	5·8	16	3·7	7·9	6·8	8·1
	s.e.m. ±	2·7	6	2·2	3	5	2	1·3	3·2	0·5	4·0	7·3	3·9	0·3	7	2·5	3·0	4·4	3·5
0·5	Mean	25	34	22	25	31	28	0·0	35·0	7·5	0·0	4·9	6·5	9·7	31·6	4·6	10·5	7·7	13·5
	s.e.m. ±	6	8	2	5	5	5	0·0	5	0·0	0·0	0·7	1·4	0·8	12·2	2·7	0·6	2·7	4·4
1·0	Mean	20	29	24	14	19	43	0·0	20	10·7	0·0	13·3	13·2	13·2	40	12·9	7·5	9·1	13·2
	s.e.m. ±	5	9	3	6	4	14	0·0	1	0·6	0·0	1·5	0·6	—	—	—	—	—	—
2·3–3·0	Mean	6·2	5·5	16	0·0	1·0	4·4	0·0	8·1	11·8	0·0	5·3	8·3	13·0	40	23	3·5	4·9	24
	s.e.m. ±	1·1	0·9	3	0·0	0·8	1·3	0·0	1·2	0·7	—	—	—	0·5	4	3	—	—	—
5·0–6·0	Mean	0·6	1·5	2·4	0·0	0·0	0·0	0·0	3·5	8·7	0·0	0·8	5·6	9·4	19	28	0·0	1·0	32
	s.e.m. ±	0·6	1·5	0·8	0·0	0·0	0·0	0·0	0·5	0·7	0·0	0·8	0·4	3·1	6	10	—	—	—

* Plasma, † Serum; S = ethionamide sulphoxide; E = ethionamide, T = "third metabolite" (see text).
As the third metabolite is unidentified, its concentrations cannot exactly be given. Derivatives of ethionamide, however, show little variation in polarographic wave height with concentration; as a rough guide, concentrations were calculated assuming for historical reasons that the metabolite is 2-ethyl-4-carbamoyl-pyridine.

TABLE 2. COMPARATIVE TABLE OF URINARY "METABOLITE SPOTS"

Metabolites	Rf values				Fluorescence ultraviolet	Colour reaction with Dragendorff reagent
	Mouse	Rat	Dog	Man		
M_1	0·16*	0·16*	0·15*	—	blue	—
M_2	—	0·32*	—	—	yellow	—
M_3	0·35	0·35	0·34	0·32	none	+
M_4	0·38*	0·38	0·38*	—	blue	—
M_5	—	0·49*	0·49*	—	blue	—
M_6	0·60*	—	0·64*	0·58	blue	—
M_7	0·64*	0·65*	—	—	quench	—
M_8	0·80	0·80	—	0·82	blue	—
M_9	0·85	0·85	0·84	0·82	none	+
M_{10}	—	—	0·91*	0·92*	quench	+

* Indicates minor "metabolite" or one intermittent in appearance.
Dosage with either ethionamide or ethionamide sulphoxide. In chromatography the solvent system was n-butanol-0·2N ammonia (1 : 1 by volume); under these circumstances reference compounds have the following Rf values—2-ethylisonicotinic acid, 0·35; ethionamide sulphoxide, 0·82; 2-ethylisonicotinamide, 0·84; ethionamide, 0·89.

TABLE 3. CHROMATOGRAPHIC BEHAVIOUR OF 2-ETHYLISONICOTINIC ACID AND M_3

Solvent systems	Rf values	
	2-ethylisonicotinic acid	M_3
Butanol-ammonia (0·2N) (1 : 1)	0·32	0·34
Butanol-pyridine-water (140 : 30 : 30)	0·39	0·42
Benzene-acetic acid-water* (125 : 72 : 3)	0·85	0·85
Methyl ethyl ketone-acetic acid-water* (50 : 1 : 49)	0·64	0·64

* Running time only 4 hr.
M_3 is a urinary metabolite detected after the administration of ethionamide or ethionamide sulphoxide (see Table 2).

through an anion exchange column (Amberlite IRA-410, 40–60 mesh, OH form, 2·5 × 15 cm) and eluting with N acetic acid had the same chromatographic properties as M_3. The nature of this isolated fraction of M_3 was established from its infrared spectrum as being 2-ethylisonicotinic acid.

Rats were dosed orally (100 mg/kg) with either 2-ethylisonicotinamide or the acid, and their urine was collected and chromatographed. The amide yielded both amide and acid from the urine, whilst only the acid was detected after its administration. Since nicotinic and isonicotinic acids give rise *in vivo* to glycine conjugates (Cuthbertson, Ireland & Wolff, 1953; Komori & Sendju, 1926), a search for similar conjugates was made in the urine of all the species. In no case was evidence of a foreign glycine conjugate found using Altman's reagent (Gaffney, Schreier, DiFerrante & Altman, 1954) on chromatograms. The chance of this being a false negative result was reduced by finding that chromatograms of the synthetic glycine conjugate of 2-ethylisonicotinic acid gave a strong positive reaction with this reagent.

Though none of the fluorescent metabolites was identified, one, M_8, was isolated from human urine by chromatography and partially purified by ion-exchange methods. It formed pale yellow crystals (m.p. 132–142°). In saturated aqueous solutions of borax it had a polarographic half-wave potential of −1·25 V. An aqueous solution had fluorescence excitation and emission maxima of 355 and 410 mμ. The ultraviolet absorption

spectra varied with pH in a manner characteristic of a 6-pyridone. Four possible structures for this compound were eliminated on chromatographic grounds (Table 4).

TABLE 4. CHROMATOGRAPHIC BEHAVIOUR OF SYNTHETIC PYRIDONES AND OF M_3

Compounds	Rf
2-Ethyl-4-carboxy-6-pyridone	0·08
2-Ethyl-4-carboxamide-6-pyridone	0·55
1-Methyl-2-ethyl-4-carboxy-6-pyridone	0·05
1-Methyl-2-ethyl-4-carboxamide-6-pyridone ..	0·36
M_8	0·82

Solvent system was butanol-ammonia 0·2N (1 : 1 by volume).
Detection was by fluorescence under 365 mμ light.
M_8 is a urinary metabolite detected after the administration of ethionamide or ethionamide sulphoxide (see Table 2).

Attempts to repeat the synthesis of 1-methyl-2-ethyl-4-thiocarbamoyl-6-pyridone by the method of Yamamoto (1961) have consistently failed. In every instance, the thiocarbamoyl group was also oxidized and the resulting product was 1-methyl-2-ethyl-4-carbamoyl-6-pyridone. This compound showed blue fluorescence, but chromatography demonstrated that it was not one of the urinary metabolites.

Excretion of sulphur. The metabolic conversion of ethionamide and ethionamide sulphoxide to the corresponding amide would involve loss of the sulphur atom. On this reasoning the excretion of inorganic sulphate by rats before and after receiving either of the two antituberculosis drugs (100 mg/kg) was determined. An increase occurred during the first day after administration, equivalent to 80% of the dose. The increase in excretion was statistically indistinguishable whichever of the two drugs was given. There was no indication of increased sulphate excretion extending into the second day after administration.

Discussion

The interconversion between ethionamide and its sulphoxide found in the rat, mouse and dog is similar to that found in man (Bieder & Mazeau, 1962). These seem to be the only instances of such an interconversion between two therapeutically active foreign substances. Other sulphur-containing compounds, chlorpromazine for example, are known to be metabolized to sulphoxides *in vivo*, but this reaction is thought to be irreversible. These other sulphoxides are of the type $\mathrm{C{-}\overset{\overset{O}{\uparrow}}{S}{-}C}$ whereas thioamide sulphoxides have the structure $\mathrm{C}(\!=\!\mathrm{S}\!\rightarrow\!\mathrm{O})(\!-\!\mathrm{N}\!<\!)$. The latter compounds have only recently been discovered (Walter & Curts, 1960).

An analysis of the blood-level figures made at each time on each species shows there is no statistical difference between the two treatments ($P > 0{\cdot}05$). In view of this, it is not surprising that the pattern of

urinary metabolites is independent of the antituberculosis compound given.

The blood levels also indicate interesting species differences in the metabolism of ethionamide and its sulphoxide, both in rate of metabolism and in the balance between ethionamide and ethionamide sulphoxide. For example, after 5 to 6 hr there are only traces of ethionamide derivatives remaining in the blood stream of the mouse but much greater amounts in both the rat and the dog; the rat demonstrates only transitory amounts of ethionamide sulphoxide, which appears only in the ¼ hr sample, whereas the ratio between the blood concentrations of the two drugs is nearer unity in the other two species.

Further examination of the figures in Table 1 shows that the polarographic wave due to the third component tends to increase to a maximum, the time of peak concentration occurring after the ethionamide peak. Although no identification of this wave has been made, it appears to be a metabolite which is formed at a later stage than the interconversion and the parent compounds. Its polarographic half-wave potential coincides with that of 2-ethylisonicotinamide, and this metabolite has been found by us in animal urine; its presence in human urine is reported by Bieder & Mazeau (1962) as well as in our results. However, chromatographic examination of ultrafiltrates of plasma in this laboratory indicates that the third wave is attributable to metabolites other than the simple amide (Law, 1963, unpublished).

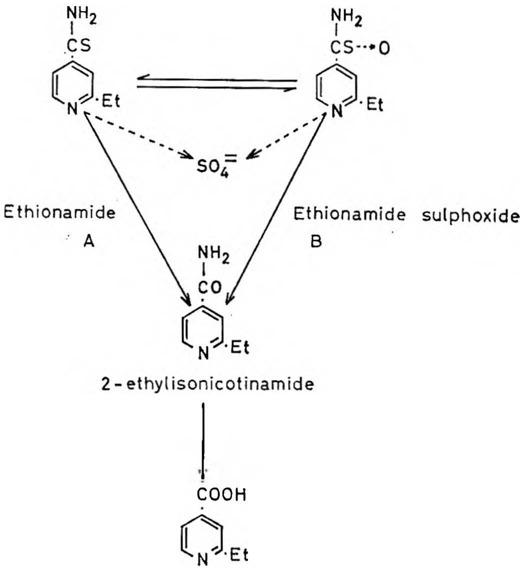
Urinary metabolites detected in the three animal species and man fall into two classes, fluorescent and non-fluorescent. Of the latter, five have been identified. Two of these are the two drugs and the others are 2-ethylisonicotinamide, 2-ethylisonicotinic acid and inorganic sulphate. There was no evidence of the corresponding glycine conjugate of the acid, an unusual contrast with nicotinic and isonicotinic acids (Cuthbertson & others, 1953; Komori & Sendju, 1926). The ready conversion of the amide into the acid, demonstrated by the feeding of 2-ethylisonicotinamide to rats, is however similar to known *in vivo* reactions of other pyridine acids and amides.*

The increased excretion of inorganic sulphate in the urine of dosed rats, provides supporting evidence for the formation of these sulphur-free metabolites.

Although none of the fluorescent metabolites has been identified, there is some evidence that these are pyridones. There is a precedent for this in that one of the metabolites of nicotinamide, 1-methyl-3-carbamoyl-6-pyridone, is fluorescent (Chang & Johnson, 1959). The pyridone claimed to have been found by Yamamoto, 1-methyl-2-ethyl-4-thiocarbamoyl-6-pyridone, could be the metabolite designated M_8 in our nomenclature on the basis of Rf values, but unfortunately we failed to confirm his synthetic route. Bieder & Mazeau describe similar fluorescent metabolites, without further identification other than fluorescence data.

* Since the completion of this work the results of a study of the metabolism of ethionamide in man have been published by Bieder, A. & Mazeau, L. (1964). *Therapie*, **19**, 897–907.

The five metabolites that we have identified are consistent with the following metabolic scheme:



There is nothing to indicate whether route A, route B or both routes are operative *in vivo*. The placing of the amide higher in the metabolic pathway than the acid is justified by the results from the experiment where both compounds were fed to rats. The acid was detected in urine in both instances, but amide was only found after dosing with amide. It is not clear at which point *N*-methylation may occur or the suggested pyridones be formed; by analogy with nicotinamide this would be at the amide stage. The reactions may, however, take place earlier or later in the metabolic pathway.

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Ionization constants and water solubilities of some aminoalkylphenothiazine tranquillizers and related compounds

A. L. GREEN

A simple graphical method is described for deriving the ionization constants of poorly soluble organic bases from the pH dependence of the water solubility. Ionization constants and water solubilities of 13 aminoalkylphenothiazines and related drugs have been determined using this procedure. Potent tranquillizing activity in this group of compounds is shown to be associated with a low water solubility at blood pH.

THE choice of a suitable method for determining the ionization constants of aminoalkylphenothiazine tranquillizers and related drugs is severely restricted by the very poor water solubility of the free bases. This low solubility prohibits the use of conductivity measurements, or of what is normally the most satisfactory method, namely potentiometric titration in aqueous solution. Furthermore, the ultraviolet spectra of the base and cation are too alike for the ionization constant to be calculated from the extinction in buffers of different pH. However, for bases which are much less soluble in water than their corresponding salts, it is possible to derive the ionization constants from the pH dependence of the water solubility. This method has been used here.

Experimental

MATERIALS

The drugs used were kindly supplied by various pharmaceutical companies as follows: amitriptyline hydrochloride (Merck, Sharp & Dohme), desipramine hydrochloride and imipramine hydrochloride (Geigy), chlorpromazine hydrochloride, pecazine hydrochloride, prochlorperazine ethanedisulphonate and trifluoperazine dihydrochloride (Smith Kline & French), fluopromazine hydrochloride (Squibb), perphenazine (Allen & Hanbury), promazine hydrochloride (Rhône-Poulenc), promethazine hydrochloride (May & Baker), thiopropazate dihydrochloride (Searle) and thioridazine hydrochloride (Sandoz).

Buffers were prepared as described by Gomori (1955) from sodium phosphate, tris, 2-amino-2-methylpropane-1,3-diol and glycine.

METHODS

Two methods were used to obtain the solubilities. In the first and simpler method, solid amine salt or free base was shaken with 10 ml of 0·01N sodium hydroxide or 0·01M buffer at room temperature (24 ± 1°). Sufficient compound was added to ensure that some undissolved material was always present. After 3 hr, this undissolved material was removed

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by centrifuging. The pH of the solution and its extinction at the wavelength of maximum absorption, generally close to 255 or 305 mμ (Warren, Eisdorfer, Thompson & Zarembo, 1966), were then measured. The extinction was converted into concentration of dissolved amine with the aid of the extinction coefficient calculated from a solution of the amine salt at known concentration in 0·01N sodium hydroxide (the compounds obeyed Beer's Law).

Prolonged centrifuging at high speed was generally needed to clarify the solutions adequately; even then the method occasionally failed to give satisfactory results. Consequently, the solubilities were also determined by the following, rather lengthier, procedure.

A series of dilutions of an amine salt in water (4·5 ml) were shaken for 45 min at room temperature (24 $\pm$ 1°) with 0·5 ml of 0·1N sodium hydroxide or 0·1M buffer. The extinction of each solution was then measured at 450 mμ. The compounds studied do not absorb light significantly at this wavelength and the observed extinction is due to the scattering of light by the turbid suspension which is formed if the concentration of the free base at the pH of the solution exceeds its solubility. A plot of extinction against concentration of amine salt generally gives two straight lines (one on the horizontal axis) intersecting at the solubility of the base. Typical plots are illustrated in Fig. 1. Occasionally, the turbidity did not

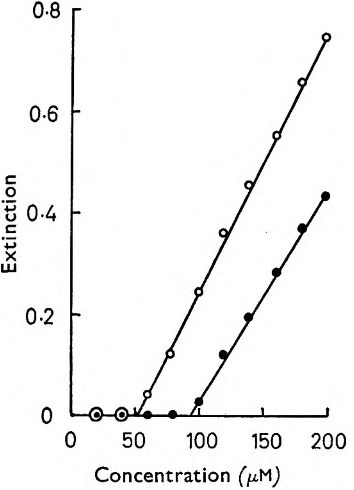

FIG. 1. Plot of extinction against concentration of amitriptyline hydrochloride at pH 9·78 (open circles) and pH 9·20 (filled circles).

vary linearly with concentration; but even so, the point of intersection could usually be determined with fair precision.

The ionization constants were derived from the pH dependence of the solubilities as follows. At any pH, the total concentration of the base plus salt ([B] + [HB$^+$]) will equal [B] (1 + [H$^+$]/K) where K is the ionization constant (K = [H$^+$] [B]/[HB$^+$]). If the free base is much less soluble

than the salt, the observed total solubility at a particular pH, S, will be related to the hydrogen ion concentration and the solubility of the free base, S_0, by the equation,

$$S = S_0(1 + [H^+]/K)$$

(when $[H^+]$ is small $S = S_0$). This can be rearranged to give

$$[H^+] = (K/S_0)S - K$$

Thus a plot of $[H^+]$ against S should give a straight line with intercepts S_0 and $-K$ on the S and $[H^+]$ axes respectively. Two typical plots are shown in Fig. 2. The solubilities in these plots were obtained by the turbidity method, but precisely similar plots were obtained when the more direct method was used.

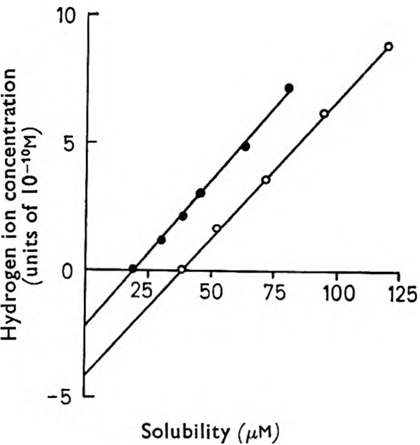

FIG. 2. Relationship between hydrogen ion concentration and solubility of pecazine (filled circles) and amitriptyline (open circles).

Results and discussion

The turbidity method for determining the solubilities gave satisfactory linear plots of hydrogen ion concentration against solubility for all the compounds studied. The more direct method gave good results with most compounds, but failed for pecazine and thiopropazate, for which only very scattered plots could be obtained. Where both methods were used, the pK_a values always agreed within $\pm 0{\cdot}2$ units, and generally within $\pm 0{\cdot}1$ units. Duplicate determinations by either method also gave values agreeing within $\pm 0{\cdot}1$ units. Mean pK_a values for ten aminoalkylphenothiazines and three related antidepressants are given in Table 1. The solubilities of the free bases listed in Table 1 are also the means of those obtained by the two methods. However, the solubilities obtained by the direct method were always lower than those found by the turbidity method. The difference tended to be proportionately greater the lower the solubility, although even with the two least soluble compounds, thioridazine and fluopromazine, the results by the two methods were

TABLE 1. WATER SOLUBILITIES AND pK_a VALUES OF AMINOALKYLPHENOTHIAZINES AND RELATED COMPOUNDS

Structure	Trivial or approved name	pKa Solubility method	pKa Chatten & Harris (1962)	Solubility (μM)	Calculated relative solubility at pH 7·4
R = H; R′ = $CH_2 \cdot CH(Me) \cdot NMe_2$	Promethazine	9·1	9·1	55	4·5
R = H; R′ = $[CH_2]_3 \cdot NMe_2$	Promazine	9·4	—	50	8·0
R = Cl; R′ = " "	Chlorpromazine	9·3	9·2	8	1·0
R = CF_3; R′ = " "	Fluopromazine	9·2	9·4	5	0·4
R = H; R′ = CH_2–(N-methylpiperidyl)	Pecazine	9·7	—	18	5·0
R = SMe; R′ = $[CH_2]_2$–(N-methylpiperidyl)	Thioridazine	9·5	9·2	1·5	0·3
R = Cl; R′ = Me	Prochlorperazine	8·1	7·5	40	0·4
R = CF_3; R′ = Me	Trifluoperazine	8·1	8·4	30	0·3
R = Cl; R′ = CH_2CH_2OH	Perphenazine	7·8	—	70	0·35
R = Cl; R′ = CH_2CH_2OCOMe	Thiopropazate	7·3	7·2	20	0·06
X = N; R = $[CH_2]_3 \cdot NHMe$	Desipramine	10·2	—	220	250
X = N; R = $[CH_2]_3 \cdot NMe_2$	Imipramine	9·5	—	65	15
X = C; R = : $CH[CH_2]_2 \cdot NMe_2$	Amitriptyline	9·4	—	35	6·0

within a factor of 1·7. Aminoalkylphenothiazines have a high surface activity and tendency to form micelles (Scholtan, 1955; Seeman & Bialy, 1963), hence some supersaturation may occur when the free base is precipitated from solution. It is consequently possible that the true solubilities may be slightly lower than those in the Table. There was one curious anomaly which could be accounted for by a supersaturation effect of this kind. Perphenazine was available both as dihydrochloride and as free base. When the dihydrochloride was used, both the direct and the turbidity methods gave the same solubility, 70 μM, but when the direct method was applied to the free base, a solubility of only 26 μM was obtained. The same pK_a value was found irrespective of whether the base or salt was used.

Schill (1965) has also obtained water solubilities and pK_a values at 20° for promazine and chlorpromazine by a procedure similar to my direct method, but by use of a non-linear instead of a linear plot. His pK_a values, 9·4 and 9·3 respectively, and his solubility for promazine (50 μM) are in agreement with my values, but not so his solubility for chlorpromazine (3·6 μM) which is considerably lower.

A common expedient for obtaining ionization constants of bases which are poorly soluble in water, but freely soluble in organic solvents, is to titrate them potentiometrically in mixtures of organic solvent and water, and then to extrapolate the pK_a values so obtained to zero organic solvent content. This procedure is open to serious errors (Albert & Serjeant, 1962) but pK_a values for some aminoalkylphenothiazines in water have been determined by extrapolation from values obtained by potentiometric titration in aqueous methanol (Marshall, 1955; Chatten & Harris, 1962). Marshall obtained values of 9·5, 9·3 and 9·0 for promazine, chlorpromazine and promethazine respectively; the values obtained by Chatten & Harris are included in Table 1. The agreement between the titration and solubility methods is generally good. The only serious discrepancy is found with prochlorperazine and trifluoperazine, which have the same pK_a values when these are determined by the solubility method, but have markedly different values when measured by titration. Since chlorpromazine has about the same pK_a as fluopromazine when measured by either method, it seems improbable that there would be any large difference between the pK_a values of the corresponding *N*-methylpiperazine analogues.

The major factor controlling the ionization constant of these compounds is the nature of the aminoalkyl side-chain. Substitution in the phenothiazine nucleus has only a minor effect on the pK_a although it causes a sharp drop in solubility. Replacement of the phenothiazine group of promazine by the dibenzazepine system in imipramine also results in only small increases in pK_a and solubility. Even replacement of the $>N—CH_2—$ group of imipramine by $>C=CH—$ in amitriptyline causes no major change in either the pK_a or the solubility. The effect of variation in the structure of the amino-group is much the same as found for other substituted aliphatic amines. Demethylation of imipramine to give desipramine increases the pK_a by 0·7 units, and replacement of the

dimethylamino-group by *N*-methylpiperidine, in pecazine and thioridazine, raises the pK_a by about 0·3 units. These differences are typical of those normally found between monomethylamino- and dimethylamino-compounds, and between dimethylamino- and *N*-methylpiperidino-compounds (Clark & Perrin, 1964; Perrin, 1965). Insufficient pK_a values have been published for piperazine derivatives to enable any quantitative correlation with the present series, but the values obtained for prochlorperazine, perphenazine and thiopropazate are consistent with the increasing electron-withdrawing properties of the substituent on the terminal nitrogen atom. Piperazine derivatives are dibasic, but as the second pK_a value is much lower than the first (Chatten & Harris, 1962), the equations on pages 11–12 need no modification for solubilities determined at pH 7 or above.

No very extensive correlation is discernable between ionization constant, water solubility and tranquillizing activity, but there is a trend for depressant activity to be associated with a low pK_a or low water solubility. A rough guide to the likely joint influence of these two factors is the water solubility at blood pH (7·4). The calculated solubilities at pH 7·4 relative to that of chlorpromazine are given in the final column of Table 1. The potent tranquillizing drugs all have relative solubilities of 1 or less, whereas the antidepressant drugs imipramine and desipramine have relative solubilities greater than 10. The antidepressant drug amitriptyline, with a relative solubility of 6, also produces sedation and can be used as a combined antidepressant-tranquillizer (Freed, 1960). Promazine and pecazine, with relative solubilities of 8 and 5, have weak tranquillizing activity, but are used far less for this purpose than the more active, ring-substituted compounds. Promethazine, which has a relative solubility of 4·5, causes some sedation, but it is not a tranquillizer. However, this drug has only 2 carbon atoms separating the phenothiazine ring from the terminal amino-group, and consequently does not conform to one of the generally accepted structural requirements for tranquillizing activity, namely a trimethylene chain between the ring system and the amino-group (Gordon, Craig & Zirkle, 1964). The relative solubility at pH 7·4 may thus provide a useful indication of whether tranquillizing or antidepressant activity will predominate in any series of compounds having appropriate structures for these types of activity; but, the converse, that bases with a low solubility at pH 7·4 will have tranquillizing properties, will generally not be true.

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Analgesically active basic anilides: stereospecificity and structure of the basic group

A. F. CASY* AND M. M. A. HASSAN

The synthesis of (*RS*)-, (*R*)- and (*S*)-*N*-(2-dimethylaminopropyl)propionanilide and the benzylmethylamino- analogues of methadone and isomethadone is described. The hot-plate activities in mice of the (*S*)- enantiomorphs of both the dimethylamino- and benzylmethylaminopropionanilides are greater than those of the corresponding (*RS*)- and (*R*)- forms, while the methadone and isomethadone analogues are inactive in the same test. These results support the view that 3-amino-1,1-diphenylpropyl- and basic anilide - analgesics differ in their modes of binding to the analgesic receptor site.

IN 1959 some analgesically active basic anilides (I) were reported which were regarded as analogues of methadone because they are formally derived from (II; R = Me) by replacing one phenyl group and its attached quaternary carbon atom with nitrogen (Wright, Brabander & Hardy, 1959). The enantiomorphic forms of diampromid (Ia) (the most active

$$\text{Me(R)N.CH(Me).CH}_2\text{.N(Ph).CO.Et} \quad \text{(I)}$$

R = a)Ph.$[CH_2]_2$ b)Ph.CH_2; c)Me.

$$\text{Me(R)N.CH(Me).CH}_2\text{—C(Ph)(Ph)—CO.Et} \quad \text{(II)}$$

methadone (R = Me)

member of the series) and its *N*-benzyl analogue (Ib) differ in their analgesic activities (Table 1), in common with (+)- and (−)-methadone and related enantiomorphic pairs. The more active enantiomorphs of several analgesics [including (−)-methadone] containing the structural feature >N·CH(Me)·R, are related to *R*-(−)-alanine (Beckett & Casy, 1965 and refs there cited). The more active forms of the anilides (Ia and b), which contain the same type of asymmetric centre as methadone are, however, related to *S*-(+)-alanine (Portoghese & Larson, 1964). This reversal of optical specificity is not without precedent in analgesics related to methadone [ethyl (+)-2,2-diphenyl-4-dimethylaminopentanoate and α-(−)-methadol, both derived from (+)-methadone, are more active in mice than their respective enantiomorphs (Eddy, Halbach & Braenden, 1956)], but in all other classes studied, groups of analgesics with related asymmetric centres have identical configurations (Beckett & Casy, 1965).

In analgesics based on the 4-phenylpiperidine skeleton it is well known that derivatives carrying *N*-2-arylethyl substituents are more active than corresponding *N*-methyl derivatives; *N*-benzyl derivatives, however, have either very low potencies or are inactive (Beckett & Casy, 1965 and refs there cited). In acyclic analgesics, such as methadone, optimum

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activity is obtained with a dimethylamino-group or with a five- or six-membered alicyclic basic group, activity falling when larger basic substituents are employed. Thus the basic functions of the anilides (Ia and b) are of a type unusual to acyclic analgesics, the benzyl group of (Ib) being, in addition, unusual even to analgesics of greater molecular rigidity.

These observations may be related to the fact that the basic anilides (Ia and b) do not conform to the configurational requirements established for methadone and related compounds, and the aim of this work was to obtain data regarding such a correlation. Two approaches were adopted: determination of (i) the stereospecificity of the basic anilide (Ic), containing the "normal" basic dimethylamino-function of acyclic analgesics, and (ii) the effect of replacing the *N*-methyl group in methadone and isomethadone by the *N*-benzyl group, a group, present in the basic anilide (Ib), a compound which has significant analgesic potency.

CHEMISTRY

(±)-*N*-[2-(Benzylmethylamino)propyl]aniline (VI), the key intermediate in this work, was prepared from an α-bromopropionyl halide (III) by a reported method (see III–VI) (Wright, Brabander & Hardy, 1961). The need to use an excess of methylbenzylamine in the conversion of the bromanilide (IV) to the basic anilide (V) was avoided by

Me·CH(Br)·CO·X (X = Br or Cl) (III) —PhNH₂→ Me·CH(Br)·CO·NH·Ph (IV) —NH(Me)·CH₂·Ph + K₂CO₃→

Me·CH(N(Me)·CH₂·Ph)·CO·NH·Ph (V) —LiAlH₄→ Me·CH(N(Me)·CH₂·Ph)·CH₂·NH·Ph (VI)

Me·CH(NH·Ph)·CO·NH·Ph (VII)

including potassium carbonate (as acid adsorbent) in the reaction mixture. Pyridine was a less satisfactory adduct in this respect, in one experiment the α-anilino-anilide (VII) (structure confirmed by nmr spectroscopy) being isolated rather than the desired anilide (V). [Pyridine presumably displaces aniline from the anilide (IV), the latter base then reacting with unchanged substrate to give (VII)]. Catalytic debenzylation of *N*-[2-(benzylmethylamino)propyl]propionanilide (Ib), derived from the diamine (VI), in the presence of formaldehyde, gave the dimethylamino-anilide (Ic): the corresponding (+)- and (−)-enantiomorphs were obtained when the optically active propionanilides (Ib) were used in this reaction. The configurational relationships of enantiomorphic forms of the dimethylamino-, benzylmethylamino- and methylphenethylamino-anilides (I) are shown in Table 1, all (+)-bases [≡(−)-base salts] in this series having identical configurations.

The racemic dimethylamino-anilide (Ic) was also prepared by alkylation of the sodio-derivative of propionanilide with 2-chloro-*NN*-dimethylpropylamine. This reaction involves a rearrangement and probably

TABLE 1. OPTICAL AND ANALGESIC ACTIVITIES OF SOME *N*-(2-AMINOPROPYL)-PROPIONANILIDES

Me(R)N·CH(Me)·CH_2·N(Ph)·CO·Et

	$[\alpha]_D^{22-25°}$ in EtOH			Pharmacological activity (mg/kg)	
R	Base	Salt[1]	Configuration	AD50 (tail flick)[2]	ED50 (hot plate)[3]
Ph·CH_2	Racemic form		—	8	15
	+41·9	−23·5	*S*	4·3	12
	−41·9	+23·0	*R*	inactive (50)	⩾ 40
Ph$[CH_2]_2$	Racemic form		—	3·7	—
	+25·2[4]	—	*S*	3·6	—
	−25·9	—	*R*	11·7	—
Me	Racemic form		—	—	50
	+17·0	−40·9	*S*	—	35
	−17·7	+41·5	R	—	⩾ 40
Pethidine				11	23

[1] Hydrochloride (R = Ph·CH_2), nitrate (R = Me).
[2] in rats by subcutaneous injection (Wright & Hardy, 1963).
[3] in mice by subcutaneous injection (Janssen & Jageneau, 1957).
[4] Wright & Hardy (1963).

proceeds via the ethyleneimonium ion (VIII) (Schultz & Sprague, 1948). It appears that steric factors govern the direction of nucleophilic attack

Me_2N^+ (ring: $\overset{2}{MeCH}$—$\overset{1}{CH_2}$) Cl^- (VIII) Me_2N·CH_2·CH(Me)·N(CO·Et)·Ph (IX)

upon this intermediate since the propionanilide (Ic) [which must result from attack at the less hindered C-1 atom of the ion (VIII)] was isolated in high yield, while the close similarity of the nmr spectra of the total alkylation product and the pure anilide (Ic) showed that little of the isomeric anilide (IX) (characterized by a *N*-methyl singlet at 132 c/sec and a methine multiplet centred at 306 c/sec) is formed in this reaction.

In contrast, alkylation of diphenylacetonitrile with *N*-(2-chloropropyl)-*N*-methylbenzylamine resulted in a 1 : 1·5 mixture of the isomeric cyanides (X and XI; R = CN) as was clearly apparent from the nmr spectrum of the total alkylation product. These cyanides, with ethyl magnesium

(Ph·CH_2)N(Me)·CH(Me)·CH_2·$C(Ph)_2$·R (X)   (Ph·CH_2)N(Me)·CH_2·CH(Me)·$C(Ph)_2$·R (XI)

bromide, gave the ketones (X and XI; R = CO.Et), the benzylmethyl-amino-analogues of methadone and isomethadone respectively; in the case of the cyanide (XI; R = CN), the hydrocarbon (XI; R = H) was isolated as a by-product of this reaction.

PHARMACOLOGY AND DISCUSSION

The analgesic activities of the racemic (*RS*)-, (*R*)-, and (*S*)-forms of the basic anilides (Ib and c) (Table 1), and of the benzylmethylamino-ketones (X and XI; R = CO.Et) were determined in mice after subcutaneous injection, using the hot-plate method (Janssen & Jageneau, 1957). The (*S*)-benzylmethylamino-anilide produced morphine-like excitation in mice and had twice the hot-plate activity of pethidine; the (*R*)-enantiomorph was much less active in this test and caused no Straub tail response. These results parallel the activities of the same isomers in rats by a tail-pressure method (Wright & Hardy, 1963).* The (*RS*)-dimethylamino-anilide was about half as active as pethidine and had morphine-like effects. The corresponding (*S*)-isomer was more potent but produced no Straub reaction, while the (*R*)-isomer was inactive; neither isomer gave behavioural effects.

Because of the low order of potency of the (*RS*)- and (*S*)- forms of the dimethylamino-anilide, and the doubts about their morphine-like action, a clear decision on the influence of basic group structure upon stereospecificity is not possible. However, accepting these limitations, it does appear that stereospecificity in the dimethylamino-anilide is the same as that found in the anilides (Ia and b), the (*S*)-isomer being the more active form in all instances. Thus, stereospecificity in diampromid and its *N*-benzyl analogue does not appear to be directly linked with the presence of an arylalkylamino-substituent in the molecule.

Pharmacological results with the benzylmethylamino-analogues of methadone and isomethadone emphasize the unusual structural requirements for activity, relating to the basic group, in the amino-anilides (I), the *N*-benzylmethadone (X; R = CO·Et) and isomethadone (XI; R = CO·Et) analogues being virtually devoid of activity in the hot-plate test.

It appears likely, therefore, that 3-amino-1,1-diphenylpropyl and basic anilide analgesics differ in their modes of binding to the analgesic receptor site, as recently proposed by Portoghese (1965). Possible reasons for binding differences will be discussed elsewhere in terms of probable conformations of the analgesic molecules.

Experimental

*α-Benzylmethylamino-*N*-phenyl-propionamide* (V). A mixture of α-bromo-*N*-phenyl-propionamide (22·8 g, 0·1 mole), methylbenzylamine (12·1 g, 0·1 mole), anhydrous potassium carbonate (41·4 g, 3 mole) and acetone (200 ml) was heated under reflux for 12 hr. The reaction mixture was filtered, the filtrate evaporated, and the solid residue crystallized from ethanol to give the basic anilide (V) (24·6 g, 92% yield), m.p. 72–73° (Wright & others, 1961, give for this a m.p. 72–74°). When potassium carbonate was replaced by pyridine (15 ml) in the above procedure,

* In rats the (*S*)/(*RS*) potency ratio was almost 2 [a value indicating the (*R*)-isomer was inactive and without influence upon the (*S*)-isomer], whereas in mice the ratio was 1·25 [indicating the (*R*)-isomer to have some activity and/or to potentiate the action of its enantiomorph]. (*S*)/(*RS*) potency ratios of just above unity have also been reported for related anilides in rats (Portoghese & Riley, 1965).

α-anilinopropionanilide (VII) (12 g), m.p. 126–128° from ethanol, was isolated (Found: C, 74·75; H, 6·65; N, 11·7; equiv. wt 246. $C_{15}H_{16}N_2O$ requires: C, 75·0; H, 6·7; N, 11·7%; equiv. wt 240). It gave a *hydrochloride*, m.p. 201–203° from ethanol-ether (Found: C, 65·0, H, 6·0; N, 10·0; equiv. wt 275. $C_{15}H_{17}ClN_2O$ requires: C, 65·1; H, 6·2; N, 10·1%; equiv. wt 277). The α-anilino-anilide (VII) had the following nmr characteristics (c/sec from TMS in $CDCl_3$): 448, 434, 404, main peaks of multiplet (10 aryl protons); 243, broad singlet (N–H); 234, centre of multiplet (C–H); 93, doublet J7 (sec-Me). The N–H signal was absent and the C–H multiplet was a 1:3:3:1 quartet (233, J7) in $CDCl_3$–D_2O.

(±)-N-[2-(*Benzylmethylamino*)*propyl*]*aniline* (*VI*) *and its resolution.* α-Benzylmethylamino-*N*-phenyl-propionamide (53·6 g) was reduced with lithium aluminium hydride (15·2 g) by the procedure of Wright & others (1961) to give the diamine (VI) (46 g), m.p. 43–44° from aqueous ethanol (Portoghese & Larson, 1962, report m.p. 42–44°). The (±)-diamine (VI) (52 g) and (+)-tartaric acid (30·8 g) in 95% ethanol (525 ml) deposited a salt (on storage at 25°) which was recrystallized four times from the same solvent to give the (+)-diamine (+)-tartrate (26·5 g), m.p. 102–103°, $[\alpha]_D^{22}$ −11·5 (*c*, 2% in H_2O) [Portoghese & Larson, 1964, report m.p. 101–103°, $[\alpha]_D^{27}$ −16·0° (*c*, 5% in H_2O)]. This salt (26 g) gave the free (+)-*diamine* (VI) (15·5 g), m.p. 60–61° from ethanol, $[\alpha]_D^{22}$ + 34·9° (*c*, 0·75% in ethanol) [Portoghese & Larson, 1964, give m.p. 59–61°, $[\alpha]_D^{27}$ + 31·2 (*c*, 5% in ethanol)]. The salt which deposited from the combined mother liquors of the above resolution (after concentration and storage at 25° for 12 hr) was recrystallized four times from water to give the (−)-diamine (+)-tartrate (12·3 g), m.p. 82–84°, $[\alpha]_D^{22}$ + 30° (*c*, 2% in H_2O) [Portoghese & Larson, 1964, give m.p. 82–84°, $[\alpha]_D^{25}$ + 30° (*c*, 2% in water)]. This salt (12 g) gave the (−)-diamine (VI) (6·8 g), m.p. 59–61°, $[\alpha]_D^{22}$ − 34° (*c*, 0·75% in ethanol) [Portoghese & Larson, 1964, give m.p. 59–60°, $[\alpha]_D^{30}$ − 30·8 (*c*, 5% in ethanol)].

(±)-, (+)- *and* (−)-N-[2-(*Benzylmethylamino*)*propyl*]*propionanilide* (*Ib*). The (±)-diamine (VI), with propionic anhydride, gave the (±)-propionanilide (Ib), b.p. 166–170°/0·4 mm, hydrochloride, m.p. 152–153° from acetone (Wright & others, 1961, give b.p. 166–170°/0·4 mm, hydrochloride, m.p. 150–151°). It formed a *methiodide*, m.p. 176·5–178·5° from ethanol-acetone (Found: C, 55·8; H, 6·85; N, 6·3; equiv. wt 450. $C_{21}H_{29}IN_2O$ requires: C, 55·75; H, 6·5; N, 6·2%; equiv. wt 452). The (+)-diamine gave the (−)-propionanilide, b.p. 178°/0·6 mm, $[\alpha]_D^{22}$ −41·9° (*c*, 5% in ethanol) [Portoghese & Larson, 1964, give b.p. 152–157°/0·3 mm, $[\alpha]_D^{32}$ −45·7 (*c*, 5% in ethanol); Wright & Hardy, 1963, give $[\alpha]_D^{25}$ −37·6° (*c*, 3—4% in ethanol)]. It formed a hydrochloride, m.p. 141–142° from acetone-ether, $[\alpha]_D^{22}$ + 23° (*c*, 1% in ethanol) [Wright & Hardy, 1963, give m.p. 141–142°, $[\alpha]_D^{25}$ + 13·8 (*c*, 3–4% in ethanol)]. The (−)-diamine gave the (+)-propionanilide, b.p. 186–188°/1 mm, $[\alpha]_D^{22}$ + 41·9° (*c*, 5% in ethanol), hydrochloride, m.p. 141–142°, $[\alpha]_D^{22}$ −23°5° (*c*, 1% in ethanol) [Wright & Hardy, 1963, give m.p. 141–142°, $[\alpha]_D^{25}$ −14·5 (*c*, 3–4% in ethanol)].

(±)-, (+)- *and* (−)-N-(2-*Dimethylaminopropyl*)*propionanilide* (*Ic*). A mixture of the (±)-benzylmethylamino-anilide (Ib) (3·1 g), formaldehyde (3·5 ml, 40% solution in water), palladized charcoal (1 g, 10%) and 90% ethanol (100 ml) was shaken with hydrogen at 60° until the theoretical volume of gas had been absorbed. The mixture was filtered, the filtrate evaporated and the residue dissolved in N HCl (30 ml) and extracted with ether. The aqueous phase was made alkaline with aqueous sodium hydroxide solution, extracted with ether and the dried (Na_2SO_4) extract evaporated to yield the (±)-dimethylamino-anilide (Ic) (2 g). A mixture of this base (2 g) in ethanol and nitric acid (4·5 ml, 10%) was evaporated under reduced pressure and the residue dried by azeotropic distillation with ethanol-benzene. The oily product solidified when triturated with dry ether and was crystallized from ethanol-ether to give a nitrate, m.p. 134–136° (Wright & others, 1961, by a different route, give m.p. 134–136°). The (−)-benzylmethylamino-anilide (6·2 g), treated as above, gave the (−)-dimethylamino-anilide (4 g), $[\alpha]_D^{22}$ −17·75° (*c*, 2% in ethanol). It formed a (+)-*nitrate*, m.p. 120–121° from ethanol-ether, $[\alpha]_D^{23}$ + 41·5 (*c*, 1% in H_2O) (Found: C, 56·1; H, 7·8; N, 14·3; equiv. wt 295. $C_{14}H_{23}N_3O_4$ requires: C, 56·5; H, 7·8; N, 14·1%; equiv. wt 297). The (−)-anilide (Ic) with hydrogen bromide, gave N-(2-*dimethylaminopropyl*)*aniline dihydrobromide*, m.p. 203–206° (Found: C, 39·6; H, 6·05; N, 8·6. $C_{11}H_{20}Br_2N_2$ requires: C, 38·85; H, 5·9; N, 8·2%). The (+)-benzylmethylamino-anilide gave the (+)-dimethylamino-anilide, $[\alpha]_D^{23}$ + 17° (*c*, 2% in ethanol). It formed a (−)-*nitrate monohydrate*, m.p. 120–121° from ethanol-ether, $[\alpha]_D^{23}$ −40·9 (*c*, 1% in H_2O) (Found: C, 53·3; H, 8·0; equiv. wt 315. $C_{14}H_{23}O_4.H_2O$ requires: C, 54·0; H, 7·9%; equiv. wt 315) ν_{max} 3300 cm^{-1} (H_2O, broad band). The (±)-dimethyl-amino-anilide was also obtained as follows: A stirred mixture of propionanilide (74·5 g), sodium hydride (24 g, 50% in oil) and xylene (one litre) was heated at 110–120° (oil-bath) for 3 hr whereupon the sodium propionanilide separated. 2-Chloro-*NN*-dimethylpropylamine (66·8 g freshly liberated from the hydrochloride salt) in xylene (400 ml) was added and the mixture heated at 120–130° for 10 hr with stirring. The cold reaction mixture was filtered, the residue washed with xylene, and the combined filtrate and washings extracted with 10% hydrochloric acid. The extract was made alkaline with aqueous ammonia and extracted with ether; the organic phase was dried (Na_2SO_4), evaporated and the residue distilled to give base A (82 g), b.p. 120–122°/0·1 mm, which formed the nitrate (Ic) (60·5 g), m.p. and mixed m.p. 135·5–137·5°. The base from this salt had the following nmr characteristics (c/sec from TMS in CCl_4): 439, main peak of multiplet (5 aromatic protons); 232, quartet J gem 14 J vic 8·5, 203, quartet J gem 14 J vic 6 (methylene protons); 128, singlet (NMe_2); 117, quartet J7 (CO.$\underline{CH_2}$.Me); 57·5; triplet J7 (CO·CH_2·$\underline{Me}$); 49, doublet J 6·5 (sec-Me). The nmr spectra of the pure propionanilide (Ic) and the total alkylation product (base A, above) were very similar; the latter displayed a small peak at 132 c/sec (NMe_2) characteristic of the isomeric anilide (IX). The latter, prepared by the method of Wright & others, 1961), was isolated as a *hydrochloride*,

m.p. 169–171° (from ethanol-ether) (Found: C, 58·5; H, 8·7. $C_{14}H_{25}ClN_2O_2$ requires: C, 58·2; H, 8·7%).

Reaction of N-(2-*chloropropyl*)-N-*methylbenzylamine with diphenylacetonitrile.* *N*-(2-Chloropropyl-*N*-methylbenzylamine (61 g) (Wilson, 1952) in benzene (60 ml) was added to a mixture of diphenylacetonitrile (60 g), sodamide (14·6 g) and benzene (200 ml) which had previously been stirred at 30–40° for 1 hr, and the product heated under reflux for 18 hr. The benzene solution was then washed with water and concentrated to give a crude mixture of cyanides (X and XI, R = CN) (103 g), present in the ratio of 1·5 (XI) to 1·0 (X) [from integrals of the two *N*-Me (129 and 124) and sec-Me (70·5 and 60·5 c/sec from TMS in $CDCl_3$) nmr signals]. Light petroleum (b.p. 60–80°) was added to the mixture and the solid which separated (47 g) recrystallized from benzene-light petroleum b.p. 40–60° to give the 2-methylpropyl cyanide (XI) (40 g), m.p. 106·5–107·5° (Wilson, 1952, gives the same m.p.). The mother liquors were concentrated and the residue acidified with methanolic hydrogen chloride, whereupon the butyl cyanide (X) hydrochloride (21 g), m.p. 210·5–212·5° from ethanol-ether, separated (Wilson, 1952, gives m.p. 206)°. The higher field chemical shifts of the *N*-Me and sec-Me groups, given above, correspond with the butyl cyanide (X), and the lower field with the isomer (XI).

6-*Benzylmethylamino*-4,4-*diphenylheptan*-3-*one.* The butyl cyanide (X) (7·1 g) in toluene (50 ml) was added to ethyl magnesium bromide in ether (40 ml) prepared from ethyl bromide (6·5 g) and magnesium (1·4 g). The ether was removed by distillation, the mixture heated under reflux for 5 hr, and then added to ice and concentrated hydrochloric acid (35 ml). The base (6 g), recovered from the aqueous phase, with hydrogen bromide gave the *ketone* (X, R = CO·Et) *hydrobromide*, m.p. 96–98° from ethanol (Found: C, 68·85; H, 7·2; N, 3·2; equiv. wt 462. $C_{27}H_{32}BrNO$ requires: C, 69·3; H, 6·9; N, 3·0%; equiv. wt 466).

6-*Benzylmethylamino*-5-*methyl*-4,4-*diphenylhexan*-3-*one.* The 2-methylpropyl cyanide (XI, R = CN) (7·1 g), treated with ethyl magnesium bromide as described above, gave the amino-ketimine (XI, R = C(NH)Et) (7·2 g), which was heated under reflux with concentrated hydrochloric acid (40 ml) and ethanol (5 ml) for 12 hr to yield the *ketone* (XI, R = CO·Et), isolated as a *hydrobromide*, m.p. 205–206° from ethanol-ether (Found: C, 69·1; H, 7·0; equiv. wt 464).

In one reaction the hydrocarbon (XI, R = H) was isolated from the crude ketimine. It gave a *maleate*, m.p. 174–176° from ethanol-ether (Found: C, 75·8; H, 7·2; N, 3·15. $C_{28}H_{31}NO_4$ requires: C, 75·5; H, 7·0; N, 3·15%). The infrared spectrum of the free base showed no significant absorption in the region 1600–1800 cm^{-1}.

The nmr spectra were recorded on a 60 Mc Varian A–60 instrument in $CDCl_3$ or CCl_4 with TMS as internal standard. We thank Miss J. Lovenack, School of Pharmacy, University of London, for carrying out these measurements, and Dr. P. Janssen for the pharmacological results.

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An *in vitro* model for soluble drug absorption

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A physico-chemical model is proposed to simulate the transfer of a drug through a lipoidal membrane. The drug is transferred from a buffer of a pH found in the gut, through an immiscible organic liquid acting as the membrane, to a buffer of the plasma pH (7·4). The transfer of salicylic acid and amidopyrine obeys the theoretical equations. The apparatus used has the advantage that there is little or no danger of emulsion formation because of the lack of disturbance at the interface and the ease of removing samples for analysis.

ABSORPTION of weakly acidic and weakly basic drugs is usually considered to be by passive diffusion of the unionized molecular species through the lipoidal membrane of the alimentary tract (Brodie, 1964). Correlation of physico-chemical data with *in vivo* absorption data has usually been limited to pK_a determinations, solubility measurements and measurement of distribution coefficients of the drug between an aqueous phase of physiological pH and an immiscible organic solvent. When considering a water-soluble drug it is probable that the rate of partitioning is of more interest than the distribution coefficient for comparison with *in vivo* absorption rates. This paper suggests the use of a partitioned cell based on a cell previously used for transport studies (Schulman & Rosano, 1960) for these kinetic comparisons. In Fig. 1 the drug, in solution at various pH values of the alimentary tract, is placed in compartment A and is transferred through the organic layer (simulating the lipoidal membrane) to a pH 7·4 buffer (blood pH) in compartment C. All phases are stirred to eliminate concentration gradients within the three compartments, leaving the transfer across the two interfaces the rate controlling steps. Considering the transfer to be a first order process and the concentration in the three compartments to be A, B, and C and the corresponding volumes V_A, V_B and V_C

$$A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} B \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C$$

where k_1 and k_2 are the first order rate constants for the forward transfer and k_{-1} and k_{-2} are the corresponding back transfer constants. The transfer rates are given by

$$V_A \frac{dA}{dt} = V_A k_1 A + V_B k_{-1} B$$

$$V_B \frac{dB}{dt} = V_A k_1 A - V_B k_{-1} B - V_B k_2 B + V_C k_{-2} C$$

$$V_C \frac{dC}{dt} = V_B k_2 B - V_C k_{-2} C.$$

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The solution of these differential equations is complex.* The equations are general and all transfers investigated in the cell should obey them. It is not suggested that the transfer constants obtained can be directly compared to absorption rates from animal work, but simple plots of concentration against time of various salts of a drug at different pH values, or various analogues of a drug under varying conditions, may yield data for the correlation of *in vivo* or *in vitro* studies with the gut. The purpose of the present work was to design an apparatus for measuring partitioning rates and to compare the results obtained with theoretical equations.

The rate controlling steps are the transfers across the two interfaces, which depend upon the pK_a of the drug, the interfacial area, the relative volumes of the phases, the pH values of the aqueous phases, the nature of the organic solvent and the distribution coefficient of the drug between water and the organic phase. The distribution coefficient C_o/C_w is only a true constant at a given temperature when considering the same species in both phases (Martin, 1960). Using this situation and considering single unionized drug molecules the ratio of concentrations of the drug in the two aqueous compartments at equilibrium is given by:

$$\frac{A}{C} = \frac{1 + 10^{(pK_a - pH_A)}}{1 + 10^{(pK_a - 7\cdot4)}} \text{ for a basic drug}$$

$$\frac{A}{C} = \frac{1 + 10^{(pH_A - pK_a)}}{1 + 10^{(7\cdot4 - pK_a)}} \text{ for an acidic drug}$$

and so is independent of the organic phase.

Experimental

APPARATUS

The apparatus (Fig. 1) consists of a box made of 6 mm Perspex, of internal dimensions 20 cm by 10 cm by 10 cm. The 8 cm high central partition divides the cell into two compartments of equal volume. The removable box is held rigidly on a three-screw Perspex levelling table by means of two corner brackets. The two aqueous compartments (A and C) are stirred by means of air-driven magnetic stirrers, and the top organic layer by means of a glass stirrer. Butyl stoppers are placed in 13 mm holes drilled in the middle of the end walls (35 mm from the base of the cell).

REAGENTS AND BUFFERS

All buffers were 0·2 molar: pH 2·0 buffer used Analar potassium chloride, pH 3·0 and pH 4·0 used Analar citric acid, and buffers of pH 5·0 to pH 8·0 used Analar monosodium dihydrogen phosphate—the final adjustment to pH being with concentrated hydrochloric acid or a concentrated solution of sodium hydroxide pH measured with a Model 23A pH meter Electronic Instruments Limited. The aminopyrine was B.D.H.

* For solution see: Frost & Pearson (1961), *Kinetics and Mechanism*, 2nd Edn, John Wiley.

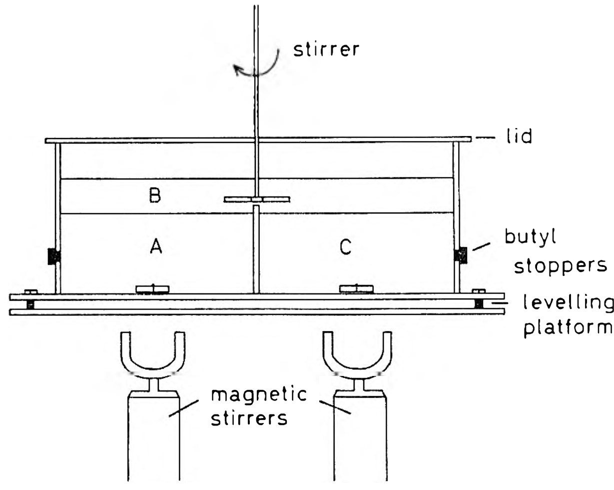

FIG. 1. Diagram of the cell.

laboratory reagent grade, the salicylic acid was Analar grade and propranolol (Inderal; 1-isopropylamino-3-(1-naphthyloxy)-2-propanol hydrochloride) was clinical trial material of I.C.I. compound 45,520. The decanol was B.D.H. laboratory reagent grade, the amyl alcohol was Analar grade and the cyclohexane B.D.H. laboratory reagent, suitable for spectroscopy.

METHOD

The cleaned cell was placed on the levelling table between the brackets and levelled by means of the three screws. Equimolar buffer solutions were prepared to prevent the cell acting as an osmometer, and the buffers were saturated with top phase to minimize transfer by one phase dissolving in the other. Approximately 20 mg of the drug was added to 1 litre of the solution to be placed in compartment A. 550 ml of this solution placed in the compartment took the level of the solution almost to the top of the central partition. A similar volume of pH 7·4 buffer was placed in compartment C. 300 ml of the top organic phase (presaturated with pH 7·4 buffer) was lowered gently onto the aqueous solutions so producing the organic "membrane" connecting the two aqueous phases. The stirring was then commenced at such a rate as to prevent vortices from forming. Samples (1·8 ml) were removed with needles and syringes via the butyl stoppers from the aqueous compartments at various time intervals. At the same time, 2 ml samples were taken from the top. Fresh top solution (2 ml) was added after removal of the top sample to prevent the level of the liquid falling below the paddles of the top stirrer. All samples were assayed by means of their ultraviolet absorption spectra using 1 cm or 4 cm microcells in the Hilger and Watts "Uvispek" spectrophotometer. The experiments were continued until equilibrium was attained or approached.

Results

EFFECT OF TOP PHASE

Fig. 2 shows the effect of the top phase on the transfer rates of propranolol (pK_a = 9·53 in ionic strength of 0·2). The free base is much more soluble in amyl alcohol than cyclohexane and so the transfer rate from the pH 8·0 phase to the organic layer is greater with the higher

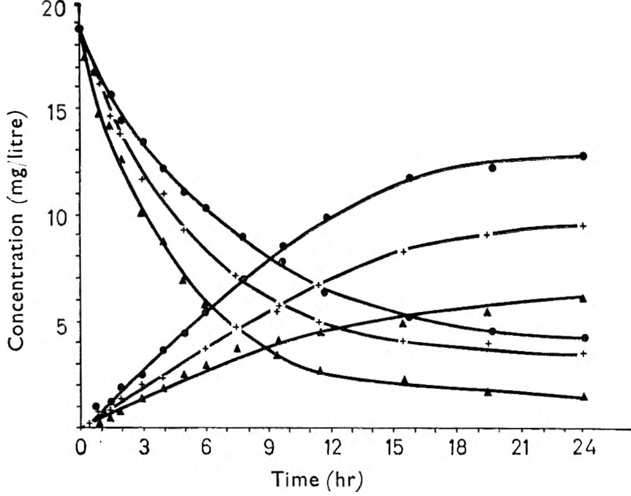

FIG. 2. Effect of the composition of the organic layer on the transfer rate of propranolol from a pH 8·0 aqueous layer to a pH 7·4 layer. ▲, 10% amyl alcohol in cyclohexane. +, 5% amyl alcohol in cyclohexane. ●, cyclohexane.

alcohol content, but the retention by this top phase is also greater and so the appearance in compartment C is slower as shown in the graph. In all instances the equilibrium ratio (3·9) of concentration in compartment C to that in compartment A was approached.

ACIDIC DRUG

Salicylic acid (pK_a 2·96 at an ionic strength of 0·21) was used and preliminary experiments suggested that 20% decanol in cyclohexane was a suitable organic phase. The distribution coefficient (63·3) was heavily in favour of the top layer. Transfer from buffers of pH 2, pH 3, pH 4, and pH 5 to a buffer of pH 7·4 was investigated. At pH 2·0 salicylic acid is present mainly as the unionized drug and as such is only sparingly soluble in the water and so quickly passes into the organic layer. However, when the drug passes to compartment C it ionizes completely and is quickly removed from B (Fig. 3), and little is retained by the top phase at equilibrium. At pH 3·0 approximately 48% of the drug is in the free acid form and this is sufficient to maintain the transfer rates. At pH values of 4 and 5 however, there is insufficient free acid to maintain the transfer rate although the drug appears in compartment C at the same

rate as at the lower pHs. It was found, with this system, that the back transfer constants are negligible and so the rate equations reduce to

$$V_A \frac{dA}{dt} = -V_A k_1 A$$

$$V_B \frac{dB}{dt} = V_A k_1 A - V_B k_2 B$$

$$V_C \frac{dC}{dt} = V_B k_2 B$$

These readily integrate to give

$$A = A_0 e^{-k_1 t}$$

$$B = \frac{550\, A_0 k_1}{300 k_2 - \frac{550}{300} k_1} \left(e^{-\frac{550}{300} k_1 t} - e^{-k_2 t}\right)$$

$$C = A_0 \left[1 - \frac{1}{k_2 - \frac{550}{300} k_1} \left(k_2\, e^{-k_1 t} - k_1 e^{-k_2 t} - \frac{550}{300} k_1 e^{-k_1 t} + k_1 e^{-\frac{550}{300} k_1 t}\right) \right]$$

where $V_C = V_A = 550$ ml and $V_B = 300$ ml. Fig. 3 shows the experimental points together with the theoretical curves obtained using the

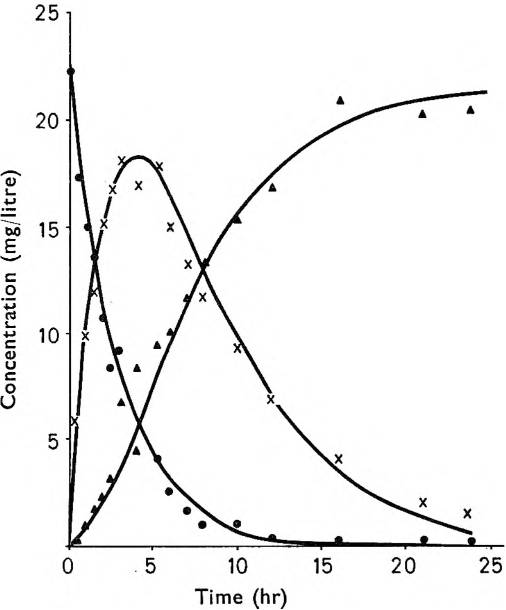

FIG. 3. Transfer of salicylic acid from pH 2·0 to pH 7·4 through a layer of 30% decanol in cyclohexane. The points are experimental and the lines are theoretical. ●, drug in pH 2·0 compartment. ×, drug in organic layer. ▲, drug in pH 7·4 compartment.

above equations in a digital computer. The computer derived rate constants are:

pH	k_1 (hr^{-1})	k_2 (hr^{-1})
2	0·34	0·22
3	0·37	0·22
4	0·12	0·22
5	0·044	0·25

BASIC DRUG

Amidopyrine (pK_a 5·10 at ionic strength 0·21) was taken as the example and cyclohexane was used as the top phase. The partition coefficient (0·213) was in favour of the water. Here the transfer rate from A to B rises as expected but the overall picture is complicated by the back transfer rate constants which are not longer negligible. This is due to the fact that the drug is more ionized in A than C and to the value of the distribution coefficient. The drug in all experiments built up quickly to a low equilibrium value in the organic layer, but the overall equilibrium was approached very slowly. Fig. 4 shows a typical set of results together

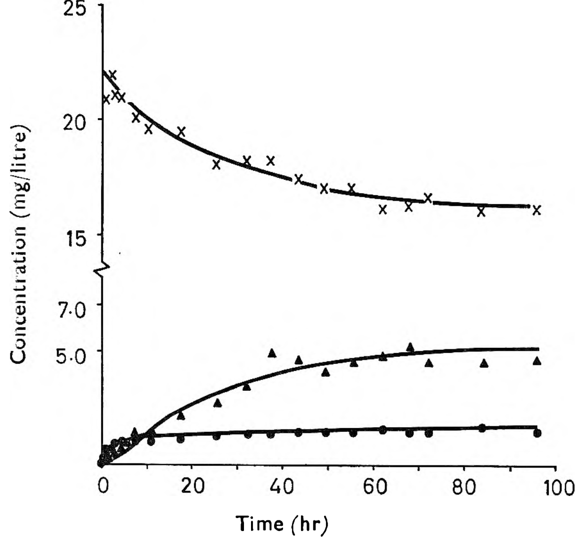

FIG. 4. Transfer of amidopyrine from pH 5·0 to pH 7·4 through a layer of cyclohexane. The points are experimental and the lines are theoretical. ×, drug in pH 5·0 compartment. ●, drug in organic layer. ▲, drug in pH 7·4 compartment.

with the theoretical curves derived using the general equations. The estimates of the transfer rates (hr^{-1}) obtained by the digital computer are:

pH	k_1	k_{-1}	k_2	k_{-2}
5	0·018	0·33	0·34	0·055
6	0·045	0·35	0·46	0·082
7	0·058	0·39	0·44	0·075

Discussion

The results show the design and construction of the apparatus to be adequate in that the data obey the theoretical equations. If the system is to be compared to drug transfer across a lipoidal membrane there should be little or no retention by the organic layer. The amount retained will be controlled by the pK_a of the drug, the pH values of the aqueous phase and the distribution coefficients. These problems are illustrated by the examples cited above and are usually more difficult to overcome with basic drugs particularly when using physiological pH in compartment A. Frequently solvents with no similarity to natural lipids, such as chloroform and cyclohexane, are used in partition studies for correlation with drug absorption data. Higher alcohols such as decanol and dodecanol can be used in this apparatus without the danger of emulsion formation, which can occur in simple partitioning and in the three phase rocking apparatus (Doluisio & Swintosky, 1964). Again this lack of emulsion formation enables natural lipids such as lecithin and cephalin to be dissolved in the top phase, and these materials are known to orientate at an oil-water interface with the polar head towards the aqueous phase. In this situation the rate controlling steps would be the transfer across this lipid interfacial "membrane," so giving a much closer comparison to the natural process. Work is continuing in this direction.

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Enzymatic decyanation of cyanocobalamin in rat tissues

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Cyanocobalamin is decyanated *in vitro* to hydroxocobalamin by rat liver and kidney. This appears to be due to an enzyme system which we have called "cyanocobalamin-decyanase". The enzyme is in the soluble fraction of the cells; it requires reduced cozymases and flavins and has an optimum pH of 7·2 under anaerobic conditions. This is consistent with the fact that conversion of cyanocobalamin to hydroxocobalamin and/or its reduced forms ($B_{12}r$, $B_{12}s$) involves a reductive process in the earliest stage of the biosynthesis of coenzyme B_{12}.

VITAMIN B_{12} coenzyme (5′-deoxyadenosyl-B_{12}, DBC-coenzyme) accounts for as much as 72% of cobamide compounds in animal liver (Toohey & Barker, 1961). Since it is less stable than the cobamide vitamins, these authors felt that the true percentage in the liver may be even higher and previous failures in its isolation may be due to its lability.

Although the presence of small amounts of hydroxocobalamin and cyanocobalamin in liver has not yet been excluded, most of the hydroxocobalamin found as "native" vitamin B_{12} very likely arises from the cleavage of the coenzyme or cyanocobalamin or both.

Structural assignments based upon the cleavage of the coenzyme by photolysis or acid hydrolysis or both, demonstrated that the coenzyme possesses all the structural features of cyanocobalamin; in the former the deoxyadenosyl moiety takes the place of the cyano-group (Smith, Mervyn, Johnson & Shaw, 1962).

The chemical pathway to the coenzyme (and probably also the enzymatic one) proceeds *via* a two-electron reduction of cyano- or hydroxocobalamin in presence of air (Johnson, Mervyn, Shaw & Smith, 1963; Smith, Mervyn, Muggleton, Johnson & Shaw, 1964; Peterkofsky & Weissbach, 1964).

Hydroxocobalamin seems therefore to play a primary role both in the cleavage of the coenzyme (Brady & Barker, 1961; Hogenkamp, 1964) and in its biosynthesis from cyanocobalamin (Fenrych, Pawelkiewicz & Magas, 1962; Górna, 1963; Pawelkiewicz, Górna, Fenrych & Magas, 1964).

We set out to elucidate the early metabolic step in the biosynthesis of the coenzyme B_{12} from cyanocobalamin. We describe the enzymatic conversion of cyano- to hydroxocobalamin in tissues of high cobalamin turnover, like liver and kidney, and discuss the different roles of these tissues in the metabolism and storage of the two cobalamins (Cima, Mastrogiacomo & Maraschin, 1965; Mastrogiacomo, Cima & Maraschin, 1965).

Experimental

MATERIALS AND METHODS

Preparation of tissues samples. All tissues samples were prepared at 0 to 5°. Male albino rats (Wistar) were killed by a blow on the head

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and tissues immediately removed and homogenized in 10 parts of 0·25 sucrose. Supernatant fractions free of unbroken cells, nuclei and mitochondria, were prepared by centrifugation of the homogenates at 9,000 × *g* for 20 min. The microsomes were obtained by centrifugation of the supernatant for 60 min at 72,000 × *g*. Microsomes were thoroughly washed with isotonic potassium chloride and recentrifuged; they were then resuspended in potassium chloride solution, so that 1 ml of suspension contained the microsomes from 1 g of tissue.

Cyanocobalamin was incubated with the tissue preparations in different concentrations, with or without cofactors, at pH values from 5 to 8; volume was made up to 3·5 ml with 0·1M phosphate buffer.

Experiments were at 0°, 25° and 37° in a metabolic shaking incubator both in air and anaerobically, using an atmosphere of nitrogen, or *in vacuo*.

Chromatography. Samples of the incubated mixtures (0·5–0·7 ml) were put directly on thin-layer chromatoplates of silica gel (Kieselgel G Merck suspension in 95% ethanol, dried at 37°) and developed with 50% ethanol. The silica areas of hydroxo- (Rf 0·08–0·1) and cyanocobalamin (Rf 0·58–0·60) were transferred into a G4 Büchner and eluted with 50% ethanol to obtain a final concentration of about 5 μg/ml. These procedures were performed in dim light or in the dark.

Determination of cobalamins. Cyanocobalamin was determined spectrophotometrically (British Pharmacopoeia, 1963). Hydroxocobalamin binds firmly to the silica (Dony & Conter, 1956; Cima & Mantovan, 1962); accordingly, its spectrophotometric evaluation in eluates was merely qualitative. It was quantitatively determined by reconverting it to cyanocobalamin. The silica powder containing the hydroxocobalamin was suspended in 5 ml of M/15 phosphate buffer, pH 6, containing potassium cyanide (1 mg/ml) in a glass-stoppered tube; after 8–10 hr the cyanocobalamin formed was eluted as above and isolated by extraction from the buffered suspension of silica and cyanide, which was vigorously shaken three times with 50% *p*-chlorophenol (0·5 ml) in carbon tetrachloride, in a glass-stoppered centrifuge tube. The mixtures were each centrifuged and the bulked lower layers collected and shaken with n-butanol–carbon tetrachloride–water (3:1·5:0·75 the bulk volume), when the cyanocobalamin passed into the aqueous layer. The organic phase was re-extracted twice with water (0·75 × its vol.) for a quantitative recovery.

The amount of hydroxocobalamin formed could also be obtained indirectly by measuring the amount of cyanocobalamin remaining in the incubated mixture.

Both methods agreed for short incubation times. We usually used the direct method because it is independent of unrelated transformation of cyanocobalamin.

Results

In preliminary experiments, aerobically incubated homogenates (1:2 w/v) of rat liver, kidney, lung, brain, heart and spleen with cyanocobalamin

at 37° for 2 hr showed no appreciable decyanation. Anaerobically some decyanation took place with kidney and spleen (2–3%), and with liver (3–6%). Liver homogenates and anaerobic conditions were therefore used.

Since oxygen inhibits decyanation and the cyano-group can be split from cyanocobalamin by reducing agents (Kaczka, Wolf & Folkers, 1949; Fricke, Lanius, Derose, Lapidus & Frost, 1950; Brockmann, Pierce, Stokstad, Broquist & Jukes, 1950; Bernhauer, Renz & Wagner, 1962), the addition of naturally-occurring reducing compounds should enhance the formation of hydroxocobalamin. This did occur in presence of a mixture of cozymase (NADH) and flavinmononucleotide (FMN). NADH alone produced no more hydroxocobalamin than the controls; an increase did occur in the presence of FMN, though this was not so high as with the mixture of coenzymes.

Controls made without tissue homogenates showed that the coenzymes had an insignificant effect on decyanation.

The existence and properties of a "cyanocobalamin-decyanase" were elucidated by studying: (i) liver intracellular distribution; (ii) optimum conditions for activity; (iii) flavin requirement; (iv) reduced cozymase requirement; (v) influence of ions and of inhibitors; (vi) activity in kidney tissue.

(i) *Liver intracellular distribution.* Cell fractions equivalent to 0·2 g of rat liver were incubated for 1 hr at 37° with 0·7 μmole of cyanocobalamin, 10 μmole of FMN and phosphate buffer M/15, pH 7·2 under nitrogen. Almost all the enzyme activity was found in the supernatant containing the soluble fraction and the microsomes. Fractionation of this supernatant showed the decyanase was localized in the soluble fraction [cozymases are mainly localized in the soluble cell fraction (Dixon & Webb, 1964)]. Liver supernatant equivalent to 0·2 g liver (wet weight) was subsequently used.

(ii) *Optimum conditions for enzyme activity.* These were determined experimentally to be: incubation of 0·5 μmole cyanocobalamin for 90 min with 1 μmole of FMN at pH 7·2 at 37°.

(iii) *Flavin requirement.* Riboflavin, FMN and FAD were tested comparatively for their abilities to restore the decyanase activity of the soluble fraction after mild acid treatment, which is known to dissociate the flavin prosthetic group from flavoprotein (Zelitch & Ochoa, 1953).

Unexpectedly, riboflavin showed the same activity as its two coenzymes. Any of the three flavins can therefore replace the prosthetic group; furthermore, when added in excess, they markedly accelerated the extent of decyanation; they were used at a concentration of 1 μmole.

The observation that cyanocobalamin-decyanase can use riboflavin, which is usually considered only a precursor of FMN or FAD, is reminiscent of the mammalian nitroreductase system; here, activity is accelerated by the addition of an excess of any one of the flavins (Fouts & Brodie, 1957).

(iv) *Reduced cozymase requirement.* Cozymase was increased in the incubation mixtures by addition of NADH or pretreatment of the experimental animals.

Cozymase added in vitro. Some decyanation occurred when the liver supernatant was replaced by NADH, but not by NAD.

In the absence of liver supernatant, using the optimum conditions for activity for 40 min, the extent of decyanation was found to be proportional to the amount of NADH added, 1 μmole causing approximately 5·6% decyanation (Table 1). In presence of the liver supernatant, this

TABLE 1. NADH REQUIREMENT

NADH (μmole)	% Decyanation		
	Supernatant omitted (*a*)	Supernatant added (*b*)	Supernatant added (*b–a*)
—	—	18·0	18·0
0·5	3·0	22·0	19·0
2·0	11·7	29·4	17·7
3·5	19·1	37·8	18·7
5·0	27·5	46·9	19·4
	Average/μmole 5·6	Average 18·5	

Anaerobic incubation for 40 min. Other conditions optimal.

became 18·5%. Decyanation to this extent might be promoted by 3·3 μmole of NADH alone. Since liver supernatant contains no more than 0·06 μmole of NADH (Ricci & Conte, 1952; Glock & McLean, 1955), its decyanating activity is about fifty times higher than expected on the basis of its NADH content. The decyanation of cyanocobalamin by NADH through reduction of the flavin therefore occurs only to a limited extent.

Effect of an increase in hepatic NADH. The *in vitro* results after NADH led us to check if an increase of liver cozymase in animals pretreated with nicotinamide increased decyanation of cyanocobalamin. Intraperitoneal administration of nicotinamide (1 g/kg) causes an average tenfold increase both of NAD and of NADH in rat liver, after 12 hr (Bonsignore & Ricci, 1948; Kaplan, Goldin, Humphreis, Ciotti & Stolzenbach, 1956; Missale & Colajacomo, 1956; Bonsignore & Ricci, 1958).

The liver supernatant from nicotinamide pretreated rats increased in decyanating ability (Table 2); this remained high for 2 hr, decreasing and

TABLE 2. INCREASE OF DECYANASE ACTIVITY IN LIVER SUPERNATANTS OF NICOTINAMIDE PRETREATED RATS

Time (hr)	Controls: Hydroxocobalamin formed (μmole)	Controls: Decyanation (%)	Nicotinamide pretreated rats: Hydroxocobalamin formed (μmole)	Nicotinamide pretreated rats: Decyanation (%)	Nicotinamide pretreated rats: Increase in decyanation (%)
½	0·0865	17·3	0·1205	24·1	39·3
1	0·1190	23·8	0·1650	33·0	38·6
2	0·1325	26·5	0·1790	35·8	35·0
3	0·1560	31·2	0·1865	37·3	19·5
4	0·1885	37·7	0·1940	38·8	2·9

Optimum conditions. Average values of three assays.

disappearing after 4 hr of incubation. The enzymatic activity after 30 min was similar to that obtained (see Table 1) when liver supernatant from

normal rats was added with 0·5 μmole of NADH, corresponding to a tenfold addition of the normal NADH content.

We sought the apparent relationship between the increased decyanation and the amount of NADH available. NADH cannot be measured spectrophotometrically in our system since the method does not differentiate the coenzyme from NADCN (Meyerhof, Ohlmeyer & Mohle, 1938; Colowick, Kaplan & Ciotti, 1951); we therefore followed the decrease in decyanation when the liver supernatants of the pretreated rats were stored at 0° in the air or anaerobically.

Table 3 shows that aerobic conditions cause a more rapid loss of the enzyme activity than anaerobic conditions.

TABLE 3. DECREASE OF DECYANASE ACTIVITY UPON STORAGE AT 0°

Time (days)	Aerobic	Anaerobic	Hydroxocobalamin formed (μmole)	Decyanation (%)	Decrease in decyanation (%)
–	–	+	0·1725	34·5	—
2	+	–	0·0875	17·5	49·2
2	–	+	0·1415	28·3	17·9
4	+	–	0·0420	8·4	75·6
4	–	+	0·1330	26·6	22·8
6	+	–	0·0255	5·1	85·2
6	–	+	0·1045	20·9	39·4

Incubation time 90 min; other conditions optimal. Liver supernatant of nicotinamide pretreated rats.

This suggests a definite role of the reduced form of cozymase, as a hydrogen donor; the reducing ability of NADH is likely to be maintained by pyridinenucleotide-dependent dehydrogenases and by oxidizable substrates present in the supernatant.

(v) *Influence of ions and of inhibitors.* To avoid precipitation of insoluble phosphates, 0·2M Tris-buffer was substituted for the phosphate buffer and riboflavin was used in place of FMN; previous experiments had shown that the results were unaffected.

From Table 4 it is evident that high concentrations of azide, sodium acetate, and, to a smaller extent, Fe^{++}, Fe^{+++} and pyrophosphate, inhibit the enzymatic decyanation of cyanocobalamin. Mg, Zn, Ca and Mu showed only weak inhibiting effects.

TABLE 4. INFLUENCE OF IONS AND INHIBITORS

Added compounds	μmole	Hydroxocobalamin formed (μmole)	Decyanation (%)	Inhibition (%)
—	—	0·128	25·6	—
Na-pyrophosphate	10	0·100	20·0	21·8
Na-azide	10	0·000	0·0	100·0
Na-EDTA	10	0·036	7·2	71·8
,,	50	0·031	6·2	75·7
$MgCl_2$	10	0·118	23·6	7·8
,,	100	0·120	24·0	6·2
$CaCl_2$	2·5	0·117	23·4	8·5
$FeCl_2$	10	0·064	12·8	50·0
$FeCl_3$	10	0·069	13·8	46·0
$MnCl_2$	2·5	0·122	24·4	4·6
$ZnCl_2$	2·5	0·125	25·0	2·3

Tris-buffer 0·2 M (pH 7·2). Anaerobic incubation for 120 min. Other conditions optimal.

For a better knowledge of the role of the ions involved in decyanase activity, a portion of liver supernatant was dialysed at O° for 48 hr and the activity was determined using as a reference a non-dialysed portion. A 50% reduction of the enzymatic activity was observed in the dialysed sample, which could not be restored by addition of Zn, Mg, Ca and Mn. When the dialysis was continued during 4 days, the decyanase activity disappeared completely and was not restored by the same additions. None of the ions tested was found to be essential or to act as a catalyst.

(vi) *Activity in kidney tissue.* The role of the kidney in the metabolism of cyanocobalamin is well known (Okuda & Chow, 1960; Wong, Yeh & Chow, 1960; Lee & Glass, 1961; Okuda, 1962), and we have recently shown (Cima & others, 1965; Mastrogiacomo & others, 1965) that the uptake of labelled cyanocobalamin is greater in the kidney soon after its administration than in the liver, but this is later reversed.

We found that kidney decyanating system, prepared as that of liver, is also active anaerobically and in presence of FMN or riboflavin, but it has only about 60% of liver activity.

Since the NADH content of kidney supernatant is the same as that of the liver (Glock & McLean, 1955), its low decyanating ability may be due to a smaller enzyme content or to a slower rate of activity.

It is difficult to correlate the observations *in vitro* with the higher uptake of cyanocobalamin by liver *in vivo*. But kidneys *in vivo* take up the vitamin only for a short time before undergoing a depletion (Okuda, 1962; Cima & others, 1965; Mastrogiacomo & others, 1965); furthermore cyanocobalamin is converted into coenzyme B_{12} at only about half the rate of that of the liver (Fenrych & others, 1962).

Discussion

The results suggest that cyanocobalamin is decyanated in the rat through a biochemical pathway, taking place mainly in the liver and to a lesser extent in the kidney. In both tissues there was a single or composite enzyme system converting cyanocobalamin to hydroxocobalamin; this we have called "cyanocobalamin-decyanase". It is localized in the cell soluble fraction; it is pyridine- and flavin-dependent and has a maximum activity at pH 7·2 at 37° under anaerobic conditions.

The liberated CN probably forms cyanide salts using ions from the medium and eventually an addition compound with NAD. This is known to be formed in some instances (Meyerhof & others, 1938; Colwick & others, 1951) and it may readily release the CN to hydroxocobalamin reconverting it into cyanocobalamin. Since both *in vitro* and *in vivo* decyanation were never complete, it seems that an equilibrium is reached which is probably related to the fate of the CN.

The pyridine hydrogen-donor is cozymase in its reduced form, while the enzyme includes a flavoprotein, whose prosthetic group can be replaced equally well by FAD, FMN or riboflavin. Addition of an excess of any one of these flavins accelerates the decyanation, suggesting that it not only conjugates to the enzyme but acts also as an artificial electron carrier between NADH and cyanocobalamin.

The non-specificity of the flavin requirements has been demonstrated in the biosynthesis of coenzyme B_{12} from cyanocobalamin by bacterial cell extract (Peterkofsky, Redfield & Weissbach, 1961). Also in this system, the simple role of the flavin as electron carrier was demonstrated, superseding a previous hypothesis of an alternative role as ribose donors.

Our demonstration of three of the main requirements of the system (NADH, flavin and anaerobic conditions) suggests that the conversion of cyanocobalamin to coenzyme B_{12} is a reductive process: this may involve the reduction of cobalt III to cobalt II.

Recent data report structural ambiguities concerning the cobalt atom in the coenzyme B_{12}. It appears trivalent on the basis of chemical reactions (Smith & others, 1962; Bernhauer, Müller & Müller, 1962; Müller & Müller, 1962) and of electron-spin resonance measurements (Hogenkamp, Barker & Mason, 1963), while its electrophoretic behaviour and paramagnetic character indicate that it is divalent (Johnson & Shaw, 1960; Nowichi & Pawelkiewicz, 1960; Bernhauer, Gaiser, Müller, Müller & Günter, 1961). On the other hand, vitamin $B_{12}r$, stable only under anaerobic conditions, is obtained by chemical one-electron reduction of vitamin B_{12} and contains divalent cobalt (Bernhauer, Gaiser, Müller & Wagner, 1960). Further, one-electron reduction yields vitamin $B_{12}s$ (hydridocobalamin), thought to contain monovalent cobalt. Vitamin $B_{12}s$ is also very unstable and it is the unique substrate for the chemical synthesis of coenzyme B_{12}. Decyanated cobalamins seem therefore to be the unique substrates for both chemical and enzymatic synthesis of coenzyme B_{12} (Smith & Mervyn, 1963; Johnson & others, 1963; Tackett, Collat & Abbott, 1963; Smith & others, 1964; Hill, Pratt & Williams, 1964).

In independent experiments in which liver supernatants were incubated anaerobically in the dark at 37°, in presence of ATP for 45 hr, we did not observe the formation of coenzyme B_{12}. However Pawelkiewicz, Górna, Fenrych & Magas (1964) were able to obtain some conversion of cyanocobalamin into coenzyme B_{12} under different conditions by incubating rat liver and kidney sediments, whereas with the respective supernatants the conversion was poor (10–12%).

These observations may support the view that the synthesis of coenzyme B_{12} from cyanocobalamin in liver occurs in two stages, taking place in two individual cellular fractions. The former, in the supernatant, is the decyanase system requiring NADH and flavin; the latter, in the sediments, is a synthetase catalysing the conversion of hydroxocobalamin (via $B_{12}r$, $B_{12}s$?) and requiring the same cofactors; this one also needs ATP as a substrate and probably as a precursor of both the base and sugar moieties of the 5′-deoxyadenosyl nucleoside.

A similar two-step reaction was postulated in the enzymatic synthesis of coenzyme B_{12} by cell free extracts of some *Propionibacteria* and *Clostridia* (Pawelkiewicz, Bartosinski & Walerych, 1964). Conversely, a single-step concerted reaction was postulated by Weissbach, Redfield & & Peterkofsky (1962), because ATP itself seems necessary also for cyanide release.

Our results using liver and kidney do not agree with the last mechanism, since the release of cyanide ion from cyanocobalamin occurs independently from the addition of ATP; this, in turn, does not increase the rate of decyanation.

Our results are therefore consistent with the existence of two enzymatic processes catalysing a two-step reaction; the over-all pathway is outlined in the following scheme.

$$(a)\ \text{cyanocobalamin} \xrightarrow[\text{flavin}]{\text{NAD(P)H}} \text{hydroxocobalamin} + \text{CN}$$

$$(b)\ \text{hydroxocobalamin} + \text{ATP} \xrightarrow{\text{reduced flavin (2 e)}} [B_{12}s] \rightarrow$$

$$\searrow \xrightarrow{\text{reduced flavin (1 e)}} [B_{12}r] \xrightarrow{\text{reduced flavin (1 e)}} \nearrow$$

$$\text{coenzyme } B_{12} + \text{tripolyphosphate}$$

Reaction *a* has been elucidated by our studies on liver and kidney tissues; reaction *b* was postulated on the basis of more general reactions for the biosynthesis of coenzyme B_{12} in micro-organisms; a sound demonstration however is still lacking.

The hypothesis of two enzymatic processes seems to be confirmed by a recent observation of Uchino, Yagiri, Yoshino, Kondo & Wakisawa (1965) *in vivo*: carbon tetrachloride primarily injures the pathway from cyano- to hydroxocobalamin, while further transformation to coenzyme B_{12} remains almost unaffected.

The importance of decyanation as an enzymatic pathway in coenzyme B_{12} synthesis is a major conclusion of the present work. It may have a more general meaning, if it is considered in the light of its participation in the formation of methylcobalamin (methyl-B_{12}), a proposed intermediate in the biosynthesis of liver methionine (Buchanan, Elford, Loughlin, McDougall & Rosenthal, 1964; Dickerman, Redfield, Bieri & Weissbach, 1964; Weissbach & Dickerman, 1965).

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A note on spectrophotometric methods for the determination of norethynodrel and mestranol in tablets

R. A. BASTOW

The method for assay of norethynodrel involves acid catalysed rearrangement of the Δ5,10 system to a Δ4,3-ketone with stronger ultraviolet absorption. Mestranol is determined from its absorption at 280 mμ after elimination of the interfering ketonic absorption by reduction with borohydride. Residual interference is allowed for by a three point correction.

NORETHYNODREL (17-hydroxy-19-nor-17-pregn-5(10)-en-20-yn-3-one) and mestranol (17-hydroxy-3-methoxy-19-nor-17-pregna-1, 3, 5 (10)-trien-20-yne) occur together in a number of tablet formulations, and as the normal levels of mestranol are in the range 0·05 to 0·15 mg per tablet a sensitive analytical method is required. The widely used Kober reaction for oestrogens has undergone various modifications to improve precision and reproducibility. It is applicable to mestranol after preliminary separation from norethynodrel by chromatography but the technique proved to be tedious and of poor precision in this laboratory. Thin-layer and gas-liquid chromatography (Schulz, 1965) have also been proposed for the determination of mestranol in tablets. Legrand, Delaroff & Smolik (1958) determined oestradiol type steroids in the presence of large amounts of ketosteroids, such as testosterone or androstanolone, by ultraviolet spectrometry after reducton of the interfering ketone with potassium borohydride. Residual absorption by the reduction products was corrected graphically using data obtained on pure reduced samples of phenolic and ketosteroids.

This reduction is applicable to norethynodrel but pure samples of this drug, needed for obtaining data on the background absorption, are difficult to obtain because, as normally manufactured, it contains up to 1·5% of mestranol. Repeated recrystallization will not normally bring this below 0·5%. An alternative method of background correction is by means of a three point correction (Morton & Stubbs, 1946) and the method to be described uses this principle.

The technique of determining norethynodrel by direct ultraviolet absorption is unsatisfactory due to the lack of characteristic absorption and for this reason Chissell (1964) proposed a colorimetric method using *m*-dinitrobenzene. Norethynodrel under acid conditions rearranges to norethisterone (17-hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one) which has the characteristic conjugated ketone absorption at about 240 mμ suitable for its quantitative estimation.

Experimental

ACID CATALYSED REARRANGEMENT OF NORETHYNODREL

A solution of norethynodrel in methanol, 100 μg/ml, was prepared and 5 ml portions heated for different time intervals in a boiling water bath

From Pfizer Ltd., Sandwich, Kent.

with N hydrochloric acid (5 ml). The resultant solution was diluted with methanol (100 ml) and examined spectroscopically. The absorption at 241 mμ, brought about by rearrangement to norethisterone, was at a maximum E (1%, 1 cm) of 560 after 5 min heating and corresponded to complete isomerization.

DERIVATION OF BACKGROUND CORRECTION FOR MESTRANOL

A pure sample of mestranol was subjected to the borohydride reduction procedure described by Legrand & others (1958) and a three point correction calculated from a plot of the ultraviolet spectrum of the resultant solution (Fig. 1).

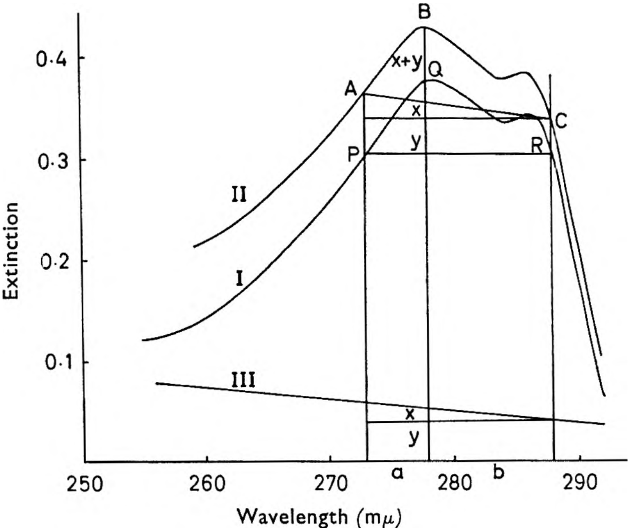

FIG. 1. Ultraviolet spectra of: I, mestranol; II, mestranol in the presence of background interference; III, background interference.

In the spectrum of pure mestranol, points P and R are of equal absorption and this absorption is a known fraction F of the absorption Q at the peak. By proportion in the similar triangles:

$$x = (A - C)\frac{b}{a + b}$$

and

$$\frac{C - y}{B - (x + y)} = \frac{R}{Q} = F$$

The required corrected absorption is B — (x + y).

Using a solution of borohydride-treated mestranol at a concentration of 38·6 μg/ml, equal extinction points at 273 mμ and 288 mμ were chosen; at these wavelengths the absorption was 0·76 (fraction F) of the absorption at the peak of 278 mμ. Substitution of these data in the above equations and solving for x and y is shown as follows:

$R = 0{\cdot}180 \quad Q = 0{\cdot}237 \qquad a = 5\ m\mu \quad b = 10\ m\mu$

$$x = (A - C)\frac{10}{15} \qquad \frac{C - y}{B - (x + y)} = \frac{0{\cdot}180}{0{\cdot}237} = 0{\cdot}76$$

$$x = 0{\cdot}667\ A - 0{\cdot}667\ C \qquad y = 2{\cdot}109\ A - 3{\cdot}164\ B + 2{\cdot}054\ C$$

$$\text{Corrected absorption} = B - (x + y) = 4{\cdot}164\ B - 2{\cdot}776\ A - 1{\cdot}387\ C$$

Application of this correction to determine the mestranol content of samples of norethynodrel after reduction with borohydride requires that the background absorption due to reduced norethynodrel should be linear over the wavelength range covered by the correction. Direct confirmation of this linearity was not possible as all samples of norethynodrel examined contained 0·5% or more of mestranol. A sample of norethynodrel was subjected to the acid catalysed rearrangement to norethisterone; this latter compound is less soluble than its precursor in polar solvents and recrystallization gave a product much lower in mestranol content. Examination of its ultraviolet spectrum after borohydride reduction (Fig. 2) showed substantial linearity over the range

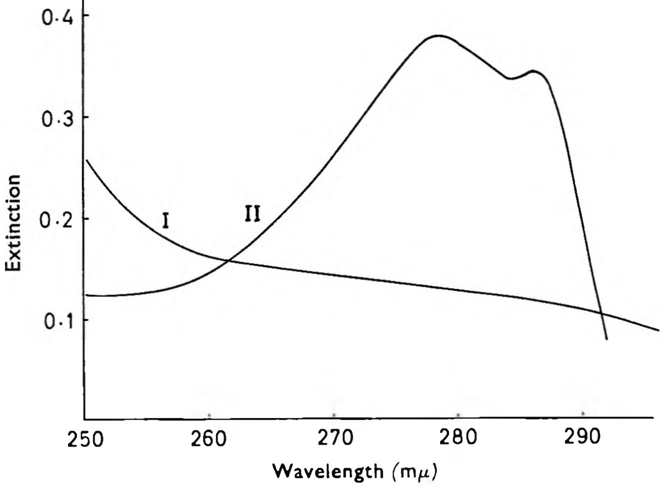

FIG. 2. I. Ultraviolet spectrum of norethisterone after borohydride reduction (137 μg/ml). II. Tablets of mestranol and norethynodrel after borohydride reduction (42 μg mestranol/ml).

260 to 300 mμ. It is believed that the borohydride reduction product of norethynodrel is identical to that of norethisterone.

GENERAL METHOD

On the basis of the foregoing experimental work the following procedure was established for the determination of norethynodrel and mestranol in tablets, alone and in combination.

Norethynodrel. Transfer a weight of powdered tablets equivalent to 10 mg of norethynodrel to a 100 ml volumetric flask and dilute with

methanol (100 ml). Stir for 15 min with a magnetic stirrer, filter and transfer 5 ml of filtrate to a 100 ml volumetric flask, add N hydrochloric acid (5 ml) and heat in a boiling water-bath for 5 min. Cool, dilute to 100 ml with methanol and determine the extinction in a 1 cm cell at the maximum at about 241 mμ against a similarly treated blank.

For calculation use an E (1%, 1 cm) of 560.

Mestranol. Reagents: Potassium borohydride, reagent grade. Methanol reagent, dissolve potassium borohydride (1·5 g) in methanol A.R. (1 litre), add N sodium hydroxide (8 ml), reflux for 4 hr and distil.

Transfer a weight of powdered tablets equivalent to 1·5 mg of mestranol to a 50 ml volumetric flask and add methanol reagent (35 ml). Shake for 30 min, add 0·1 N sodium hydroxide (4 ml) containing potassium borohydride (200 mg) and stand overnight. Add 0·1 N hydrochloric acid (4 ml), shake gently to eliminate dissolved gases and dilute to 50 ml with methanol reagent. Determine the extinction in a 1 cm cell at 273, 278 and 288 mμ, against a similarly treated blank.

Corrected extinction = 4·164 extinction at 278 mμ—2·776 extinction at 273 mμ—1·387 extinction at 288 mμ. For the calculation use an E (1%, 1 cm) of 70.

Results and discussion

Table 1 summarizes results obtained using the method described on tablets in which the excipients and lubricants were lactose, starch, alginic acid, magnesium stearate and stearic acid. No interference was experienced from these vehicles. The relatively low E (1%, 1 cm) of 70 for mestranol at the wavelength of determination requires the extraction of 1 to 2 g of tablets giving a high level of any methanol soluble substances present in the formulation. The borohydride reduction and subsequent correction procedure are likely to contribute towards the elimination of interference from tablet excipients but it is possible that alternative extraction procedures may be required for other formulations.

TABLE 1. RESULTS WITH TABLETS IN WHICH THE EXCIPIENTS AND LUBRICANTS WERE LACTOSE, STARCH, ALGINIC ACID, MAGNESIUM STEARATE AND STEARIC ACID

Sample tablets	Norethynodrel content mg tablet		Mestranol content mg tablet	
	Nominal	Found	Nominal	Found
1	0	—	0·1	0·099
2	0	—	0·1	0·108
3	0	—	0·1	0·103
4	3·50	3·54	0·14	0·12
5	5·00	5·08	0·075	0·073
6	5·00	5·08	0·075	0·076
7	5·00	5·00	0·075	0·079

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Pharmacological properties of tetrahydropapaveroline

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Tetrahydropapaveroline (THP) exerts β-sympathomimetic effects similar to those of isoprenaline. On guinea-pig isolated atria, THP elicits positive inotropic and chronotropic activities which are not abolished by previous reserpinization of the animals; on isolated mammalian heart these effects are associated with an increase in coronary flow. In the dog, THP increases myocardial contractile force and rate, elicits a hypotensive effect and stimulates respiratory activity in normal and reserpinized animals; when injected intra-arterially the drug causes vasodilatation. All the effects are prevented by the β-adrenergic blocking agents propranolol, dichloroisoprenaline and pronethalol. Structure-activity relationships between tetrahydroisoquinoline derivatives and their open-ring phenylethylamine congeners, which are closely related to sympathomimetic drugs, are discussed.

THE pharmacological properties of tetrahydropapaveroline (THP), described by Laidlaw (1910), have been reviewed recently by Holtz, Stock & Westermann (1964) and by Santi, Bruni, Luciani & others, (1964) and Fassina, Tóth & Santi (1965). THP produces β-sympathomimetic effects similar to those of isoprenaline.

Holtz, Stock & Westermann (1963) obtained experimental evidence that THP may be a naturally occurring substance, since it is formed *in vitro* by guinea-pig liver mitochondria by condensation of dopamine, the precursor of adrenaline, with its primary product of oxidative deamination, dihydroxyphenylacetic aldehyde, but the *in vivo* formation of THP has not yet been demonstrated. It therefore seemed worthwhile comparing the pharmacological behaviour of THP, papaverine and isoprenaline.

Chemically, tetrahydropapaveroline is 1-benzyl-3′,4′,6,7-tetrahydroxy-1,2,3,4-tetrahydroisoquinoline (I).

(I)

Experimental

Acute toxicity was assessed in mice of either sex, 20–22 g, by intraperitoneal injection of THP suspended in 5% acacia gum. The LD50 was determined according to Weil (1952) by using 6 doses and 5 animals per dose; the animals were observed for 5 days after drug administration.

Spasmolytic activity was examined on isolated guinea-pig and rabbit ileum suspended in a 30 ml bath containing Tyrode solution at 37°

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bubbled with air. THP was compared with papaverine against acetylcholine (10^{-7}), histamine (10^{-7}) and barium chloride (10^{-4})-induced contractions of the guinea-pig ileum. A dose cycle of 10 min was used, the spasmolytic drugs being allowed to act for 3 min. The effect of THP and isoprenaline on spontaneous contractions and tone was tested on rabbit duodenum.

Effect on myocardial activity. Guinea-pig isolated atria were suspended vertically in a 30 ml bath containing oxygenated Webb (1950) solution at 29°.

Reserpinized atria were obtained by pretreating the animals with reserpine 2 mg/kg i.p. daily for two days.

Guinea-pig isolated hearts were perfused with the conventional Langendorff technique. Coronary flow rates were measured by a flowmeter.

The effects on myocardium *in vivo* were examined in open chest dogs by means of the strain gauge technique described by Boniface, Brodie & Walton (1953). Some experiments were made on dogs reserpinized according to Paasonen & Krayer (1958).

Vasodilator effects on the hind limb of the dog were obtained by recording flow through the femoral artery with a Shipley & Wilson (1951) rotameter. The effects on vascular smooth muscle in the rabbit isolated ear perfused according to the technique of Krawkow & Pissemski (Ther, 1949) were also examined.

Blood pressure and respiration effects were assessed in dogs and cats anaesthetized with pentobarbitone sodium, 30 mg/kg i.v., and guinea-pigs anaesthetized with urethane, 1–1·2 g/kg. Carotid blood pressure was recorded with a mercury manometer or a Sanborn pressure transducer model 267 B. THP was administered intravenously at doses ranging from 0·005 to 5 mg/kg. In the cat, effects on the nictitating membrane stimulated through the right cervical sympathetic chain by rectangular pulses (0·2 msec duration at 10 pulses/sec, for 10 sec, every 10 min) were also examined.

Short-circuit current and potential difference changes of the isolated frog skin were measured according to the technique of Ussing & Zerahn (1951).

Oxidative phosphorylation. The effect of THP, isoprenaline and papaverine on oxidative phosphorylation was tested using rat liver mitochondria, isolated according to Hogeboom (1955). Measurement of oxygen uptake was made by the conventional Warburg technique at 30°. Inorganic phosphate was determined by the procedure of Fiske & Subbarow (1925); mitochondrial protein was measured as described by Gornall, Bardawill & David (1949).

DRUGS

Drugs used were: tetrahydropapaveroline hydrochloride (Inst. Pharm. Chem. Padua), papaverine hydrochloride (Hoffmann La Roche), isoprenaline sulphate (Abbott), adrenaline bitartrate (Recordati), acetylcholine hydrobromide (Farmitalia), bradykinin (Sandoz), histamine

hydrochloride (Hoffmann La Roche), propranolol hydrochloride, pronethalol hydrocloride (I.C.I.), reserpine (CIBA), heparin (Vister), dichloroisoprenaline (SKF).

Results

ACUTE TOXICITY

The intraperitoneal LD50 of THP in mice was 703 mg/kg (fiducial limits 586 and 843 mg/kg). With doses up to 200 mg/kg the animals showed no untoward effects; larger doses caused marked sedation, with eyelid ptosis, bradypnoea followed, sometimes, by apnoea and death. The LD50 of papaverine given i.p. in rats was 89 mg/kg (fiducial limits 18 and 101 mg/kg).

SPASMOLYTIC ACTIVITY

On the guinea-pig ileum the spasmolytic activity of THP was less than that of papaverine. Concentrations of 1×10^{-6} THP produced a 10–50% inhibition of contractions caused by acetylcholine, histamine or barium chloride, but with increasing concentrations of THP there was a decrease in the amount of inhibition produced (Fig. 1). Unlike

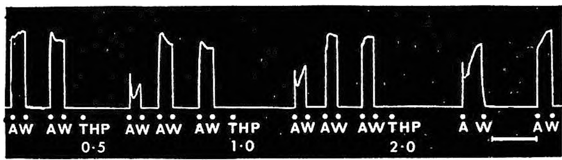
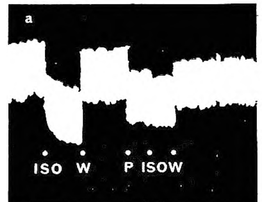


FIG. 1. Isolated guinea-pig ileum. Antispasmodic effect of tetrahydropapaveroline (THP, $\times 10^{-5}$) with a decrease in effect after repeated increasing doses. A, acetylcholine 2×10^{-7}. W, wash. Time scale = 3 min.

papaverine, THP caused a similar inhibition of the rapid and the tonic phases of the contractions to the agonist drugs.

On the rabbit duodenum THP reduced both tone and spontaneous contractions. The effects of 2×10^{-7} THP were comparable to those obtained with 4×10^{-8} isoprenaline and in each case the inhibition was reduced by propranolol 1×10^{-6} (Fig. 2). However this result is difficult to interpret because propranolol itself inhibited the tone and spontaneous contractions of the intestine.



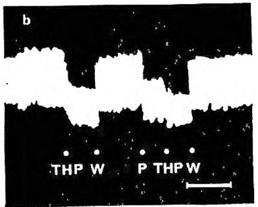


FIG. 2. Isolated rabbit duodenum. (a) effect of isoprenaline (ISO, 4×10^{-8}) before and after propranolol (P, 10^{-6}). (b) effect of tetrahydropapaveroline (THP, 2×10^{-7}) before and after propranolol. W, wash. Time scale = 3 min.

EFFECTS ON MYOCARDIAL ACTIVITY

THP in concentrations of 5×10^{-8} to 5×10^{-7} increased both rate and force of contraction of guinea-pig atria (Fig. 3). THP was approxi-

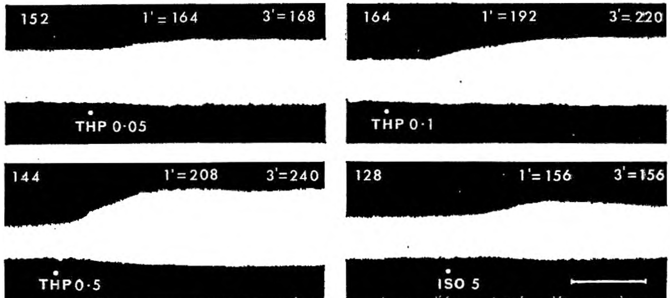


FIG. 3. Isolated guinea-pig atria. Stimulant effects of tetrahydropapaveroline (THP, $\times 10^{-6}$) and isoprenaline (ISO, $\times 10^{-9}$). Time scale = 1 min.

mately 20 times less potent than isoprenaline. Atria from animals pretreated with reserpine were unresponsive to tyramine but more responsive to THP, which was effective at concentrations as low as 5×10^{-9}. Furthermore, pronethalol (1×10^{-7}), dichloroisoprenaline (1×10^{-7} g/ml) or propranolol (1×10^{-7} g/ml) blocked the effect of THP and of isoprenaline.

In the guinea-pig perfused heart, THP at concentrations from 2 to 8×10^{-8} increased amplitude and rate of contraction, with a simultaneous increase of coronary flow (Fig. 4).

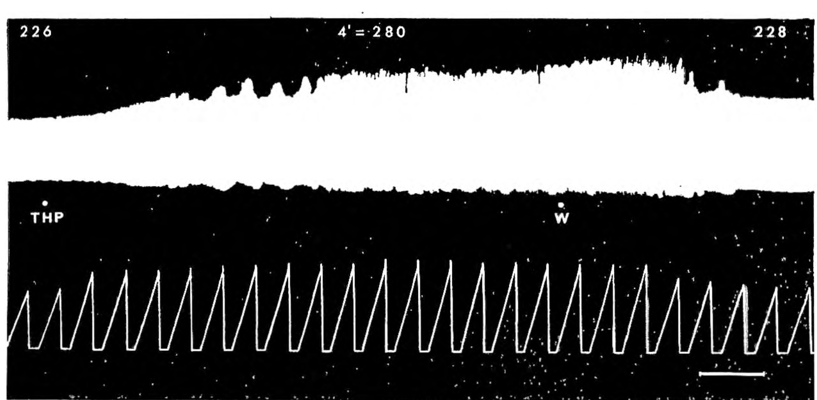


FIG. 4. Perfusion of guinea-pig heart by the Langendorff technique. Effects of tetrahydropapaveroline (THP, 5×10^{-8}) on contractile activity and coronary flow. Time scale = 1 min.

The intravenous administration of 5 μg/kg of THP to the dog increased the force (70–80%) and the rate (15–20%) of the cardiac contraction. THP was about 50 times less active than isoprenaline and 200 times more active than papaverine. Propranolol (0·25 mg/kg) abolished the increase in contractile force caused by isoprenaline (0·05 μg/kg), THP

(5 μg/kg), or papaverine (1 mg/kg) but did not prevent the fall in blood pressure produced by papaverine. In dogs pretreated with reserpine, THP and papaverine maintained their activity, whereas tyramine was ineffective.

EFFECTS ON FLOW THROUGH THE FEMORAL ARTERY AND RABBIT ISOLATED EAR

THP injected intra-arterially in the dog at doses ranging from 3 to 15 μg/kg increased the femoral blood flow by 25–100%. The effect was about 50 times less than that of isoprenaline, but twice that of papaverine. Propranolol (0·25 mg/kg i.v.) blocked the increase in flow caused by THP or isoprenaline but failed to block the effect of papaverine.

The vasoconstrictor effect induced by continuous perfusion of the rabbit ear with 5×10^{-8} adrenaline was abolished by the injection into the lateral cannula of THP, 500 μg; this effect lasted 1–2 min. The effect of papaverine was about the same but more persistent (2–4 min).

EFFECT ON BLOOD PRESSURE AND RESPIRATION

In agreement with the findings of Laidlaw (1910) THP gave a clear hypotensive effect in dogs, cats, guinea-pigs and rabbits, at doses ranging from 0·005 to 5 mg/kg i.v. A significant stimulation of the respiration accompanying the fall of blood pressure, was also confirmed. Dichloroisoprenaline (5 mg/kg), pronethalol (3 mg/kg) or propranolol (0·25 mg/kg) blocked the hypotensive effect of THP (Fig. 5) but in similar experiments the hypotension caused by papaverine was unaffected.

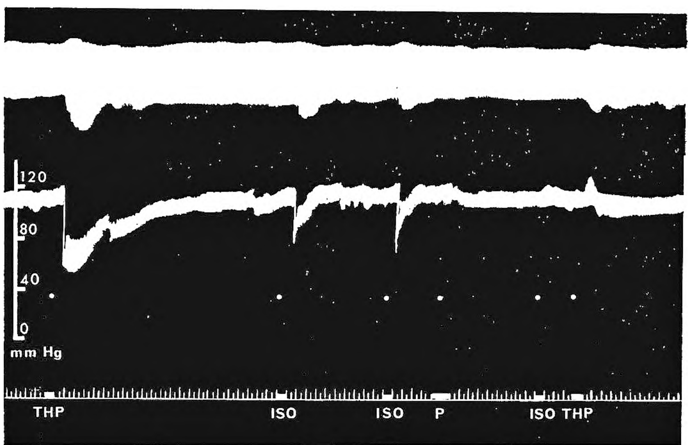


FIG. 5. Mongrel dog, male, 10 kg, under pentobarbitone sodium anaesthesia. Effects of tetrahydropapaveroline (THP, 0·05 mg/kg) and isoprenaline (ISO, 0·1 μg/kg) before and after propranolol (P, 0·25 mg/kg) on carotid blood pressure and on respiration. Time in min.

SUPERIOR CERVICAL GANGLION-NICTITATING MEMBRANE

THP in doses causing a pronounced fall in blood pressure (5 mg/kg i.v.) failed to impair the responses of the cat nictitating membrane to electrical stimulation of the preganglionic cervical sympathetic nerves.

SHORT-CIRCUIT CURRENT AND POTENTIAL IN THE ISOLATED FROG SKIN

THP and isoprenaline ($1 \cdot 5 \times 10^{-4}$M) induced a significant increase of the short-circuit current but the slight increase of the potential difference was not statistically significant. Papaverine ($2 \cdot 7 \times 10^{-4}$M) on the contrary elicits a statistically significant fall in both the short-circuit current and the potential difference (Table 1). The effects of both

TABLE 1. FROG SKIN POTENTIAL AND SHORT-CIRCUIT CURRENT RESPONSE TO TETRAHYDROPAPAVEROLINE, ISOPRENALINE AND PAPAVERINE

Number of skins	Treatment	Skin potential difference ΔmV	%	P	Short circuit current difference ΔμA	%	P	Time (min) Potential	Time (min) Current
	Tetrahydro-papaveroline								
10	none	0±6	—	—	0±17	—	—	60	60
5	$4 \cdot 2 \times 10^{-5}$M	+13±5	(30)	>0·20*	+ 70±20	(26)	<0·05*	18± 5	24± 5
5	$1 \cdot 5 \times 10^{-4}$M	+ 7±3	(12)	>0·40	+ 68± 6	(37)	<0·02	19±11	37±10
3	$3 \cdot 0 \times 10^{-4}$M	+10±3	(17)	>0·20	+107±22	(67)	<0·05	37± 9	53± 7
	Isoprenaline								
5	none	0±6	—	—	0±12	—	—	60	60
3	$1 \cdot 5 \times 10^{-4}$M	+4±1	(11)	>0·50	+66±15	(34)	<0·05	3±2	17±6
	Papaverine								
7	none	0±3	—	—	0±7	—	—	60	60
7	$2 \cdot 7 \times 10^{-4}$M	−24±4	(57)	<0·01	−77±20	(68)	<0·01	128±38	133±38

Skin of *Rana esculenta* was mounted in a lucite chamber and attached to a short circuiting apparatus equivalent to that described by Ussing & Zerahn (1951). Short circuit current and skin potential are expressed as differences as well as percent variations from pretreatment values. Times reported are those at which response reached its maximum. Control skin values were measured for 60 min. Means ± s.e. * P = significance of difference from untreated controls. The drugs were added to the Ringer solution bathing the two sides of the skin.

THP and isoprenaline were prevented by 10^{-3}M pronethalol and 10^{-4}M propranolol.

EFFECTS ON OXIDATIVE PHOSPHORYLATION

THP or isoprenaline failed to inhibit the uptake of oxygen or phosphate using either glutamate or succinate as substrate whereas papaverine 0·1 mM abolished both oxygen and phosphate uptake with glutamate as substrate, without affecting the oxidation of succinate (Santi, Ferrari & Contessa, 1964).

Discussion

The findings suggest that the effects of THP are of a β-sympathomimetic nature. This statement is supported mainly by the close similarities of the effects displayed by isoprenaline and THP and by the antagonism of these drugs by β-adrenergic blocking agents.

These conclusions agree with the previous findings of Holtz & others (1964) and are consistent with the results of the investigations on molecular interaction on lipolysis *in vitro*, clearly indicating an affinity of THP for the same receptor system as isoprenaline (Fassina & others, 1965). Quantitatively, THP appears to be from 1/10 to 1/50 as active as isoprenaline, depending on the method of assessment. However, on isolated frog skin THP was as active as isoprenaline in increasing the short-circuit current.

In spite of their structural similarity, remarkable pharmacological

differences are evident between THP and papaverine; the β-sympathomimetic mechanism is not involved in the hypotensive effects of papaverine, as demonstrated by the results obtained with propranolol and dichloroisoprenaline. Furthermore the difference in behaviour between papaverine and THP is evident in the experiments on oxidative phosphorylation, isolated frog skin and spasmolytic action.

It seems that both the hydrogenated isoquinoline ring and the hydroxy groups are essential for the sympathomimetic activity of THP, because the unsaturated compound papaveroline, 3,4-dihydropapaveroline and tetrahydropapaverine are devoid of any sympathomimetic activity (Tóth, Ferrari, Contessa & Santi, 1966). The study of these isoquinoline compounds on lipolysis *in vitro* leads to the same conclusion (Fassina & others, 1965).

In the extensive investigation of Hjort, deBeer & Fassett (1938, 1940) and Fassett & Hjort (1938), the close similarity of activity of the β-phenethylamines and their cyclized 1,2,3,4-tetrahydroisoquinoline derivatives is pointed out. In the light of these conclusions, THP, which has hydroxy-groups in positions 6 and 7, might be considered a cyclized derivative of epinine. This could account for the direct sympathomimetic activity of THP, even if it does not explain its selective β-sympathomimetic activity.

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Some pharmacological effects and chemical properties of *N*-propargylnoratropine

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N-Propargylnoratropine has been prepared from noratropine, and its structure proved by hydrogenation followed by alkaline hydrolysis. Some pharmacological effects of this compound have been compared with atropine. The central and peripheral cholinolytic activity is some 50% lower than that of atropine. There is a fourfold increase in the analgesic-disorientating action of atropine.

SUBSTITUTION of the *N*-methyl group of atropine by other alkyl groupings may lead to marked alterations in the pharmacological properties of the parent compound (György, Dóda & Nádor, 1965). *N*-Allyl-noratropine (N-728) (Nádor, György & Dóda, 1961; Decsi & Nádor, 1963) had a preferential inhibitory effect on cholinergic receptors in the central nervous system with only a very slight influence on those in peripheral structures (Soyka & Unna, 1961; Dal Ri & Schmidt, 1961). On continuing these experiments we thought it worthwhile to investigate the pharmacological properties of noratropine with an *N*-acetylenic substituent, namely propargyl ($CH{\equiv}C{-}CH_2$).

Experimental

The pharmacological methods have been described elsewhere (Decsi & Nádor, 1963; Decsi, Várszegi & Méhes, 1961, 1963) and a comparison of *N*-propargylnoratropine (N-1084) with atropine is made in Table 1.

TABLE 1. PHARMACOLOGICAL PROPERTIES OF *N*-PROPARGYLNORATROPINE

Compound	Cholinolytic activity		Anti-tremorine effect, ED50 mg/kg	Analgesic disorientating effect ED50 mg/kg	Acetyl-choline-depleting effect, ED50* mg/kg
	Central ED50 mg/kg	Peripheral Relative activity			
Atropine	0·92	1·0	1·51	2·06	11·2
N-Propargylnoratropine (N-1084)	1·80	0·64	4·10	0·55	14·1

* An intraperitoneal dose causing 50% decrease of the cerebral acetylcholine level in the mouse.

The LD50 of N-1084 in the mouse was 150 mg/kg when given by the intraperitoneal route and 60 mg/kg in the rat when administered intravenously. In addition to the effects demonstrated in the Table, the compound had a central excitatory action shown by slightly increased spontaneous motility of mice. On the isolated intestine, it showed a musculotropic spasmolytic action about half that of papaverine.

The potencies of the central and peripheral cholinolytic effects of N-1084 were about one half of those of atropine. On the other hand, there was a fourfold increase in the analgesic-disorientating action,

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probably a manifestation of the psychotomimetic action of anticholinergic drugs (Decsi, Várszegi & Nádor, 1966), but also the depletion of cerebral acetylcholine was less than that due to atropine.

The exchange of the *N*-methyl group of atropine for the triple bond-containing propargyl radical produces only slight alteration in the pharmacological properties of the parent compound. Of these changes, the increase in disorientating effect may deserve attention, in view of the psychotomimetic activity of some cholinolytic drugs.

Preparation of N*-propargylnoratropine.* A solution of propargyl bromide (9·3 g, 0·078 mole) in benzene (90 ml) was added dropwise over 30 min to a solution of noratropine (43 g = 0·156 mole) in ten volumes of benzene at 60°. After stirring for 1 hr at this temperature the solution was cooled and the benzene extracted with 5 × 50 ml of water. The benzene was extracted with 4 × 25 ml of dilute hydrochloric acid and *N*-propargylnoratropine base liberated by concentrated ammonia. The base was extracted by methylene chloride. Yield: 21 g (86%). *N*-Propargylnoratropine was obtained in colourless crystals, m.p. 116–117°, after two recrystallizations from light petroleum (b.p. 100–120°). (Found, C, 72·9; H, 7·5; N, 4·3%. $C_{19}H_{23}NO_3$ requires C, 72·9; H, 7·3; N, 4·5%.)

Thin-layer chromatography (silica gel with a 1 : 1 mixture of ethanol and hydrochloric acid as solvent and with Dragendorff reagent as developer) showed the compound to be homogeneous. The picrate salt prepared in the usual way melted at 168°. (Found: C, 55·5; H, 4·8; N, 10·5%. $C_{25}H_{26}N_4O_{10}$ requires C, 55·35; H, 4·8; N, 10·3%.)

Transformation into N*-n-propyl-noratropine.* *N*-Propargylnoratropine (1 g) in methanol (25 ml) was hydrogenated at atmospheric pressure in the presence of a small amount of 9·6% Pd-charcoal. Hydrogenation was complete in 80 min. After filtration, the methanol solution was evaporated. The residue (0·983 g) of *N*-n-propyl-noratropine was dissolved in a small amount of ethanol and transformed into the hydrochloride by dry HCl in ether. Recrystallization from ethanol-ether gave a product m.p. 168–169°. The mixed melting point with an authentic sample (Nádor & Gaál, 1962) was 168–169°.

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Inhibition of the dual amine uptake-concentration mechanisms of the adrenergic neurons by ϵ-aminocaproic acid

Sir,—Two different amine uptake-concentration mechanisms of the adrenergic neurons, namely, the amine transport through the nerve cell membrane, the "membrane pump", and subsequent incorporation in the storage granule complex have been demonstrated (Carlsson, Hillarp & Waldeck, 1963; Hillarp & Malmfors, 1964; Malmfors, 1965; Carlsson & Waldeck, 1965). The former mechanism can be selectively blocked with such agents as protriptyline and desipramine while the latter mechanism can be selectively blocked with such agents as reserpine and prenylamine (segontin) (Carlsson & Waldeck, 1965; Malmfors, 1965). Guanethidine inhibits both these mechanisms (Lindmar & Muscholl, 1964; Shore & Giachetti, 1966; Carlsson & Waldeck, 1966).

ϵ-Aminocaproic acid (EACA), a 6-carbon acyclic carboxylamine compound, has been shown to cause almost complete depletion of noradrenaline from the heart of rats (Lippmann & Wishnick, 1965; Andén, Henning & Obianwu, in preparation). This compound has many pharmacological properties similar to those of guanethidine (Andén, Henning & Obianwu, in preparation), though there are important differences. For example, unlike guanethidine, the adrenergic nerve blockade induced by EACA is accompanied by a measurable loss of tissue noradrenaline.

The ability of EACA to inhibit the dual amine uptake-concentration mechanisms of the adrenergic neurons is reported below. For comparison, substances whose actions on these mechanisms have been previously demonstrated are included in the studies.

As an indicator of amine uptake, ^{3}H-metaraminol was used. Metaraminol appears to utilize similar transport and storage mechanisms to noradrenaline (see Carlsson & Waldeck, 1966). Substances such as reserpine and prenylamine which impair the storage mechanism of the amine granules do not significantly affect the uptake of ^{3}H-metaraminol 30 min after its administration whereas substances such as desipramine and guanethidine greatly inhibit its uptake at this time. But both these groups of substances reduce the amount of ^{3}H-metaraminol retained after 3 hr. By estimating the degree of inhibition of ^{3}H-metaraminol 30 min and 3 hr after its administration it is possible to determine which of the two uptake-concentration mechanisms is inhibited by a drug.

Male Sprague-Dawley rats 200–250 g were used. The test substances were administered in the doses indicated in Fig. 1 by intraperitoneal injection and 60 min later, ^{3}H-metaraminol (10 μg/kg) was administered intravenously into the tail; the animals were killed 30 or 180 min after ^{3}H-metaraminol. ^{3}H-Metaraminol was administered 18 hr after in rats given reserpine. In another series of experiments, the jugular veins were cannulated under light ether anaesthesia and the rats were used 2–3 days later. ^{3}H-Metaraminol (10 μg/kg) was administered 15 min after desipramine (0·5 mg/kg) and the rats killed 30 or 180 min later. Both drugs were injected via the cannula. The controls from this series of experiments gave results similar to those from the former series. ^{3}H-Metaraminol in the hearts was estimated by the method previously described by Carlsson & Waldeck (1965). The results are presented in Fig. 1. The initial uptake of ^{3}H-metaraminol (30 min after i.v. injection) was not significantly affected by reserpine and prenylamine but was reduced to about 50% of the control levels by guanethidine, EACA and desipramine. This represents inhibition of the membrane pump mechanism. Three hr after ^{3}H-metaraminol all the substances except desipramine showed clearcut reduction of the amount of ^{3}H-metaraminol retained. This inhibition represents inhibition of the storage

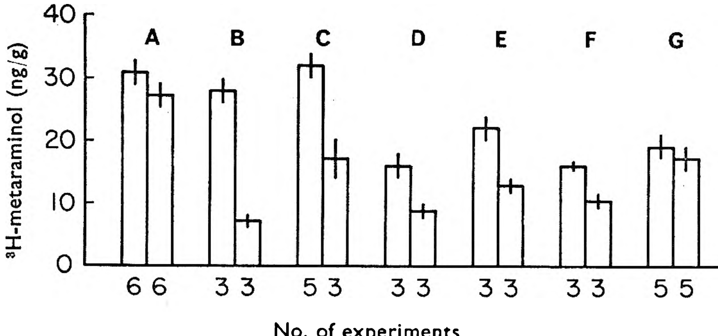

FIG. 1. Effect of various drugs on the uptake of ^{3}H-metaraminol by rat heart. The rats were given guanethidine, EACA or prenylamine 60 min, in the case of reserpine and desipramine 18 hr and 15 min respectively before i.v. administration of ^{3}H-metaraminol (10 μg/kg). The animals were killed 30 or 180 min after ^{3}H-metaraminol. The control rats received saline. The left column of each pair represents the level of ^{3}H-metaraminol 30 min and the right one 180 min after its administration. A, control. B, reserpine, 5 mg/kg. C, prenylamine, 30 mg/kg. D, guanethidine, 15 mg/kg. E, EACA, 500 mg/kg. F, EACA, 1000 mg/kg. G, desipramine, 0·5 mg/kg.

mechanism. The level of ^{3}H-metaraminol 180 min after its administration was not significantly different from that after 30 min in rats treated with desipramine. In fact, the difference (2·5 ng/g) was less than that of the control (3·7 ng/g). This indicates that desipramine, a potent inhibitor of the membrane pumps has no significant effect on the storage mechanism of the amine granules.

EACA in a dose of 500 mg/kg caused only a moderate inhibition of the membrane pump mechanism. This dose also caused only a moderate sympathetic blockade (Andén, Henning & Obianwu, in preparation). However, in a dose of 1000 mg/kg (LD50 = 7·0 g/kg) which had a more pronounced sympathetic blockade, the membrane pump mechanism was inhibited to the same extent as that caused by guanethidine. The present studies demonstrate that EACA inhibits the dual uptake-concentration mechanisms of the adrenergic neurons and provide further evidence in support of the view that reserpine and prenylamine inhibit the storage mechanism while guanethidine inhibits both the membrane pump and the storage mechanisms of the adrenergic neurons.

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Effects of inhibition and induction of the liver microsomal enzyme system on the narcotic activity of ethanol in mice

SIR,—It is generally accepted that ethanol is largely metabolized in the liver by alcohol dehydrogenase. However, Forney, Hughes, Hulpieu & Clark (1962) have reported that ethanol is metabolized more rapidly during the first 30 min after administration than during subsequent 30 min periods, and they have discussed the possibility that this could be because during this time ethanol is not metabolized by the dehydrogenase route alone, but also by the liver microsomal enzyme system. This has also been suggested from results of *in vitro* experiments (Orme-Johnson & Ziegler, 1965). We therefore considered the possibility that drugs known to affect this microsomal enzyme system, either by inhibiting or inducing activity, might thereby alter the pharmacological effectiveness of ethanol. Induction of ethanol-metabolizing activity of liver slices has recently been reported as a result of prior treatment with ethanol (Ryan & Cornish, 1966).

A series of experiments was made to determine the effect of various pretreatments on the narcotic activity of ethanol in mice as measured by the sleeping time. Groups of 10 Schneider female mice were used for each treatment and they were kept at 32° throughout the experiment, sleeping times being measured at this temperature. Ethanol was injected intraperitoneally as 20% v/v solution in water.

An inhibitor of liver microsomal enzymes, SKF 525A (β-diethylaminoethyl-diphenylpropyl acetate) (Brodie 1956), injected in a dose of 25 mg/kg intraperitoneally 45 min before the ethanol, resulted in a fourfold prolongation of sleeping time, a potentiation which was significant at the 95% probability level (Table 1). This was associated with a potency increase of 1·1 (1·083–1·139).

TABLE 1. THE PROLONGATION OF ETHANOL SLEEPING TIME IN MICE BY SKF 525A, 25 MG/KG I.P.

Treatment	Ethanol, g/kg i.p.	Sleeping time, min
SKF 525A + ethanol	4·5	11·3 ± 2·5
SKF 525A + ethanol	5·0	30·5 ± 3·6
Ethanol only	5·0	7·0 ± 1·1
Ethanol only	5·25	24·0 ± 3·9

TABLE 2. EFFECT OF OVERNIGHT PRETREATMENT WITH VARIOUS DRUGS ON THE SLEEPING TIME DUE TO ETHANOL IN MICE

Pretreatment			Sleeping time, min	
Drug	Dose mg/kg i.p.	Ethanol g/kg i.p.	Treated	Control
Chlorpromazine ..	2	5·5	18·7 ± 3·2	43·5 ± 6·1
Pentobarbitone ..	50	5·5	22·4 ± 4·7	43·5 ± 6·1
Amitriptyline ..	10	5·75	43·0 ± 5·9	55·4 ± 6·4
Amitriptyline ..	20	5·75	10·9 ± 3·3	44·7 ± 6·4
Imipramine ..	40	5·75	26·3 ± 4·5	54·6 ± 7·6

The great increase in sleeping time associated with only a 10% increase in potency reflects the steepness of the dose-response relation for ethanol in mice (Fig. 1). Thus, only a small alteration in enzyme activity is necessary to alter considerably the effectiveness of ethanol.

Numerous agents have been shown to induce liver microsomal enzyme activity, attenuating the effectiveness of other drugs metabolized by this system (Fouts, 1965). These inducing agents include barbiturates and chlorpromazine (Kato & Chiesara 1962). These drugs were given as a pretreatment 21 hr before ethanol in an attempt to affect the sleeping times by enzyme induction (Table 2).

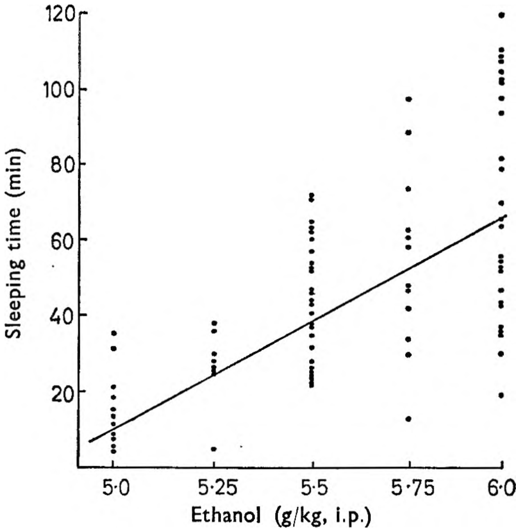

FIG. 1. The sleeping time, in min, of mice injected intraperitoneally with various doses (g/kg) of ethanol in 20% v/v solution in water. Slope of log dose-response relation, $b = 710$; $S_b = 78·1$; $P < 0·001$ ($n = 120$).

The sleeping time due to ethanol was much shortened by pretreatment with chlorpromazine, 2 mg/kg, and pentobarbitone, 50 mg/kg. Amitriptyline at 20 mg/kg also shortened ethanol sleeping times in mice although imipramine at 40 mg/kg did not cause a significant effect. Kato & Chiesara (1962) reported that imipramine (dose not stated) did not shorten pentobarbitone sleeping time in rats. We have found however, (unpublished) that both imipramine at 5 mg/kg, i.p. and amitriptyline at 20 mg/kg, shortened pentobarbitone sleeping times in mice.

These results support the suggestion that the non-specific oxidizing enzyme system of the liver microsomes contributes to inactivation of ethanol in mice and that agents known to inhibit or induce the activity of this system can cause significant potentiation or reduction in the narcotic effectiveness of ethanol.

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Histamine liberators and melanophores of *Rana tigrina*

SIR,—Adrenaline, noradrenaline and melatonin are known to turn frogs yellow by centripetal movement of melanin granules in the individual skin melanophores (Burgers & Van Oordt, 1962). We have now found that substances known to release histamine from mammalian tissues also turn conscious and anaesthetized frogs yellow.

Adult conscious *Rana tigrina* of either sex (weight 150–350 g) were observed in daylight in glass jars containing water. The frogs were treated with drugs dissolved in water and injected into the abdominal cavity.

Frogs which were anaesthetized were given sodium pentobarbitone (50 mg/kg) injected into the abdominal cavity. Drugs, dissolved in 0·6% saline, were injected through a polyethylene cannula tied into the left branch of the thoracic aorta. Histamine liberators which were comparatively less potent or painful on injection were administered only to anaesthetized frogs.

The skin colour was observed with the naked eye and the melanophores of the web skin of the hind limbs by microscope. Changes in distribution of melanin in melanophores were recorded in grades (Hogben & Slome, 1931).

Rana tigrina usually maintains a brown-black colour of the skin (melanophores of grade 5 or 4) during handling, injection or anaesthesia. Twenty-nine diverse substances (including digoxin, sodium cyanide, dibenzyline, ATP, oxytocin) which are not known to release histamine in the mammal did not affect the melanophores of the anaesthetized frog.

Histamine liberators turned conscious frogs deep yellow within 1 hr (Table 1). At this stage melanophores appeared under the microscope (× 60) as punctate spots (grade 1 or 2). Within 4–6 hr the frogs turned brown again. Throughout the experiment the animals appeared otherwise normal and healthy, and responded again if reinjected after 4 days' interval. In anaesthetized frogs, histamine liberators produced a similar change though in smaller doses (Table 1) and of shorter duration.

TABLE 1. DOSES OF HISTAMINE LIBERATORS WHICH PRODUCE MELANOPHORE CHANGES IN ANAESTHETIZED AND CONSCIOUS FROGS

Drug	Anaesthetized frogs, effective dose mg or ml/kg (No. of experiments)		Conscious frogs, effective dose mg or ml/kg (No. of experiments)	
Compound 48/80	0·01	(15)	2·0	(8)
Polymyxin B sulphate	0·05	(11)	2·5	(5)
Tubocurarine chloride	0·04	(10)	4·0	(6)
Propamidine isethionate	1·00	(13)	8·0	(2)
Polysorbate 80	0·003 ml	(22)	0·05 ml	(24)
Gallamine triethiodide	7·5	(7)	—	
Peptone	25·0	(8)	—	
50% v/v fresh egg-white solution	1·5 ml	(10)	—	
Horse serum	2·0 ml	(7)	—	
Russell's viper venom	1·00	(5)	—	
Trypsin	25·0	(7)	—	
Histamine acid phosphate	11·0	(9)	—	
Noradrenaline	0·5	(10)	—	
Adrenaline	2·0	(5)	—	

Also, histamine liberators were effective in decapitated frogs and when injected into subcutaneous lymph sacs of isolated limbs of anaesthetized frogs. This suggests a direct action on skin melanophores.

The doses of potent histamine liberators compare favourably with that of noradrenaline (Table 1). In 2 groups each of 10 anaesthetized frogs, dibenzyline (25 mg/kg) blocked the action of effective doses of noradrenaline and adrenaline (Table 1) on frog melanophores. In another group of 9 anaesthetized frogs dibenzyline failed to block the action of effective doses of histamine liberators

(tubocurarine, compound 48/80 and polysorbate 80) on melanophores. This suggests that the histamine liberators act on melanophores by a mechanism which is different to that of noradrenaline or adrenaline.

Histamine acted like the histamine liberators except that even with much higher doses (200 mg/kg as histamine acid phosphate) the degree of melanin concentration was less than that induced by histamine liberators. This dose of histamine was well-tolerated by the frogs which are known to be resistant to it (Rocha e Silva, 1955).

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β-Adrenergic auto-inhibition of the effect of noradrenaline on avian pulmonary artery

Sir,—We have previously reported that the predominantly β-adrenergic amine, isoprenaline, exerts both β-adrenergic vasodilator and α-adrenergic vasoconstrictor effects on isolated vascular smooth muscle (Somlyo & Somlyo, 1964, 1966a). In contrast, noradrenaline is a predominantly α-adrenergic vasoconstrictor amine, and its β-adrenergic vasodilator effects have previously been demonstrated only after α-adrenergic blockade, *in vivo* (Brick, Hutchison & Roddie, 1966). In the absence of α-adrenergic blockade, noradrenaline is one of the most potent vasoconstrictors of large and medium vessels (Somlyo, Sandberg & Somlyo, 1965a; Somlyo & Somlyo, 1966b) when potency is judged by maximum isotonic response. We now find that, in certain types of vascular smooth muscle, noradrenaline, in the absence of α-adrenergic blocking agents, can exert sufficient β-adrenergic vasodilator activity to produce auto-inhibition of the α-adrenergic vasoconstrictor effect.

Right and left main branches of the pulmonary artery were obtained from rapidly exsanguinated chickens. The preparation of helically-cut vascular strips and recording methods employed in our laboratory have been reported in detail (Somlyo & Somlyo, 1964; Somlyo, Sandberg & Somlyo, 1965a,b; Somlyo, Woo & Somlyo, 1965; Woo & Somlyo, 1966). The temperature for the present experiments was maintained at $41{\cdot}5 \pm 0{\cdot}5°$. Loading tensions applied were 2 g for pulmonary and 3 g for sciatic artery strips.

Fig. 1 shows the effect of the β-adrenergic blocking agent, pronethalol, on cumulative dose-response curves of pulmonary (1A) and sciatic (1B) artery strips to noradrenaline. Auto-inhibition of α- by β-adrenergic effect in pulmonary vascular smooth muscle is indicated by the maximum contractile effect, which is increased by β-adrenergic blockade, being depressed. Similar results were obtained in another group of 5 pulmonary arteries, suspended in Mg-free Krebs solution. The maximum isotonic response of the two pooled groups

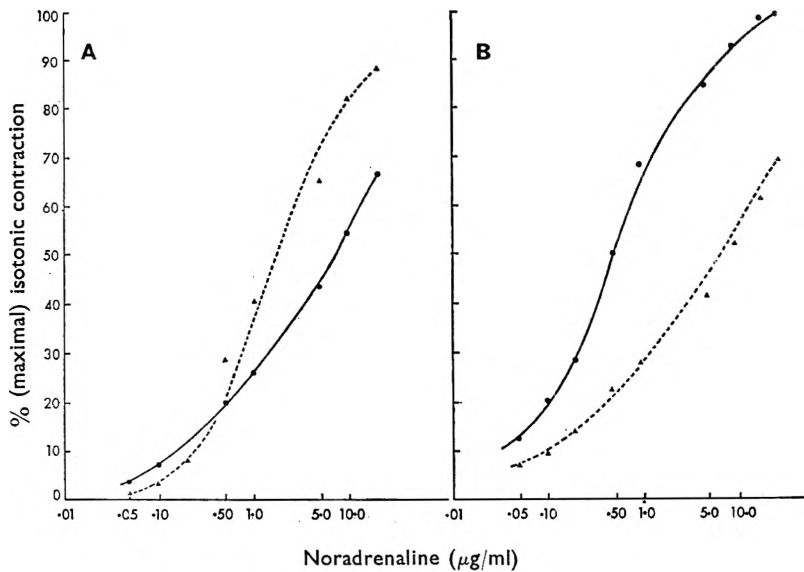

FIG. 1. Cumulative dose-response curves of chicken pulmonary (A) and sciatic (B) artery strips to noradrenaline: effect of pronethalol. Circles represent control responses, triangles the responses in the presence of pronethalol 10 μg/ml. Each curve represents the mean of five experiments.

(10 strips) to noradrenaline was 36·6% ±10·2 greater in the presence of pronethalol 10 μg/ml (P <0·01). The dose-response curves of sciatic artery strips (1B) exhibited no auto-inhibition with the same concentrations of noradrenaline. In these preparations a non-specific, depressant effect of pronethalol significantly (P <0·01) depressed the contractile response to noradrenaline. A depressant action of pronethalol on intestinal smooth muscle has also been demonstrated (Woo & Somlyo, 1966). This non-specific effect of pronethalol was presumably responsible for the diminished responses of pulmonary artery strips to low (<0·5 μg/ml) concentrations of noradrenaline and the absence of a pure parallel shift in Fig. 1A.

Individual (rather than cumulative) dose-response curves obtained with pulmonary arterial strips showed the descending limb configuration characteristic of auto-inhibition (Bijlsma, Werff & Julius-Bijlsma, 1961; Ariens & Simonis, 1964).

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Antihypertensive effect of methyldopa in metacorticoid immunosympathectomized rats

SIR,—It is now generally accepted that methyldopa (α-methyldihydroxyphenylalanine) is an effective antihypertensive agent. Day & Rand (1963) proposed that methyldopa lowered the blood pressure by acting as a weak false sympathetic neurotransmitter. This hypothesis is inconsistent with the observation that administration of methyldopa did not inhibit the effect of sympathetic nerve stimulation (Stone, Ross, Wenger, Ludden, Blessing, Totaro & Porter, 1962; Varma & Benfey, 1963) and did not reduce the release of noradrenaline after stimulation of sympathetic nerves (Davies, 1966). Indeed, Nickerson (1965) pointed out that "the role of catecholamine depletion or, indeed, of any action on catecholamine metabolism in the antihypertensive effect of methyldopa, requires re-evaluation".

Since almost complete destruction of the peripheral sympathetic system can be produced in mammals by immunosympathectomy (Levi-Montalcini & Booker, 1960; Levi-Montalcini & Angeletti, 1962), it became possible to test whether the antihypertensive action of methyldopa is due to a reduction in peripheral sympathetic activity and whether a fully active sympathetic system is essential for experimental hypertension.

Immunosympathectomy was produced by subcutaneous injection of 0·2 ml of 61,000 anti-units/ml of bovine anti-serum to nerve-growth factor (kindly supplied by Dr. R. K. Richards, Abbott Laboratories, Chicago) in 1–2 days-old Sprague-Dawley rats. The effectiveness of this treatment producing immunosympathectomy has been described by Iversen, Glowinski & Axelrod (1966). The treated and untreated litter mate controls were raised together. Noradrenaline (equivalent) was assayed biologically on isolated rabbit aortic strip (Helmer, 1961). Treated rats exhibited marked ptosis of the eye lids. The daily urinary excretion of catecholamine (as noradrenaline equivalent) was 2·1 $\pm$0·5 μg/kg in treated rats and 5·6 $\pm$1·2 μg/kg in normal rats. Myocardial noradrenaline in 3 treated rats was 0·22 $\pm$0·22 μg/g and in 3 normal controls was 1·26 $\pm$0·23 μg/g. The responses of the isolated atria of untreated rats (6 preparations) to tyramine were negligible. Approximately 2 months after birth, the rats were used for inducing metacorticoid hypertension. Rats were anaesthetized with an intraperitoneal injection of pentobarbitone sodium (30 mg/kg), one kidney was removed and a 20 mg desoxycorticosterone acetate pellet contained in 50 mg beeswax was implanted under the skin. Animals were maintained on 1% sodium chloride instead of water. The systolic blood pressure in the unanaesthetized rat was determined by the tail cuff method by means of an Electrosphygmograph (E & M Instruments). Methyldopa (200

mg/kg, once daily) was injected intraperitoneally into the hypertensive rats and the effect of this treatment on the blood pressure was measured (Table 1).

The control blood pressures of the immunosympathectomized rats and their litter mate controls were not different from each other. The incidence of hypertension in the immunosympathectomized rats was higher and the severity of hypertension in the 2 groups was identical. Methyldopa lowered the blood pressure in all metacorticoid hypertensive rats. The antihypertensive effect of methyldopa in both groups was also similar. After stopping the administration of methyldopa, the recovery of the blood pressure to pretreatment level was faster in control rats than in immunosympathectomized rats. The sedative effect of methyldopa was apparent in both groups of animals.

TABLE 1. EFFECT OF METHYLDOPA ON THE SYSTOLIC PRESSURE OF NORMAL AND IMMUNOSYMPATHECTOMIZED METACORTICOID HYPERTENSIVE RATS

	Immunosympathectomized	Normal control
No. of rats	12	12
Mean control systolic pressure, mm Hg	121 ± 7·2	123 ± 5·3
No. of hypertensive rats	7	3
Mean systolic pressure, mm Hg	194 ± 5·4	182 ± 1·5
Mean systolic pressure after treatment with methyldopa*, mm Hg	128 ± 5·3	142 ± 7·0
Mean systolic pressure 2 weeks after stopping methyldopa	175 ± 4·8	200 ± 5·7

* Only 6 rats were treated with methyldopa (200 mg/kg/day i.p. for 7 days).

These results suggest that experimental hypertension can be produced after almost complete destruction of the peripheral sympathetic system which follows immunosympathectomy. Since methyldopa lowered the blood pressure in metacorticoid hypertensive rats and since its antihypertensive action was not reduced by the absence of an active peripheral sympathetic system, it is suggested that the antihypertensive action of methyldopa, at least in part, is unrelated to the peripheral sympathetic system. The possibility that the residual sympathetic system in the immunosympathectomized rats (Levi-Montalcini & Angeletti, 1962; Iversen & others, 1966) may account for the observed effects of methyldopa is unlikely but cannot be excluded.

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Effect of desipramine and reserpine on the *in vivo* β-hydroxylation of α-methyl-*m*-tyramine and α-methyldopamine

SIR,—It has been shown that desipramine and related compounds block the amine-uptake mechanism of the adrenergic neuron at the level of the cell membrane ("the membrane pump"). On the other hand reserpine blocks the amine storage mechanism of the amine granules (Hillarp & Malmfors, 1964; Malmfors, 1965; Carlsson & Waldeck, 1965). The enzyme dopamine-β-hydroxylase, which converts dopamine to noradrenaline, appears to be located within the adrenergic neuron (Carlsson & Waldeck, 1963; Fischer, Musacchio, Kopin & Axelrod, 1964) and evidence has been presented that this enzyme is attached to the amine-storing granules (Kirschner, 1962; Potter & Axelrod, 1963).

On the basis of *in vitro* evidence it has been proposed that reserpine inhibits the β-hydroxylation of dopamine (Kirschner, 1962; Weiner & Rutledge, 1966; Rutledge, 1966; Stjärne, 1966). The investigation of this problem *in vivo* is complicated by the fact that any noradrenaline formed in the adrenergic nerve fibres of reserpine-treated animals will probably be deaminated rapidly by intraneuronal monoamine oxidase and the deaminated metabolites promptly escape into the general circulation. We have therefore examined the effect of reserpine on the β-hydroxylation of dopamine analogues containing a methyl group in the α-position which are thus resistant to monoamine oxidase. In this investigation we have also included desipramine. This drug might be expected to inhibit β-hydroxylation of the administered amines by preventing their entry into the adrenergic nerve fibres.

We used tritium-labelled α-methyldopamine and α-methyl-*m*-tyramine. These compounds are good substrates for dopamine β-hydroxylase (Creveling, 1963), and their β-hydroxylation *in vivo* has been demonstrated (Carlsson & Lindqvist, 1962).

In one experimental series, ^{3}H-α-methyldopamine or ^{3}H-α-methyl-*m*-tyramine, synthesized by Hallhagen & Waldeck (to be published) according to the principles of Birkhofer & Hempel (1963), were injected intravenously into mice, some of which had received pretreatment with desipramine (Table 1). The animals were killed 30 min after the injection of the labelled compounds and the ^{3}H-α-methyldopamine and ^{3}H-α-methylnoradrenaline or ^{3}H-α-methyltyramine and ^{3}H-metaraminol in the hearts were isolated by ion-exchange chromatography and measured by liquid scintillation counting. In the hearts of animals given ^{3}H-α-methyltyramine alone the amount of the corresponding β-hydroxylated compound, ^{3}H-metaraminol, was about 20% of the precursor at the time interval

TABLE 1. EFFECT OF DESIPRAMINE ON THE β-HYDROXYLATION OF ^{3}H-α-METHYL-*m*-TYRAMINE AND ^{3}H-α-METHYLDOPAMINE IN THE MOUSE HEART. Desipramine 10 mg/kg was given i.v. 5 min before the i.v. injection of ^{3}H-α-methyltyramine (20 μg/kg) or ^{3}H-α-methyldopamine (40 μg/kg). The animals were killed 30 min after. Controls received labelled compounds only. Each value represents the level in 6 pooled hearts.

	^{3}H-α-methyltyramine injected		^{3}H-α-methyldopamine injected	
Treatment	^{3}H-α-methyl-tyramine ng/g	^{3}H-metaraminol ng/g	^{3}H-α-methyl-dopamine ng/g	^{3}H-α-methylnor-adrenaline ng/g
Control	12·6	2·4	63·0	25·6
	11·9	1·9	36·7	31·2
Desipramine	1·7	0·6	3·2	4·7
	2·0	0·0	1·2	0·7

TABLE 2. EFFECT OF RESERPINE ON THE β-HYDROXYLATION OF ^{3}H-α-METHYL-*m*-TYRAMINE IN THE MOUSE HEART. Reserpine (10 mg/kg) was given i.p. 6 hr before the i.v. injection of ^{3}H-α-methyltyramine (20 μg/kg). The animals were killed 15, 30 and 60 min after. Controls received ^{3}H-α-methyltyramine only. Each value represents the level in 6 pooled hearts.

Time after ^{3}H-α-methyl-tyramine (min)	Control		Reserpine	
	^{3}H-α-methyl-tyramine ng/g	^{3}H-metaraminol ng/g	^{3}H-α-methyl-tyramine ng/g	^{3}H-metaraminol ng/g
15	21·0	2·7	12·4	1·8
	16·8	1·2	12·5	2·1
30	12·3	2·9	6·9	2·0
	—	—	6·2	2·0
60	11·8	4·2	4·2	2·0
	10·0	3·9	4·3	1·8

studied. When ^{3}H-α-methyldopamine was given alone the amount of ^{3}H-α-methylnoradrenaline was about 50% of the precursor. Desipramine given before either labelled compound much diminished the amount of the precursor in the heart as well as that of the product.

In another experimental series, mice pretreated with reserpine were given ^{3}H-α-methyltyramine (Table 2). The animals were killed at different time intervals thereafter. Fifteen min after the injection of ^{3}H-α-methyltyramine its concentration in the hearts of reserpine-treated animals was about 30% lower than in the control hearts. The amount of ^{3}H-metaraminol formed appeared to be about the same in the two groups. From 15 to 60 min after the injection, ^{3}H-α-methyltyramine in the reserpine-treated animals disappeared more rapidly than in the controls. During the same time ^{3}H-metaraminol remained approximately constant in the reserpine-treated animals whereas there was a twofold increase in the controls.

Normally the α-methylated compounds used in the present investigation will be transported into the neuron by the "membrane pump" where the β-hydroxylation probably takes place in or at the amine storage granules. Blockade of the "membrane pump" by desipramine prevented the entrance of ^{3}H-α-methyltyramine and ^{3}H-α-methyldopamine to the β-hydroxylase. Hence little or no ^{3}H-metaraminol or ^{3}H-α-methylnoradrenaline could be detected. In the reserpine-treated animals ^{3}H-α-methyltyramine was still taken up by the "membrane pump" but disappeared more rapidly than in the controls because the granular storage mechanism was blocked. As long as ^{3}H-α-methyltyramine was present in sufficient amounts the β-hydroxylation continued and ^{3}H-metaraminol accumulated. Similar results were obtained with ^{3}H-α-methyldopamine (unpublished data). Inhibition of the β-hydroxylase by a specific inhibitor blocked the formation of metaraminol and α-methylnoradrenaline while there was a simultaneous increase of α-methyltyramine and α-methyldopamine, respectively (unpublished data).

Thus it would appear that β-hydroxylation of α-methyl-*m*-tyramine and α-methyldopamine takes place despite the blockade of the storage mechanism by reserpine. In other words, these α-methylated amines reach the dopamine β-hydroxylase without being incorporated in the reserpine-sensitive storage complex. Evidently more work is needed to clarify the possible relationship between amine storage and β-hydroxylation.

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Similar pharmacological properties of ergometrine and methysergide

SIR,—Ergometrine (*N*-[1-(hydroxymethyl)ethyl]-D-lysergamide) and methysergide (*N*-[1-(hydroxymethyl)propyl]-1-methyl-D-lysergamide) are closely related chemically, and we find that they share anti-5-hydroxytryptamine (5-HT) and oxytocic activities.

With the uterus of the rat in di-oestrus, suspended in de Jalon solution at 30°, approximately equal contractions were obtained with 1·0 μg ergometrine and 3·5 μg methysergide. A similar potency ratio was observed using the ileum of the guinea-pig but the doses required to produce contractions were ergometrine 10–50 μg and methysergide 20–200 μg.

In experiments made on three preparations from rats in oestrus, the amounts of the two substances which inhibited 5-HT contractions of the isolated uterus were, methysergide 0·15, 0·2, and 0·3 μg, and ergometrine 0·5, 0·5, and 0·7 μg. The mean potency ratio from these three experiments was methysergide : ergometrine = 2·7 : 1.

These experiments on the uterus of the rat show that ergometrine possesses appreciable anti-5-HT activity, being only 2·7 times less potent than methysergide, while methysergide itself is only 3·5 times less potent than ergometrine in oxytocic activity.

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The action of commercial preparations of oxytocin and vasopressin on the smooth muscle of the gut

SIR,—Reports of the actions of oxytocin and vasopressin on the smooth muscle of the gut are variable, but a fair conspectus of the published work is that vasopressin often causes contraction and oxytocin has no action. Yet in a recent publication, Levy (1963) presented evidence that both these hormones actually inhibit isolated preparations of rabbit and guinea-pig gut. Commercial preparations were used by Levy and these contained chlorbutol (0·5%) as a preservative. Since we believed that this preservative might produce these unexpected results we compared the activities of Syntocinon (Sandoz) and Pitressin (Parke Davis) with lysine vasopressin, prepared from porcine neurohypophyses (U.S. National Institutes of Health Endocrinology Study Section), and a preparation of synthetic oxytocin (kindly supplied by Professor J. Rudinger) on rabbit and guinea-pig ileum. Only the first two of these preparations contained the preservative. The effects of chlorbutol in a concentration equivalent to that in Syntocinon and Pitressin were also studied.

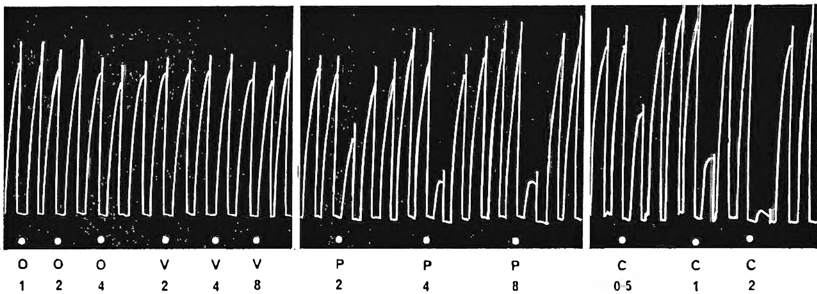

FIG. 1. The effect of Pitressin (P) compared with preservative-free lysine vasopressin (V), preservative-free synthetic oxytocin (O), and chlorbutol (C) on contractions of guinea-pig ileum after acetylcholine (2×10^{-7}). Four units of Pitressin are equivalent to 1 mg of chlorbutol. Neither pure vasopressin nor pure oxytocin inhibit the contractions caused by acetylcholine. Inhibition of the contractions seen after addition of Pitressin is due to chlorbutol and not to vasopressin. Doses of the hormones are in units and of chlorbutol in mg.

Only the commercial preparations of the hormones manifested any inhibitory effects, and these effects could be mimicked by chlorbutol. The effect for Pitressin on guinea-pig ileum is illustrated in Fig. 1. Our results suggest that any inhibitory effects shown by these commercial preparations are probably due, at least in high doses, to chlorbutol and not to a pharmacological action of the hormones.

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Reference

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Drug retention by the isolated diaphragm preparation

SIR,—Experiments have been made using the isolated rat phrenic nerve diaphragm preparation (Bulbring, 1946) to investigate protection by a reversible carbamate anticholinesterase from the effects of the irreversible organophosphate compound, paraoxon. The effects of paraoxon alone on the rat diaphragm preparation have been described by Barnes & Duff (1953).

Hemi-diaphragms from male albino rats of 300–400 g were prepared and the phrenic nerve supported by a Perspex assembly designed for use with the nerve electrodes immersed in the bath fluid. This support was constructed of sections of Perspex cemented together with wires to the electrodes lying in the cavities between the sections. Krebs perfusion fluid at 37° was used in all experiments. The organ bath volume was 100 ml. A twitch response to 50/sec stimulation followed by failure to hold tetanus was used as an indication of a severe degree of cholinesterase inhibition.

In these experiments it was found that 2×10^{-6}M of paraoxon alone produced a complete failure to hold a 50/sec tetanus within 30 min. After three washings at 5 min intervals no recovery was observed during the ensuing 2–3 hr. But if the diaphragm was exposed to 2×10^{-6}M of 3-isopropyl-*N*-methyl carbamate for 30 min, then washed several times, the anticholinesterase effects of 2×10^{-6}M paraoxon given subsequently could be reversed by washing.

However, this recovery from paraoxon poisoning was followed by a gradual secondary failure over 2 hr. The secondary failure was thought to be due to retention of paraoxon by the apparatus or diaphragm despite washing.

Experiments were made to identify where retention occurred.

Experiment (*i*). A hemi-diaphragm was set up in the bath containing 2×10^{-6}M paraoxon, while the other hemi-diaphragm from the same rat was kept in oxygenated Krebs at 37°. After 30 min the bath was washed three times. The hemi-diaphragm showed complete failure to hold a 50/sec tetanus $2\frac{1}{2}$ hr after washing. It was replaced by the other hemi-diaphragm which, in the same bath fluid, showed complete failure to hold a 50/sec tetanus $1\frac{1}{2}$ hr later.

Experiment (*ii*). The bath fluid contained 2×10^{-6}M paraoxon and the diaphragm-nerve holder was immersed in it but no diaphragm was set up. After 30 min the bath was washed three times and a hemi-diaphragm set up in it. This preparation showed a partial failure to hold a 50/sec tetanus after 2 hr. The results of this experiment showed that paraoxon was being retained by the apparatus despite washing.

Experiment (*iii*). In this the diaphragm-nerve holder was not immersed in the bath fluid containing the paraoxon. After three washes of the bath a hemi-diaphragm was set up in the bath fluid and this did not show any signs of failure after 2 hr. Thus the diaphragm-nerve support was shown to be the most likely place retaining the paraoxon.

Experiments (ii) and (iii) were repeated and the bath fluid assayed for paraoxon by a sensitive colorimetric method (Aldridge, 1964) $2\frac{1}{2}$ and 6 hr after setting up the hemi-diaphragm. In addition, another experiment (iv) was done in the same way as (ii) except that a dummy diaphragm-nerve holder, without electrodes, made of solid Perspex was used. Experiment (v) was done as experiment (iii) except that no hemi-diaphragm was set up after washing.

From a comparison of the results (Table 1) of experiments (v) and (iii) it appears that the diaphragm itself retains some paraoxon during washing but this amount is small compared with that retained by the original Perspex holder. The lower concentrations of paraoxon at 6 hr were thought to be due to degradation of paraoxon by the nerve.

TABLE 1.

Experiment	Concentration of paraoxon in the bath fluid	
	2½ hr after setting up the diaphragm	6 hr after setting up the diaphragm
(ii) Diaphragm-nerve holder immersed in the bath fluid containing 2×10^{-6}M paraoxon	1.75×10^{-8}M	not measured
(iii) As above but holder not immersed in fluid	4×10^{-9}M	2×10^{-9}M
(iv) Dummy holder made of Perspex in one piece immersed in bath fluid containing 2×10^{-6}M paraoxon	2.5×10^{-9}M	1×10^{-9}M
(v) As expt (iii) but no diaphragm set up after washing ..	5×10^{-9}M	4.3×10^{-9}M

It is therefore suggested that the diaphragm-nerve holder be constructed of solid Perspex or glass, so that drugs cannot diffuse into the cavities containing the electrode connections.

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Disulfiram and the effect of catecholamines on neuroleptic-induced catalepsy in mice and rats

Sir,—We have found that chlorpromazine- or haloperidol-induced catalepsy in mice and rats could be reversed by dopa or monoamine oxidase- and catechol-*O*-methyltransferase inhibitors or both (Maj & Zebrowska, 1966a,b). We therefore wished to know whether noradrenaline or dopamine was involved in this anticataleptic action. For this purpose we used disulfiram which inhibits the β-hydroxylation of dopamine to noradrenaline in various tissues (Goldstein, Anagnoste, Lauber & McKereghan, 1964; Musacchio, Goldstein, Anagnoste, Poch & Kopin, 1966).

Catalepsy was examined in white mice according to Zetler & Moog (1958) and in Wistar rats according to Courvoisier, Ducrot & Julou (1957). In mice, reserpine was given intraperitoneally 3·5 hr, chlorpromazine and haloperidol subcutaneously 1·5 hr, disulfiram intraperitoneally 2 hr and DL-dopa, intraperitoneally 0·5 hr before the experiment. Rats were given reserpine intraperitoneally 3·5 hr, chlorpromazine and haloperidol subcutaneously 1 hr and disulfiram intraperitoneally 2 hr and DL-dopa intraperitoneally immediately before the test. Nialamide was injected in both species 18 hr before the experiment. Observations were made at 5 min intervals for 1 hr (13 observations) in mice and at 10 min intervals (7 observations) in rats. The number of cataleptic animals and the number of cataleptic responses were recorded. The animal was considered to be cataleptic after 7 or more positive responses in mice, or 4 or more in rats.

Dopa and nialamide were not antagonistic towards reserpine-chlorpromazine or haloperidol-induced catalepsy in mice pretreated with disulfiram (Table 1). In experiments with chlorpromazine catalepsy was not seen after disulfiram, since in 5 mice the righting reflex had been abolished.

In rats (Table 2) the administration of dopa and nialamide counteracted the cataleptic action of reserpine and haloperidol. Only in animals receiving

TABLE 1. EFFECT OF DOPA, NIALAMIDE AND DISULFIRAM ON THE NEUROLEPTIC-INDUCED CATALEPSY IN GROUPS OF 10 MICE

Group No.	Compound and dose (mg/kg)				Number of cataleptic mice	Number of cataleptic responses	
	Neuro-leptic	Dopa	Nial-amide	Disul-firam		Mean	P
I	Reserpine	—	—	—	8	9·0 (±1·2)	—
II	1	200	10	—	1	1·7 (±0·9)	<0·001 (I : II)
III		200	10	400	7	8·4 (±1·0)	<0·001 (II : III)
IV	Chlor-	—	—	—	10	11·3 (±0·4)	—
V	prom-	200	10	—	2	3·5 (±1·2)	<0·001 (IV : V)
VI	azine 3	200	10	400	*	— —	—
VII	Haloperi-	—	—	—	9	10·3 (±0·9)	—
VIII	dol 1	200	10	—	2	3·9 (±1·1)	<0·001 (VII : VIII)
IX		200	10	400	8	9·1 (±1·3)	<0·01 (VIII : IX)

* 4 mice were cataleptic, in 5 mice was the righting reflex abolished.

TABLE 2. EFFECT OF DOPA, NIALAMIDE AND DISULFIRAM ON THE NEUROLEPTIC-INDUCED CATALEPSY IN GROUPS OF 8 RATS

Group No.	Compound and dose (mg/kg)				Number of cataleptic rats	Number of cataleptic responses	
	Neuro-leptic	Dopa	Nial-amide	Disul-firam		Mean	P
I	Reser-	—	—	—	8	5·8 (±0·4)	—
II	pine 7·5	200	20	—	1	1·8 (±0·6)	<0·001 (I : II)
III		200	20	400	6	4·6 (±0·8)	<0·02 (II : III)
IV	Chlor-	—	—	—	6	4·4 (±0·7)	—
V	prom-	200	10	—	3	3·5 (±0·7)	>0·4 (IV : V)
VI	azine 10	200	10	400	7	6·3 (±0·8)	<0·02 (V : VI)
VII	Haloperi-	—	—	—	7	5·9 (±0·5)	—
VIII	dol 3	200	10	—	3	3·5 (±0·9)	<0·05 (VII:VIII)
IX		200	10	400	8	6·9 (±0·1)	<0·01 (VIII : IX)

chlorpromazine was the difference not statistically significant. We did not observe this anticataleptic effect in rats treated with disulfiram. In the control mice and rats, when injections of disulfiram alone or with dopa or with dopa and nialamide, but without neuroleptics, catalepsy was not seen.

Disulfiram has been found to decrease the noradrenaline level and to increase the dopamine level in the brains of mice injected with dopa (Hashimoto, Ohi & Imaizumi, 1965), as well as in the brains (or some structures of them), of rats treated with catecholamine releasers and dopa (Goldstein & Nakajima, 1966). Similar changes in the catecholamine content were obtained in normal rats after diethyldithiocarbamate, the active metabolite of disulfiram (Carlsson, Lindquist, Fuxe & Hökfelt, 1966). Therefore our results seem to indicate that the anticataleptic effect of dopa and nialamide could be ascribed to an increase in the brain noradrenaline level but not to an increase in the dopamine level, as may be assumed, at least in reserpinized animals, on the basis of facts known from literature.

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The effect of purgative drugs on the intestinal absorption of glucose

SIR,—We have examined the effects of several purgatives on the *in vivo* absorption of glucose by the small intestine of the rat. In control experiments 20 ml of 0·9% saline containing 0·1% D-glucose and 5·0% ethanol was perfused through the lumen of the proximal 60 cm of the small intestine of an anaesthetized rat for 20 min. At the end of the experiment the rat was killed, the perfusate collected, its volume measured and the glucose concentration determined. The drugs were dissolved in the perfusion fluid, with ethanol as solvent.

The results (Table 1) show that inhibition of glucose absorption occurred with low concentration of all the purgatives except the anthraquinone derivatives. Oxyphenisatin produced the greatest inhibition and was more active than phloridzin which was included as a reference drug. Dioctyl was included because of the known inhibitory activity of other surface-active agents on the absorption of nutrients (Nissim, 1960).

TABLE 1. EFFECT OF PURGATIVES ON THE ABSORPTION OF GLUCOSE

Compound				Absorption %	
Chemical name	Name	Conc.	No. of rats	Mean ± s.e.	P value
Controls			10	87·28 ± 1·76	
	Sennoside "A"	10^{-4}	4	87·37 ± 1·43	<0·98
1,3,8-Trihydroxy-6-methylanthraquinone	Emodin	10^{-4}	4	83·72 ± 4·21	<0·4
1,8-Dihydroxyanthraquinone	Danthron	10^{-4}	4	90·92 ± 1·19	<0·3
2,3-Indolinedione	Isatin	10^{-4}	4	76·70 ± 1·41	<0·005
Dioxyphenylisatin	Oxyphenisatin	10^{-4}	6	20·50 ± 0·80	<0·001
Di-(4-acetoxyphenyl)-2-pyridylmethane	Bisacodyl	10^{-4}	4	49·38 ± 2·44	<0·001
2,2-Di(*p*-hydroxyphenyl)phthalide	Phenolphthalein	10^{-4}	4	61·45 ± 1·45	<0·001
Phloretin-2′-β-glucoside	Phloridzin	10^{-4}	4	40·24 ± 3·53	<0·001
Di-(2-ethylhexyl) sodium sulphosuccinate	Dioctyl sodium sulphosuccinate	$2 \cdot 10^{-8}$	4	62·42 ± 1·36	<0·001

The inhibition observed with phenolphthalein confirms the results of Hand, Sanford & Smyth (1966) who demonstrated inhibition of glucose transport in an *in vitro* preparation of rat small intestine. Bisacodyl has been reported to be without action in the small intestine (Macgregor, 1960) but the significant inhibition of glucose absorption obtained in the present study confirms the report by Forth, Baldauf & Rummel (1963) that this drug is capable of blocking nutrient absorption.

The observation that certain purgatives are capable of blocking glucose absorption in the small intestine raises two questions. Firstly, how do these drugs affect glucose absorption and will their study be of value in the elucidation of the transport mechanism? Secondly, of what significance is this activity in the normal purgative action of these drugs?

One experiment has been made which demonstrates that the mode of action of oxyphenisatin is not the same as that of phloridzin. When oxyphenisatin, at a concentration of 10^{-4}, was perfused in glucose-free 0·9% saline for 1 hr through the lumen of rat small intestine, glucose appeared in the perfusate. The mean concentration in two experiments was 13 mg % whereas with saline alone, or with saline containing phloridzin, 10^{-4}, no glucose was detected. The demonstration of glucose reversal with oxyphenisatin indicates that this drug is blocking the active transport stage of glucose absorption and not merely the entry of glucose into the mucosal cell, as occurs with phloridzin (Newey, Parsons & Smyth, 1959).

Bisacodyl and oxyphenisatin, the two most active drugs in the present study, are known to be capable of producing faecal evacuation within 1 hr when given as suppositories (Krebs, 1958; Sullivan, Dickinson & Wilson, 1963). This suggests that the purgatives have a direct action on the nerves or muscles of the rectum. However, these drugs may also act by inhibiting the absorption of nutrients from the lumen. The inhibition of glucose absorption, and associated retention of water, would increase the bulk within the lumen of the intestine and lead to increased peristalsis and more rapid passage of the contents.

The inactivity of the anthraquinone derivatives might be anticipated from the results of Straub & Triendl (1937) which indicated that these compounds are absorbed from the small intestine and secreted in the large intestine where they then stimulate peristalsis. The effect of these compounds on water absorption in the large intestine is under investigation.

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Book Review

STANDARD METHODS OF CHEMICAL ANALYSIS. INSTRUMENTAL ANALYSIS. 6th edition. Edited by Frank J. Welcher. Volume IIIA, pp. xviii + 1–974; volume IIIB, pp. xi + 975–2018 (including index). D. Van Nostrand Co. Ltd., London, 1966. Single volume, £18 18s.; if part of complete set, £16 16s.

Volumes IIIA and IIIB of Standard Methods of Chemical Analysis are concerned entirely with instrumental methods of chemical analysis: the volumes are under the general editorship of Professor F. J. Welcher and have 84 American authors as contributors.

The first of these books is devoted to instrumental techniques *per se* and these vary from a selection of spectroscopic methods through various electrochemical procedures to such aspects as gas chromatography, thin-layer chromatography and sedimentation analysis. The second volume sees the application of such techniques to various disciplines and the sixteen sections covered range from instrumental methods in clinical medicine to the determination of water, dealing en route with such diverse topics as soaps and synthetic detergents, semi-conductors, paper, wood and pulp, and pesticide residue analysis. The avowed editorial plan for each chapter is to deal first with the principles upon which the technique is based, then to outline the arrangement and operation of the instruments, thirdly to provide detailed directions for each step in the analytical method from the introduction of the sample to the evaluation of the final result, and finally to give detailed descriptions of representative applications followed by a tabulation of the entire range of applications. These are formidable aims and in assessing how far they have been achieved a reviewer can do little more than refer to a few of the methods with which he may be familiar. From a comparison of some six chapters the first point to note is that the standard of writing and the field covered vary considerably. Thus 42 pages are devoted to thin-layer chromatography whilst but 7 pages are devoted to mass spectrometry. Although the intending user would find much of value in the former chapter, one wonders how much useful information would be derived from the latter. In the section on thin-layer chromatography, the reader will find a detailed description of most of the commercially available apparatus together with a discussion of coating materials, solvents, and applications; this chapter may fairly be said to meet the publishers claims. For mass spectrometry on the other hand, the writer of this chapter wisely confines his remarks to generalities and directs the reader's attention to more specialist volumes. This is the only possible approach, of course, with such sophisticated techniques and is the one adopted moreover for nuclear magnetic and electron spin resonance. Indeed if to these already mentioned there are added chapters on X-ray diffraction (12 pages), X-ray emission and absorption (14 pages), electron microscopy (5 pages), it is obvious that for many of the methods the practising analyst would require access to other sources of information and to specialist workers. For certain other less sophisticated methods, however, e.g. gas chromatography and polarimetry, the ground covered gives a not unreasonable picture of the field although it is doubted if the *entire* range of applications is ever covered. It seems to the reviewer that the present volumes are more useful as a *review* of instrumental methods in general use rather than as a "complete reference book on instrumental analysis" for the laboratory—this appears to be the publisher's claim. At the price, however, it is an expensive review, and the books appear to fall between the two extremes of review and detailed manuals for each technique.

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