Journal of Pharmacy and Pharmacology

Published by
The Pharmaceutical Society
of Great Britain

Volume 19 No. 5
May 1967

Journal of Pharmacy and Pharmacology

Published by THE PHARMACEUTICAL SOCIETY OF GREAT BRITAIN

17 Bloomsbury Square, London, W.C.1. HOLborn 8967

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J. Pharm. Pharmac., 1967, **19**, 273–294 Received February 28, 1967

Routine detection and identification in urine of stimulants and other drugs, some of which may be used to modify performance in sport

A. H. BECKETT, G. T. TUCKER AND A. C. MOFFAT

A general procedure for the analysis in urine of basic drugs (and their metabolites), some of which may be misused as stimulants in sport, has been developed. The techniques used include gas-liquid and thin-layer chromatography and linked gas-liquid chromatography—mass spectroscopy. It is recommended that international control of drug-taking in sport be based primarily upon urine analysis by gas-liquid chromatography systems and also derivative formation followed by gas-liquid chromatography. The principles outlined in the procedure can be applied in a much wider forensic context.

IT is now generally accepted that the sensitivity and specificity of analytical procedures based on gas-liquid chromatography (GLC) make this the technique of choice for the detection of many drugs in body fluids. Following general work on the GLC of biologically important amines (Fales & Pisano, 1962; Brochmann-Hanssen & Svendsen, 1962; Parker, Fontan & Kirk, 1962; Vanden Heuvel, Gardiner & Horning, 1964; and others) several specific procedures for the detection of amphetamine or methylamphetamine, or both, the drugs most commonly misused in human sporting activities, have been reported. Of these, the methods most relevant to the problem of control include those of Cartoni & De Stefano (1963), Beckett & Rowland (1965a), Venerando & De Sio (1964), Lebbé & Lafarge (1965, 1966), Kolb & Patt (1965) and Greco, Paolucci & Taponeco (1965).

The purpose of the present communication is to outline a comprehensive analytical procedure for drugs likely to be misused in sport, with emphasis on the confirmation of results obtained from preliminary GLC screening. This procedure has evolved as the result of experience obtained from tests made during several major international sporting events held in the United Kingdom (Tour of Britain Cycle Races, 1965, 1966; World Cup Football Championship, 1966). It relies largely on GLC techniques but also makes use of thin-layer chromatography (TLC) and instrumental methods (e.g. mass spectroscopy) for supporting information on 'positives'. Sampling procedures, which are as important as the analytical procedures in the overall approach to the problem of detecting drug misuse have been reported elsewhere (Beckett, Tucker & James, 1966).

As well as the inherent sensitivity and specificity of the analytical procedures there are also other factors which can influence the successful detection of a 'positive'.

CHOICE OF SAMPLE

Urine is the most convenient biological sample; also, for the drugs examined, urine offers the advantage of a high concentration of drug

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compared with that in a blood sample in which low concentrations may result from extravascular concentration of the drug.

SELECTION OF DRUGS FOR SCREENING

The use of drugs in sporting activities is usually, in man, intended to increase performance. Therefore, in the present procedure, analysis for drugs with stimulant action is emphasized.

Ariëns (1964) has distinguished between long-term and short-term pharmacological conditioning for sporting events. The former implies the use of drugs, such as hormones and anabolic steroids, during the course of training. The effectiveness of these compounds taken during training is questionable; what is certain so far is that they can produce undesirable side-effects. This use of steroids by sportsmen is an insidious form of drug abuse which would be most difficult to control, at least from a sampling point of view. In short-term conditioning, there is a direct relation between the time the drugs are taken and physical effort. A wide variety of drugs, some of which are apparently effective and others which are valueless under the conditions in which they have been taken, have been and are being used. Of these drugs, the amphetamines are certainly the most common and are also amongst the most dangerous (Venerando, 1963). For these reasons their detection is emphasized in the proposed analytical scheme. Strychnine should be included in the analytical scheme, although its misuse in sport is now not prevalent. Like the amphetamines, caffeine has a stimulant effect on the central nervous system but since it is a constituent of normal beverages it is difficult, if not impossible, to distinguish between its normal use and misuse.

Ariëns (1964) also discusses several classes of compounds which may be used with intent to increase performance but these probably offer no advantage over normal physiological compensation mechanisms or metabolic processes. Such compounds include vitamins, natural metabolites and metabolite intermediates, adrenaline and sympathomimetics, analeptics and vasodilator drugs. Of these, the analeptics (e.g. nikethamide) and sympathomimetics with vasoconstrictor and cardiac stimulant actions are the most amenable to routine analysis.

As far as possible, the analytical procedure should also allow for the inclusion of new drugs having actions making their misuse in sport a possibility.

DRUG METABOLISM AND EXCRETION

The existence of a sensitive and specific analytical procedure for a particular drug is no guarantee that it can be used for the detection of that drug in biological fluids. Fundamental information about drug absorption, distribution and elimination, and particularly the time-course of these processes, is required if the analytical scheme is to be realistic. Unfortunately, for many drugs such information is inadequate. Therefore, ideally, the validity of the assay procedure for each drug considered should be tested by analysis of samples from volunteers to whom the drugs have been administered in normal doses.

A further complication is the fact that the elimination of many drugs is markedly dependent on urinary pH and for some drugs on urine volume

(Milne, Scribner & Crawford, 1958; Peters, 1960; Weiner & Mudge, 1964; Braun, Hesse & Malorry, 1963; Beckett & Rowland, 1964, 1965c; Asatoor, Galman & others, 1965; Beckett & Wilkinson, 1965a). Since urinary pH and output fluctuate throughout the day, these parameters, in addition to the time factor, can influence substantially the concentrations of unchanged drug and of its metabolites in urine. For instance, about 30–40% of a dose of amphetamine is excreted in the urine as unchanged drug over 48 hr under normal conditions (fluctuating urinary pH). However if the urine is rendered acidic (pH ca 5·0) for the same period the proportion of unchanged drug excreted increases to 60–70%. If the urine is rendered alkaline (pH ca 8·0) this percentage falls to below 10% (Beckett & Rowland, 1964, 1965b).

Thus the pH of urine samples should be measured as part of the method. Usually, since exercise tends to produce acidosis, the pH of a participant's urine is relatively acidic, although the use of special diets may complicate the situation. Sodium bicarbonate may sometimes be taken to offset the fatigue resulting from this acidosis. In large doses it will produce an alkaline urine of about pH 8·0 which would reduce the likelihood of detecting a basic drug such as amphetamine because the urinary excretion of this drug is accordingly much reduced. At the same time the subject would experience a more prolonged pharmacological effect from the drug.

These observations emphasize the need for sufficient sensitivity in the analytical methods to allow detection of the unchanged drug and, if possible, of drug metabolites under all conditions. The detection of metabolites also affords a means of distinguishing between drug that has passed through the body and drug that for some purpose may have been added directly to urine. The latter situation may arise when the sampling procedures lack security, or when a control sample has been added to a batch of test samples.

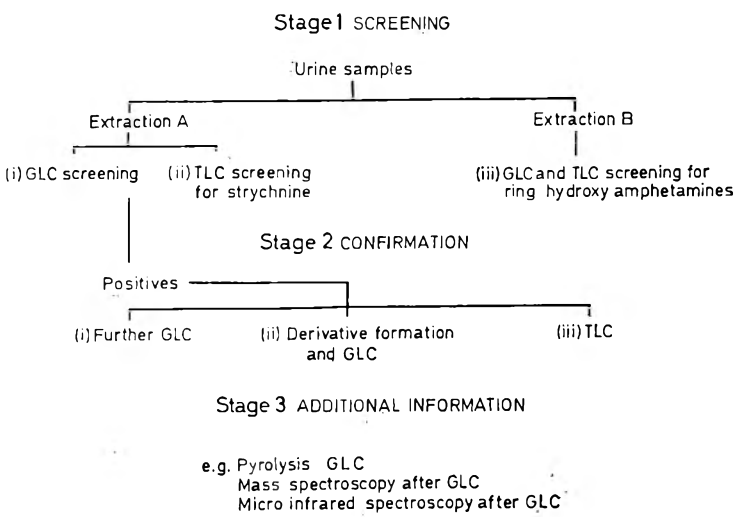


FIG. 1. Scheme of urine analysis.

METHODS

A suitable scheme of analysis, summarized in Fig. 1, involves three consecutive stages; not all stages are required routinely. Stage 1 is a routine screening procedure in which the emphasis is placed on GLC; the assay, by one worker, of 16 urine samples can be completed within a few hours of receipt. In general, implementing Stage 2 is sufficient to identify a drug giving a positive result in Stage 1. Stage 3 provides further support to the identification should this be required.

Stage 1: SCREENING

(i) GAS-LIQUID CHROMATOGRAPHY

The method is based on previously reported procedures for the determination of amphetamines (Beckett & Rowland, 1965a) and ephedrines (Beckett & Wilkinson, 1965b) in urine, and is capable of detecting these drugs for periods of up to 48 hr after normal therapeutic doses.

Extraction procedure A. Urine (1 to 5 ml) is pipetted into a glass-stoppered centrifuge tube together with 0·5 ml 20% sodium hydroxide solution. The urine is then extracted with 2 × 2·5 ml freshly distilled Analar diethylether using a mechanical tilt-shaker, centrifuged, and the ether extracts transferred to a 15 ml Quickfit test tube with a finely tapered base. The extract is then concentrated to about 50 μl on a water bath at 40°.

TABLE 1. GLC SYSTEMS USED IN STAGES 1 AND 2

System	Tubing	Liquid phase	Solid support	Oven temp. (°C)	N_2	H_2 (lb. in^{-2})	Air
A	3 m ss $\frac{1}{8}$ in. o.d.	5% Carbowax 6000 5% KOH	Chromosorb G A/W DMCS treated 80–100 mesh	155	20	20	25
B	1 m ss $\frac{1}{8}$ in. o.d.	2% Carbowax 20M 5% KOH	,,	140 & 180	15	15	25
C	2 m glass $\frac{1}{4}$ in. o.d.	2·5% SE-30	,,	120 & 160	20	24	30
D	2 m ss $\frac{1}{8}$ in. o.d.	10% Apiezon L 10% KOH	,,	155	20	20	25

Perkin-Elmer F11 gas-chromatographs with hydrogen flame-ionization detectors and Leeds & Northrup Speedomax type G (0–5mV) and Hitachi 159 (0–2·5mV) recorders were used.
Stream splitters (ratios approx. 1:5) were used with systems A, B and D.
Columns were conditioned at their operating temperatures for 24 hr before use and silanized with hexamethyldisilazane.

Approximately 3 to 5 μl of the concentrate is injected into each of two gas-chromatographic systems designated A and B (see Table 1). This combination detects most of the more commonly used central nervous system stimulants which, in general, are relatively small molecules based on the amphetamine structure. Systems B, C or D operated at higher temperatures can be used, if required, to screen many compounds of larger

molecular weight (unpublished observations) although modification of the extraction procedure may be necessary; for example, examination for analgesics like codeine and methadone, local anaesthetics like cocaine and lignocaine, caffeine and many antihistamines.

The retention times of the compounds screened using systems A and B are listed in Table 2; composite chromatograms are represented in Figs 2 and 3.

A 'positive' result at this point would be shown by the presence of a chromatographic peak, obtained on analysis of a test sample, with a retention time comparable to that of one of the drugs listed in Table 2. For instance, the chromatogram obtained on analysis of a urine sample from a competitive racing cyclist and reproduced in Fig. 4 indicates the presence of methylamphetamine and its metabolite amphetamine; confirmation requires the preparation of some of the derivatives listed in Table 2.

Discussion. In general, as little as 0·1 μg drug base per ml of urine may be determined by the above method, without interference from normal urinary constituents. Possible interference from breakdown products derived from such constituents on prolonged storage is minimized by refrigeration. Amphetamine itself is stable in refrigerated urine (4°) for at least three months.

Only basic compounds would be eluted from the alkaline columns used in systems A and B, and no interference is encountered from larger molecular weight molecules such as alkaloids and tranquillizers.

It was convenient to use separate isothermal systems for the present work since several gas chromatographs were available. However, a single 2 metre column as used in system B of Table 1, together with a temperature programming unit, could be used to detect the compounds listed in Table 2 with reasonable retention times for each. [Although the column materials used in systems A and B are essentially similar, the latter is more flexible since it employs Carbowax 20M as the liquid phase, which has a much higher operating temperature limit (ca 200°) than the Carbowax 6000 used in system A (limit 175°)]. Alternatively, a dual column instrument fitted with a 3 metre and a 1 metre type A or B column, and operated under isothermal conditions (oven temperature 150–160°) could be used. The retention times reported in Table 2 using systems A and B were reproducible over a period of at least six months.

Since some athletes are tobacco smokers, nicotine has been included in Table 2 so that a peak due to this compound is not confused with those from other materials being screened.

Most of the compounds investigated gave a single, symmetrical peak using systems A and B. Only phenoxypropazine gave more than one major peak; diethylpropion, methylphenidate and pyrovalerone gave single major peaks with small shoulders indicating decomposition. The possibility of enolization in these latter compounds on the potassium hydroxide-coated support material could explain these effects. Single peaks were obtained using system C.

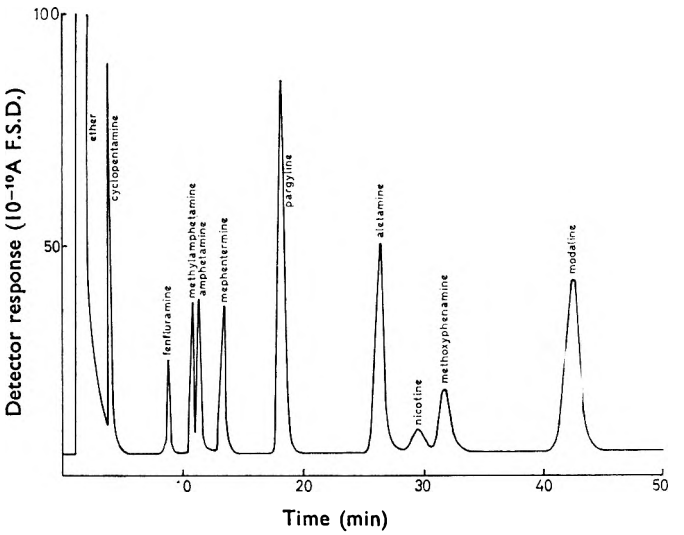


FIG. 2. Composite chromatogram of some stimulants and related compounds on Column "A".

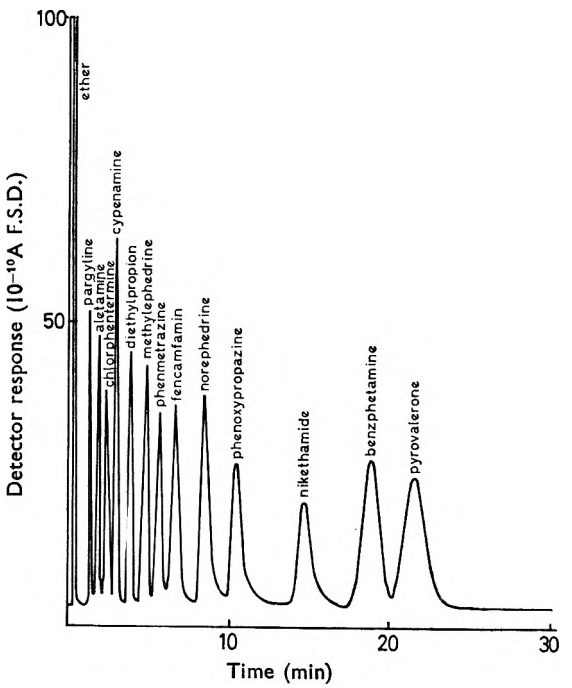


FIG. 3. Composite chromatogram of some stimulants and related compounds on Column "B".

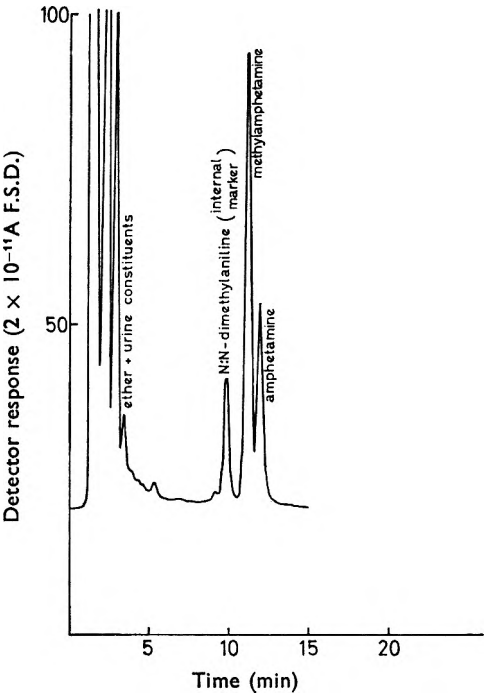


FIG. 4. Reproduction of a chromatogram obtained on analysis of a racing cyclist's urine showing the presence of methylamphetamine and its metabolite amphetamine in the urine.

(ii) THIN-LAYER CHROMATOGRAPHIC SCREENING FOR STRYCHNINE

Urine (10–20 ml) is taken through extraction procedure A (p. 276), using 2 × 5–10 ml ether. Portions of the final ether concentrate are applied to thin-layer plates which are then developed using at least two different solvent systems (see Table 4). In each instance reference strychnine is run on the same plates. A 'positive' result is recorded if, in each system, a spot is obtained with an Rf value comparable to that obtained for the reference compound.

The method was evaluated on 20 ml of a 1–3 hr urine sample obtained from a volunteer who took 4 mg strychnine hydrochloride. TLC systems IV and VI were used (see Table 4). The interpretation of the chromatograms was not complicated by the presence of normal urinary constituents, and a positive result was recorded.

(iii) GAS-LIQUID AND THIN-LAYER CHROMATOGRAPHIC SCREENING FOR RING-HYDROXY AMPHETAMINES

This group of compounds includes *p*-hydroxyamphetamine, *p*-hydroxymethylamphetamine, phenylephrine and metaraminol. Although all reports on the clinical use of these drugs emphasize the absence of central stimulation (Goodman & Gilman, 1965) their potential misuse in the present context cannot be excluded.

TABLE 2. GLC AND URINARY EXCRETION DATA FOR SOME STIMULANTS AND RELATED COMPOUNDS

Compound	Formula No. (p.283)	Retention time (min)** System A (155°)	Retention time (min)** System B (140°)	Retention time (min)** System C (120°)	Retention time (min)** System D (155°)	Excretion in urine: Unchanged	Excretion in urine: Metabolite(s)¶	Derivative retention time (min)§ System A (155°) Acetone	System B (140°) Acetone	System B (180°) *N*-Acetyl	System B (180°) *N*-Propionyl	System C (120°) Acetone	System C (160°) *N*-Acetyl	System C (160°) *N*-Propionyl
2-Aminoheptane	1	2·2	—	0·9	ND	NT		2·8	—	0·75	0·75	1·7	1·7	2·1
Methylaminomethylheptane	2	2·4	—	1·4	ND	NT		S	S	0·75	0·75	S	2·8	3·5
Isomethepten	3	3·1	—	1·8	ND	NT		S	S	1·0	1·0	S	3·2	3·9
Cyclopentamine	4	3·9	—	1·8	4·6	NT		S	S	1·3	1·3	S	4·0	5·0
Isopropylhexedrine	5	5·2	—	2·9	8·2	NT		S	S	1·7	1·7	S	5·6	7·1
Propylhexedrine	6	5·2	—	2·9	ND	NT		S	S	1·7	1·7	S	5·6	7·1
Fenfluramine	7	8·8	—	3·9	ND	Yes	N	S	S	2·1	2·1	S	5·5	6·9
Norfenfluramine	8	10·0	—	2·8	ND	Yes		9·0	—	3·2	3·3	4·3	3·7	4·4
Methylamphetamine*	9	10·8	—	3·4	8·2	Yes	A	S	S	3·1	3·2	S	5·6	6·9
Dimethylamphetamine*	10	10·8	—	4·2	ND	Yes	MA + A	T	T	T	T	T	T	T
N-Ethylamphetamine*	11	11·0	—	4·4	10·2	Yes	A	S	S	3·0	3·0	S	6·4	8·2
Phentermine	12	11·2	—	2·8	7·5	Yes		Not formed	Not formed	2·8	2·8	Not formed	3·8	5·3
Amphetamine*	13	11·4	—	2·4	6·1	Yes		12·5	—	4·0	4·1	4·7	4·0	4·9
Mephentermine	14	13·4	—	4·3	ND	Yes		S	S	2·5	2·6	S	5·6	7·6
Pargyline	15	18·4	1·4	3·6	9·1	Yes		T	T	T	T	T	T	T
Aletamine	16	26·2	1·9	5·2	ND	NT		21·8	1·6	5·6	5·6	8·8	7·0	8·8
Tranylcypromine	17	28·6	2·0	3·8	ND	NT		43·0	3·4	15·6	16·2	9·5	7·6	9·6
Nicotine	18	29·4	2·1	7·1	17·7	Yes		T	T	T	T	T	T	T
Methoxyphenamine	19	31·7	2·4	7·7	ND	NT		S	S	7·0	7·1	S	11·4	13·6
Chlorphentermine	20	32·6	2·4	6·5	21·7	Yes		Not formed	Not formed	7·4	7·4	Not formed	8·1	11·0
Modaline†	21	42·4	3·1	10·2	ND	NT		T	T	T	T	T	T	T
Cypenamine†	22	42·4	3·1	7·5	ND	NT		56·3	3·3	13·5	12·7	15·4	11·1	14·0
Diethylpropion*	23	—	3·6	13·6	43·0	Yes	D	T	T	T	T	T	T	T
Phendimetrazine*	24	—	3·6	11·6	35·7	Yes	PH	T	T	T	T	T	T	T
Prolintane	25	—	3·7	29·2	ND	NT		T	T	T	T	T	T	T
Methylephedrine*	26	—	4·7	9·5	25·2	Yes	E + NE	T	T	T	T	T	T	T
Phenmetrazine*	27	—	5·7	10·5	31·3	Yes		S	S	11·2	11·5	S	12·6	16·3
Ephedrine*	28	—	6·3	8·4	19·4	Yes	NE	—	2·7	17·8	19·2	11·6	9·9	13·1
Pseudoephedrine	28	—	6·3	8·4	ND	Yes	NP	—	2·5	16·0	16·0	10·4	10·0	13·1
Ethylephedrine*	29	—	6·5	13·0	ND	Yes	E + NE + DM	T	T	T	T	T	T	T
Fencamfamin	30	—	6·7	31·3	ND	NT		S	S	16·8	16·8	S	37·4	47·0
Norpseudoephedrine	31	—	8·4	6·9	ND	Yes		—	3·0	29·5	30·8	7·8	8·3	10·7
Norephedrine	31	—	8·6	6·9	16·1	Yes		—	2·8	28·6	30·0	8·8	8·6	10·5
Encyprate†	32	—	9·5	26·5	ND	NT		T	T	T	T	T	T	T

TABLE 2—*continued*

Compound	Formula No.	Retention time (min)** System A (155°)	B (140°)	C (120°)	D (155°)	Excretion in urine: Unchanged	Metabolite(s)¶	Derivative retention time (min)§ System A (155°) Acetone	B (140°) Acetone	B (180°) N-Acetyl	B (180°) N-Propionyl	C (120°) Acetone	C (160°) N-Acetyl	C (160°) N-Propionyl
Furfurylamphetamine	33	—	10·2	26·0	ND	NT		S	S	16·8	17·2	S	24·0	28·7
Furfurylmethylamphetamine	34	—	10·2	35·0	ND	Not detected	M + A	T	T	T	T	T	T	T
Phenoxypropazine	35	—	10·1	12·4	ND	NT		—	7·4	13·0	13·8	15·5	13·0	14·6
Nikethamide*	36	—	17·6	16·0	33·7	NT		T	T	T	T	T	T	T
Benzphetamine	37	—	19·0	74·0	ND	Yes (small amounts)	BA + MA + A	T	T	T	T	T	T	T
Methylphenidate*	38	—	20·9	46·4	ND	Yes		S	S	31·0	32·0	S	40·0	49·6
Pyrovalerone†	39	—	21·6	87·0	ND	NT		T	T	T	T	T	T	
Leptazol*	40	—	52·0	15·8	ND	NT		T	T	T	T	T	T	T

* Stimulant drugs most likely to be used as doping agents.

† Examples of newer drugs with central stimulant actions. Compounds listed also include other amphetamine analogues mainly used for their vascular and bronchial effects or as anoretics, reputedly with much less or no central stimulant action. Pargyline, tranylcypromine and phenoxypropazine are listed as examples of the monoamine oxidase inhibitor type of stimulant.

S = secondary amine. T = tertiary amine. ND = not determined. NT = not taken by volunteer(s)

** Determined with reference to amphetamine (system A) and to ephedrine (systems B and C).

§ Determined with reference to the amphetamine acetone derivative (system A); the ephedrine acetone derivative (system B, 140° and system C, 120°) and the acyl-amphetamine derivatives (system B, 180° and system C, 160°).

¶N norfenfluramine; A amphetamine; MA methylamphetamine; P phentermine; D de-ethylated analogue(s); PH phenmetrazine; E ephedrine; NE norephedrine; NP norpseudoephedrine; DM demethylated analogue of ethylephedrine; BA benzylamphetamine.

TABLE 3. GLC DATA FOR SOME AMPHETAMINE DERIVATIVES (Retention times in min.)

System	Oven temp (°C)	Free amphetamine	Reagent: A	MEK	MIK	MPK	IMK	CP	CHX	CHT	BMK	AA	HFB	CS
A	155	11·4	12·3	14·6	14·9	19·6	21·9	47·9	66·9	—	—	—	—	24·9
B	140	0·85	0·95	1·1	1·2	1·5	1·8	3·6	5·0	8·2	26·0	24·1	1·9	6·1
C	120	2·4	4·3	6·5	8·2	9·8	12·9	16·8	21·5	44·6	—	—	6·3	11·2

A acetone; MEK methyl ethyl ketone; MIK methyl isopropyl ketone; MPK methyl n-propyl ketone; IMK isobutyl methyl ketone; CP cyclopentanone; CHX cyclohexanone; CHT cycloheptanone; BMK benzyl methyl ketone; AA acetyl acetone; HFB heptaflurobutyric anhydride; CS carbon disulphide.

The presence of the phenolic group in these compounds necessitates modification of both extraction and chromatographic procedures; a suitable procedure for the detection of *p*-hydroxyamphetamine is as follows.

Extraction procedure B. Urine (10 ml) is rendered alkaline (pH 9–10) by the addition of solid sodium carbonate and then extracted with 3 × 5 ml portions of freshly distilled Analar diethyl ether. The combined ether extracts are concentrated as in extraction procedure A (p. 276). The concentrate is then analysed by GLC and TLC. The extracted urine is then neutralized and heated at 80–100° with 2 ml of 6N hydrochloric acid for 1 hr, to hydrolyse the conjugated drug (glucuronide or ethereal sulphate). After cooling, the pH is adjusted to 9–10 by the addition of 2 ml of 6N sodium hydroxide and solid sodium carbonate. The urine is then extracted as before and analysed by GLC.

GLC *analysis.* 1 μl of the final concentrated ether extracts from the unhydrolysed and hydrolysed urine is analysed using GLC system C at 160° (the use of an alkali-coated support in systems A and B prevents the elution of compounds containing acidic or phenolic groups). A 'positive' result is recorded if a peak is obtained with a retention time comparable to that of reference *p*-hydroxyamphetamine (t_R = 3·3 min).

Confirmation is obtained by chromatography of the acetyl derivative (t_R = 18·0 min) and a trimethylsilyl (TMS) derivative (t_R = 4·3 min) (see Stage 2 for methods of preparation).

TLC *analysis.* Portions of the concentrated ether extract from the unhydrolysed urine are applied to thin-layer plates which are then developed using at least two different solvent systems (see Table 4). In each instance, reference *p*-hydroxyamphetamine is run on the same plates. A 'positive' result is recorded if, in each system, a spot is obtained with an Rf value comparable to that obtained from the reference compound. Interpretation of chromatograms obtained on running the concentrated ether extract from hydrolysed urine is complicated by interference from normal urinary constituents. This is less of a problem in the GLC analysis.

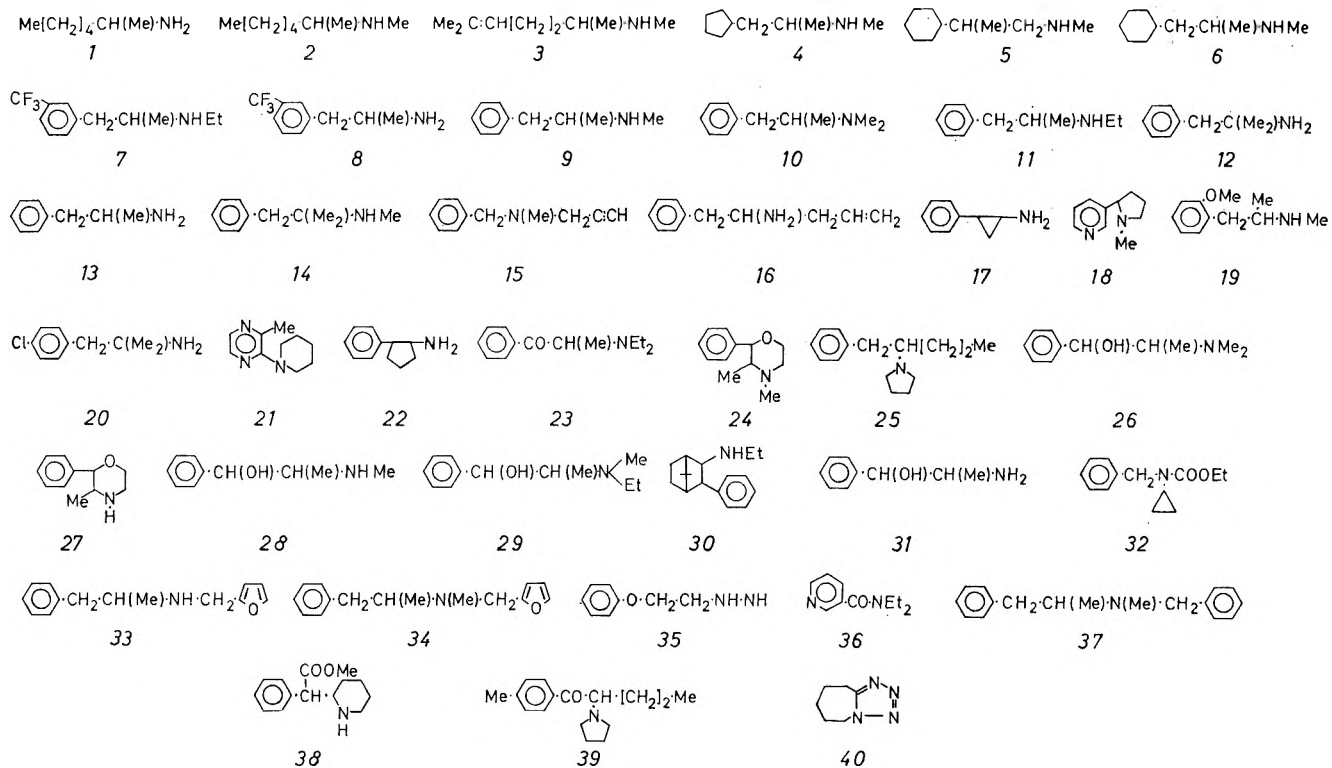
The method was evaluated on 10 ml of a 2–4 hr urine sample from a volunteer who had ingested 60 mg *p*-hydroxyamphetamine hydrobromide. A large amount of the drug was detected in the urine and the results indicated that a considerable proportion was present in a conjugated form.

Stage 2: CONFIRMATION

In this stage, conclusive identification is obtained of 'positives' recorded in Stage 1. This confirmation is based primarily on the results of further GLC, i.e. the use of different systems, and the preparation and chromatography of simple derivatives. TLC is also used as an auxilliary technique although in general it is less sensitive and specific than the GLC methods.

(i) FURTHER GLC

(a). Portions of the concentrated ethereal extract obtained in Stage 1 are injected into GLC systems C and D and the chromatograms obtained



Structural formulae of compounds listed in Table 2.

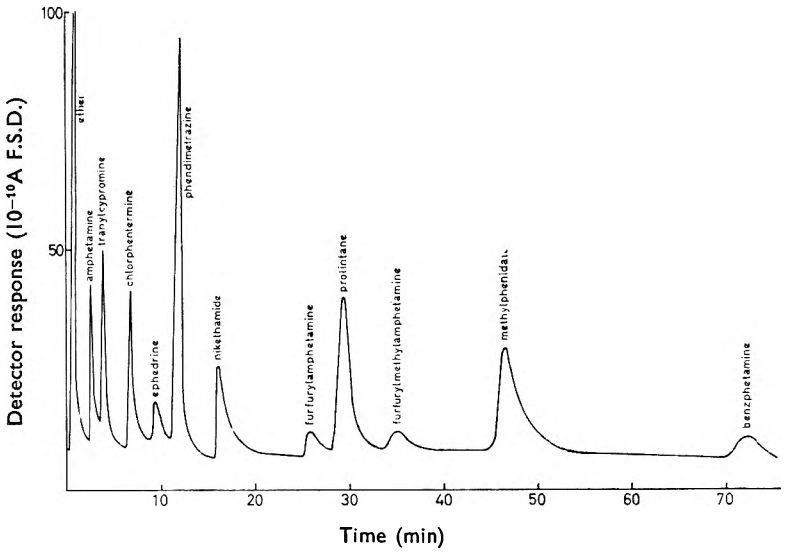


FIG. 5. Composite chromatogram of some stimulants and related compounds on Column "C".

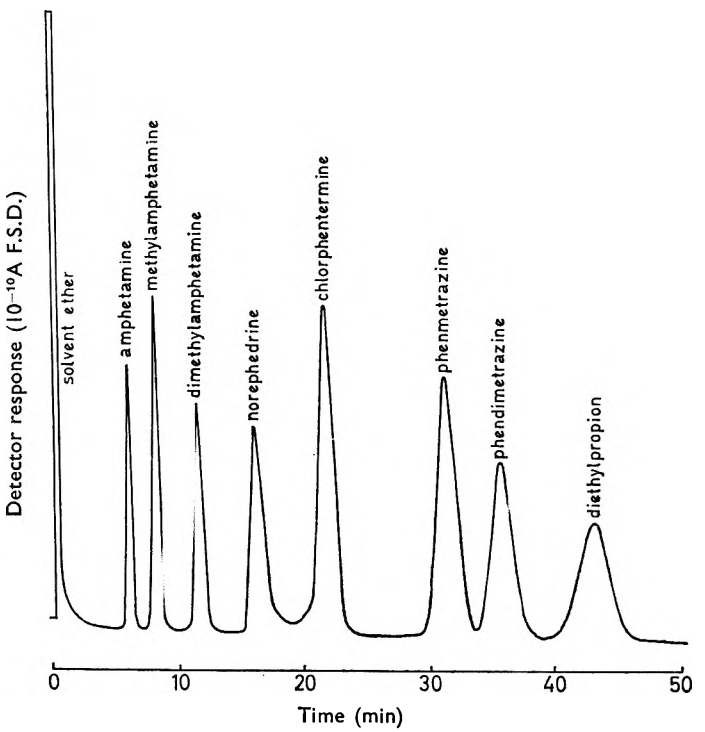


FIG. 6. Composite chromatogram of some stimulants and related compounds on Column "D".

are compared with reference chromatograms (see Figs 5 and 6). Preliminary identification, supporting the evidence obtained in Stage 1, is made by comparing retention times of the peak(s) obtained with those given by the reference compounds (see Table 2).

(b). The *relative* retention time of a compound, giving rise to a peak noted in (a), is determined with respect to an appropriate internal marker, using systems A or B, and D. (Any reference compound giving a peak with a retention time close to, but distinct from, the peak due to the compound under investigation can be used.) Identification of the compound is made by comparing its relative retention time with that determined under the same conditions using known reference compounds.

(c). The quantity of drug, identified in (b), in a 'positive' sample may be determined using the internal standard technique (see Beckett & Rowland, 1965a; Beckett & Wilkinson, 1965b).

The use of systems C and D allows a different and in many instances a greater separation of the compounds screened in Stage 1. The order of elution of several pairs of compounds (e.g. amphetamine and methylamphetamine) is the reverse of that obtained using systems A or B. A limitation of system C is that the time between sample injection and peak maximum increases and the peak tends to broaden, as the sample size is decreased. For example, 1 μg of nicotine injected in 1 μl of ether has a retention time of 7·1 min, but this is increased to 7·6 min when the concentration is decreased to 0·2 μg/μl. Therefore, it is necessary to compare retention times of the reference compounds with that of a suspected 'positive' at similar peak heights. The retention times listed in Table 2, using system C, were determined with solutions containing approximately 1 μg base per μl of ether. Retention times determined using system D (see also Lebbé & Lafarge, 1965, 1966) are independent of sample size and the use of this system is preferable, for the confirmation of 'positives'.

(ii) DERIVATIVE FORMATION AND GLC

The retention times of the primary and secondary amines, listed in Table 2, may be shifted by the formation of simple derivatives using selective reagents. Thus, having treated concentrated ethereal urine extracts from a Stage 1 'positive' with appropriate reagents, as described below, aliquots of the reaction mixtures are then injected into the GLC systems. Identification of the 'positive' is made on the basis of a comparison between the chromatograms produced with those obtained for derivatives of the reference compounds (see Table 2). Retention times relative to an appropriate internal marker can be used as the basis of identification.

This 'peak-shift technique' (Langer & Pantages, 1961) has received much attention in recent years (Brochmann-Hanssen & Svendsen, 1962; Anders & Mannering, 1962; Brooks & Horning, 1964; Vanden Heuvel, Gardiner & Horning, 1964; Capella & Horning, 1966; and others). To be of value a derivative must be formed easily and in good yield, and have a retention

TABLE 4. TLC OF SOME STIMULANT DRUGS AND RELATED COMPOUNDS

Support		Solvent v/v	Rf values × 10²											
			A	MA	*p*OHA	E	ME	NE	P	D	N	S	C	Nic
Alumina	I	$CHCl_3$–MeOH (50:50)[1]	57	81	37	69	84	13	89	92	87	93	93	91
Silica Gel	II	$CHCl_3$–MeOH (50:50)[2]	29	20	16	23	30	19	52	81	89	21	86	72
,,	III	$CHCl_3$–diethylamine (9:1)[3]	74	79	25	35	64	23	67	97	89	70	79	84
,,	IV	$CHCl_3$–acetone–diethylamine (5:4:1)[3]	84	70	46	33	69	83	60	92	82	61	71	81
,,	V	n-butanol–acetic acid–water (5:4:1)[4]	60	49	57	50	41	55	51	37	56	15	57	22
,,	VI	MeOH–acetone (50:50)[5]	59	24	51	30	36	67	45	79	75	12	74	56
,,	VII	MeOH–acetone–NH_3 (35%) (47·5:47·5:5)[6]	85	56	81	78	67	92	77	97	87	49	85	79
,,	VIII	MeOH–acetone–triethanolamine (1:1:0·03)[6]	63	27	49	28	42	71	53	79	80	14	76	63
,,	IX	Isopropanol–NH_3 (5%) (10:1)[7]	46	32	40	29	39	44	51	82	70	24	57	65
,,	X	Dimethylformamide/ethylacetate/+3 drops n-octanol (1:9)[8]	21	16	9	20	38	18	26	75	67	9	68	59

[1] Noirfalise, 1966; [2]Noirfalise, 1965, 1966; [3]Waldi, 1964; [4]Debackere & Massart-Leen, 1965; [5]Moerman, 1964; [6]Baumler & others, 1964; [7]Ristic & Thomas, 1962; [8]Eberhardt & Debackere, 1965.

A, amphetamine; MA, methylamphetamine; *p*OHA, *p*-hydroxyamphetamine; E, ephedrine; ME, methylephedrine; NE, norephedrine; P, phenmetrazine; D, diethylpropion; N, nikethamide; S, strychnine; C, caffeine; Nic, nicotine.

DRUG SOLUTIONS. Ethereal solutions of the basic forms of each reference compound were prepared by extraction of alkaline solutions of the salts. Approximately 20–30 μg of each drug was applied to thin-layer plates using a 10 μl Hamilton syringe. A standard solution of (+)-amphetamine sulphate in methanol was used for the determination of spray sensitivities.

PREPARATION OF PLATES. Alumina G (Merck) or Silica Gel G (Merck) (30 g) was mixed with water (60 ml), containing sodium fluorescein (0·04%), by stirring for 2 min in a mortar and spread on to 20 × 20 cm glass plates in a layer 0·25 mm thick. The plates were allowed to dry in the air for 15 min and then for 2 hr in an oven at 80°, after which they were placed in a desiccator to cool before use. Plates were run at ambient room temperature.

DETECTION OF SPOTS. Initially spots were located using ultraviolet light (254 and 350 mμ). All the reference compounds could be visualized in this way. The following spray reagents were used:

(*a*) Iodoplatinate reagent: 3 ml 10% solution of chloroplatinic acid treated with 97 ml water and 100 ml aqueous 6% KI solution added. Stored in a brown glass bottle.
Reference compounds appeared as pale yellow or brown spots except strychine which showed as a distinctive deep violet spot and *p*-hydroxyamphetamine and caffeine which were not detected.
Sensitivity (amphetamine) about 5 μg.

(*b*) i. Freshly diazotized *p*-nitroaniline (Wickström & Salvesen, 1952). ii. 0·5 N NaOH in ethanol.
Reference compounds appeared as yellowish spots except amphetamine which showed as a distinctive pink spot and *p*-hydroxyamphetamine which gave a brown spot. Strychnine, nicotine and caffeine were not detected.
Sensitivity (amphetamine)—about 5 μg.

(*c*) Ninhydrin reagent (Dole & others, 1966): 0·4% ninhydrin in acetone prepared within 30 min of use. After warming under the ultraviolet lamp the reference compounds appeared as pale blue spots except amphetamine, ephedrine and norephedrine which showed as distinctive violet spots. Caffeine and strychnine were not detected.
Sensitivity (amphetamine) about 1 μg.

Note: plates without added fluoroscein were used to determine the above spot colours.

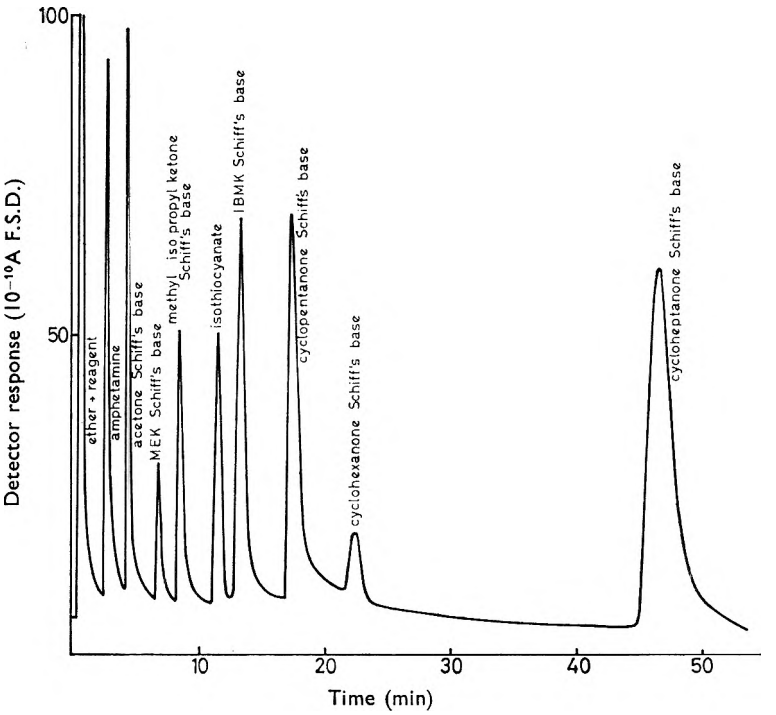


FIG. 7. Composite chromatogram of amphetamine and some of its derivatives on column "C".

time distinct from the parent compound. The following are examples of suitable derivatives.

1. *Acetone Schiff's bases and isothiocyanate derivatives.* (Acetone derivatives are only formed by primary amines. Both primary and secondary amines will react with CS_2 to give dithiocarbamates, but only in the former case will this derivative be converted to the isothiocyanate which will give a peak by GLC.)

The reagent (0·5 ml), acetone or CS_2, is added to the 50 μl concentrated ethereal urine extract. The whole is evaporated to 50 μl on a 60° water bath. In both instances, about 90% conversion is usual under these conditions.

2. *Other Schiff's bases.* (Formed by primary amines.) The ketone reagent (50 μl) is added to the concentrated ethereal urine extract and incubated in a closed evaporating tube for 10 min on a 50° water bath. In general, the yield of derivative is smaller with larger molecular weight ketones. In these instances the reaction may be allowed to proceed for a longer time to increase the yield of the derivative.

3. *Benzyl methyl ketone Schiff's bases.* (Formed by primary amines.) Larger ketones give chromatographic peaks which may interfere with those of the simpler amphetamines. Therefore, method 2 is modified when using

higher molecular weight ketones as reagents. The derivative is formed by dissolving 100 mg of the ketone in 1 ml of ether and using 50 μl of this solution for the reaction as in 2.

4. *Oxazolidines.* [Secondary amines with β-hydroxy groups (e.g. ephedrine) form oxazolidines with ketones (Capella & Horning, 1966).] The ketone (0·5 ml) is added to the concentrated ethereal urine extract, left at room temperature for 2 hr, and then evaporated to 50 μl.

5. *Carbinolamines.* [Formed by reaction of secondary amines with cyclic ketones (Capella & Horning, 1966).] Prepared as in method 2.

6. *Acetyl, propionyl and heptafluorobutyryl derivatives.* (Formed by primary and secondary amines.) The appropriate anhydride (5 μl) is added to the concentrated ethereal urine extract, and 5 μl of the mixture is injected into the gas chromatograph. Immediate 100% acylation is usually achieved.

7. *Trimethylsilyl derivatives.* (Formed when the compound contains an aromatic ring-hydroxy group.) An equal volume of reagent (2 parts hexamethyldisilizane, 1 part trimethylchlorosilizane and 10 parts dry distilled pyridine) is added to the concentrated ethereal urine extract and mixed thoroughly. A quantitative reaction is usually achieved after allowing the mixture to stand for 5 min.

Retention times of the acetone, acetyl and propionyl derivatives of appropriate reference compounds, using three of the GLC systems, are listed in Table 2, while Table 3 and Fig. 7 record the retention times of many derivatives which could be prepared, if necessary, from a single compound, i.e. amphetamine.

The value of derivative formation as a method of resolving compounds with similar retention times on the screening columns is shown by a comparison of the data for norfenfluramine, methylamphetamine and dimethylamphetamine (see Table 2). Norfenfluramine (a primary amine) can be separated from the others by the formation of its acetone derivative. Both norfenfluramine and methylamphetamine (a secondary amine) can be acylated, while dimethylamphetamine (a tertiary amine) does not give either of these simple reactions.

All the primary amines studied readily formed Schiff's bases, with the exception of phentermine and chlorphentermine. The two α-methyl groups in these compounds may hinder the reaction with ketones.

Under the conditions used for the formation of acyl derivatives, only the *N*-acyl derivatives would be produced since traces of water in the concentrated ethereal urine extracts would hydrolyse any *O*-acyl groups formed. Thus, when norephedrine base in dry ether was treated with acetic anhydride and chromatographed using system C at 160°, two derivative peaks were obtained at 8·6 and 10·0 min. On addition of water to the reaction mixture and further chromatography, the second peak (presumably either the *N*-acetyl, *O*-acetyl and/or the *O*-acetyl compound) disappeared.

The different rates of reaction of ephedrine and pseudoephedrine with acetone may be used to distinguish between them (Brochmann-Hanssen &

Svendsen, 1962); using method 4, approximately 80–90% of pseudoephedrine is converted to the oxazolidine, but only about 5–10% conversion is achieved with ephedrine.

(iii) THIN-LAYER CHROMATOGRAPHY

Portions of the final ethereal concentrate from 20 ml urine (extraction procedure A, p. 276) are applied to thin-layer plates which are then developed using at least two different solvent systems. Appropriate reference drugs are applied to the same plates. A 'positive' result is recorded if a spot is obtained, in each system, with a comparable Rf value and colour to that obtained from the relevant reference compound. TLC systems and reference drugs are chosen according to the suspected identity of the 'positive'.

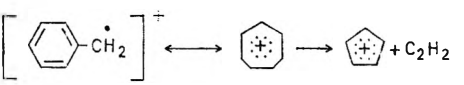
Ten TLC systems, reported in the literature, for the identification of the commonly used amphetamines were investigated using several reference compounds (see Table 4). It is essential to run solutions of reference drugs on each plate since Rf values are not very reproducible. The values quoted in Table 4 serve only to indicate the general order of separation achieved with each system.

Urine extracts from volunteers who had received normal doses of some of the reference drugs were run on all systems (amphetamine and methylamphetamine); on systems II and IX (ephedrine); on systems IV and VI (strychnine) and on systems II and VI (*p*-hydroxyamphetamine). A 'positive' result was recorded in each instance with no interference from normal urine constituents.

Stage 3: ADDITIONAL INFORMATION

The use of the two previous stages in all examples to date has led to the unequivocal identification in human urine of most of the drugs listed in Table 2. However, should additional information be required, techniques such as mass spectroscopy and micro infrared spectroscopy can be used.

For instance, Fig. 9 shows the mass spectra of amphetamine and methylamphetamine (extracted from urine), determined by combined GLC—mass spectroscopy. Ion abundance peaks which were absent from, or significantly greater than, those in the "background" spectrum are labelled in mass units. Although the spectra do not show significant amounts of parent molecular ions at masses of 135 (amphetamine) and 149 (methylamphetamine), differentiation of these closely related compounds is relatively simple by examination of their fragmentation patterns. For example, cleavage pattern b (see Fig. 8) produces an abundant ion, $[Me_3{\cdot}CH{\cdot}NH_2]^+$, of mass 44 in the spectrum of amphetamine, whereas similar cleavage of methylamphetamine gives rise to a large peak at mass 58, due to $[Me{\cdot}CH{\cdot}NH{\cdot}Me]^+$. The removal of the α-methyl group of amphetamine and methylamphetamine (cleavage pattern c in Fig. 8) is indicated by relatively abundant ions of masses 120 and 134 respectively. The peaks, in both spectra, at masses 77 and 91 are due to $[Ph]^+$ and $[Ph{\cdot}CH_2]^+$ respectively (see cleavage patterns a and b in Fig. 8), while the presence of an abundant ion of mass 65 may be accounted for as follows:



mass 91 produced by cleavage pattern b. tropylium ion mass 65

(see Grubb & Meyerson, 1963).

The mass spectrum of β-phenethylamine, on the other hand, does not have a large peak at 58 mass units, which distinguishes it from methylamphetamine, while a peak at 30 mass units (cleavage pattern b in Fig. 8) readily distinguishes it from amphetamine. The spectrum of methyl amphetamine also shows a significant peak at 30 mass units, probably resulting from cleavage d (see Fig. 8), to give $[NH{\cdot}Me]^+$. Unlike the spectra of the two drugs, the spectrum of β-phenethylamine contains a significant peak for the parent molecular ion, at 121 mass units. Spectra obtained after direct introduction of samples into the ionization chamber of the mass spectrometer are qualitatively identical to those obtained after GLC of the bases extracted from urine.

Although the spectra shown in Fig. 9 were obtained on analysis of approximately 10–20 μg of drug (extracts of 5 ml aliquots of urine containing 4 μg drug base per ml were used, i.e. concentrations of the order likely

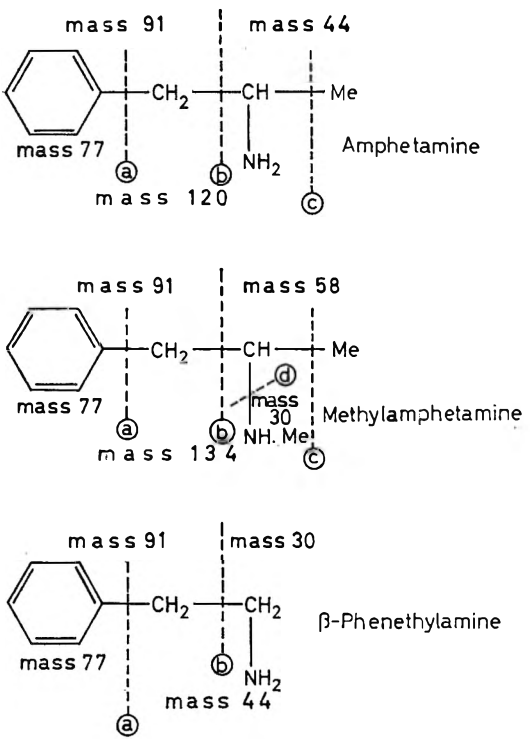


FIG. 8. Main cleavage patterns giving rise to ion abundance peaks in the mass spectra of amphetamine, methylamphetamine and β-phenethylamine.

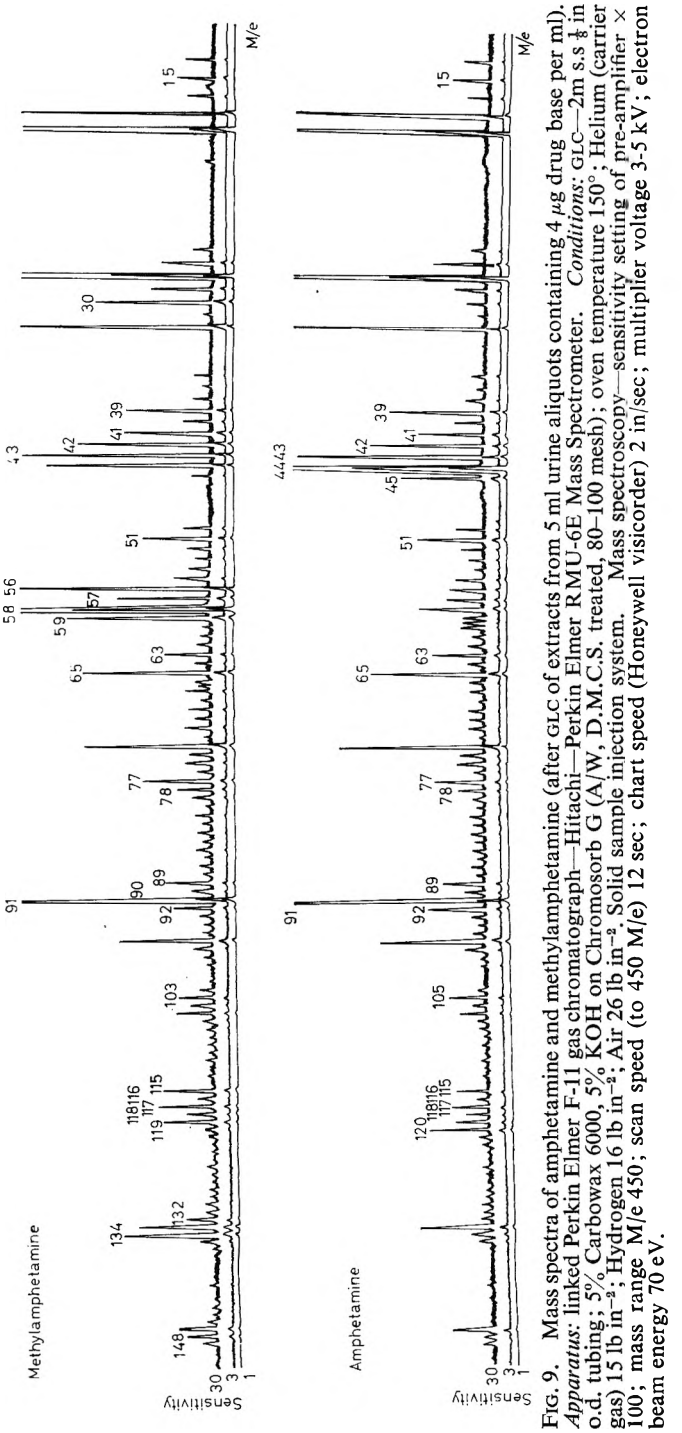


FIG. 9. Mass spectra of amphetamine and methylamphetamine (after GLC of extracts from 5 ml urine aliquots containing 4 μg drug base per ml). *Apparatus:* linked Perkin Elmer F-11 gas chromatograph—Hitachi—Perkin Elmer RMU-6E Mass Spectrometer. *Conditions:* GLC—2m s.s $\frac{1}{4}$" o.d. tubing; 5% Carbowax 6000, 5% KOH on Chromosorb G (A/W, D.M.C.S. treated, 80–100 mesh); oven temperature 150°; Helium (carrier gas) 15 lb in^{-2}; Hydrogen 16 lb in^{-2}; Air 26 lb in^{-2}. Solid sample injection system. Mass spectroscopy—sensitivity setting of pre-amplifier × 100; mass range M/e 450; scan speed (to 450 M/e) 12 sec; chart speed (Honeywell visicorder) 2 in/sec; multiplier voltage 3-5 kV; electron beam energy 70 eV.

after drug taking), much smaller amounts may be detected by this method. At present the limits of detection using linked GLC–infrared systems or micro-preparative GLC followed by micro–infrared analysis are somewhat higher (50–100 μg of compound).

Discussion

The scheme of analysis described is effective in distinguishing between the amines of Table 2, some of which are, and some of which may be misused in sport. Constituents in the urine do not interfere with this scheme of identification.

It has been held by some analysts that the presence of a drug is not proved unless the drug is identified by more than one type of analytical technique. We have therefore, included TLC in this scheme of analysis although, in general, we have found it to be much less sensitive than GLC for the detection of the amines examined. We recommend that international control of drug taking in sport be based upon the adoption of urine analysis involving GLC with different systems combined with derivative formation followed by GLC. The range of derivatives is capable of extension and it should thus be possible to draw up a list of retention times of agreed derivatives for each drug, and metabolites where applicable, relative to those for standard compounds, which would constitute acceptable proof of identity of an administered drug; the application to amphetamine (see Table 3) illustrates such an approach to the problem.

When, as well as the drug, its metabolites are excreted in urine, the GLC characteristics of the metabolites and derivatives of the metabolites using different systems, afford additional proof of the presence of the ingested drug, e.g. administered methylephedrine yields not only this compound in the urine but also ephedrine and norephedrine (Beckett & Wilkinson, 1965c) which can be characterized by the use of various columns and derivatives (see Table 2). The above scheme is capable of progressive refinement as more information is acquired about the elimination of drugs. Furthermore it is unlikely that new stimulants will now be marketed in the absence of information on their metabolism in man.

The sensitivity of the GLC method, in general, makes it possible to detect the drug and its metabolites in urine for as long as 48 hr after the ingestion of a normal dose. Thus such a drug can be detected in a participant's urine on one day and then, if necessary, a further urine sample can be requested on the following day to remove any doubt about the sampling procedure. Analysis of this sample will also furnish a check against a challenge that deliberate addition of a drug to a participant's urine has occurred.

Although the scheme was devised to detect the misuse of stimulants in sport, it is equally applicable to their detection in other circumstances. Thus it is now possible by objective tests to establish the extent of the problem of abuse of stimulants (and narcotics, which can be determined by similar techniques) provided urine collection from subjects is authorized.

Acknowledgements. The authors acknowledge the technical assistance of Mr. J. H. McAndrew. Mass spectra were run by Mr. F. Banyard and Miss C. Babb of Perkin Elmer Ltd., Beaconsfield, Berks. We also wish to thank the various pharmaceutical companies who supplied many of the drugs: Abbott Labs. Ltd., Queenborough, Kent, for Encyprate; Allen & Hanburys Ltd., Ware, Herts., for Fencamfamin HCl; Ayerst, McKenna & Harrison Ltd. for Phendimetrazine; Boehringer Ingelheim Ltd., Isleworth, Middlesex, for Phenmetrazine (Preludin) and Prolintane; Ciba Labs., Horsham, Sussex for Methylphenidate (Ritalin); Dorsey Labs., Lincoln, Nebraska for Pyrovalerone; Labs. Diamant, Paris for Furfurylmethylamphetamine (Furfenorex) and Furfurylamphetamine; Knoll Pharmaceutical Co., Orange, NJ for Methylaminomethylheptan HCl and Isomethepten mucate; Knoll AG., Ludwigshafen-am-Rhein for Isopropylhexedrine HCl (Eventin); Eli Lilly and Co. Ltd., Basingstoke, Hants. for Tuamine Sulphate and Cyclopentamine HCl; Labs. Medial-Riker, Lyon for *N*-ethylamphetamine (Adiparthrol); Wm. S. Merrell Co., Cincinnati, Ohio for Cypenamine; Merrell-National (Labs.) Ltd., London for Diethylpropion (Tenuate) National Drug Co., Philadelphia, Pa. for Aletamine; Smith, Kline & French Labs. Ltd., Welwyn Garden City, Herts, for Propylhexedrine (Benzedrex) and Tranylcypromine (Parnate); Smith & Nephew Ltd., Welwyn Garden City, Herts. for Phenoxypropazine (Drazine); Selpharm Ltd., London for Fenfluramine (Ponderax) and Norfenfluramine; Temmler-Werke, Marburg/Lahn for Dimethylamphetamine (constituent of Metrotonin); Walker Labs., Mt. Vernon N.Y., for Ethylephedrine; Warner-Lambert International, Morris Plains, N.J. for Modaline Sulphate; Wm. R. Warner & Co. Ltd., Eastleigh, Hants. for Chlorphentermine; J. Wyeth & Brother Ltd., Havant, Hants. for Mephentermine; Upjohn Ltd., Crawley, Sussex for Methoxyphenamine (Orthoxine); Upjohn Co., Kalamazoo, Mich. for Benzphetamine.

One of us (G.T.T.) thanks the Pharmaceutical Society for a research scholarship; G.T.T. and A.C.M. also thank the Science Research Council for research studentships.

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J. Pharm. Pharmac., 1967, **19**, 295–304 Received November 25, 1966

Infrared identification of pharmaceutically important sulphonamides with particular reference to the occurrence of polymorphism

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The infrared absorption spectra of sulphonamides, when compared with the spectra of Authentic Specimens, provide a simple and complete means of identification, provided the effects of polymorphism are excluded. Of 18 substances examined, twelve showed evidence of polymorphism. Limited solubility prevents the use of solution spectra, and specified solvent treatments, details of which are given, may therefore be necessary with these substances to ensure reproducible spectra.

IN a number of monographs in the British Pharmacopoeia and the British Pharmaceutical Codex an identification test is included in which the infrared absorption spectrum of the sample under examination is compared with that of an Authentic Specimen, supplied for this purpose. The original collection of Authentic Specimens consisted mainly of steroids, but is being extended to include a number of sulphonamides and other substances. For the purposes of this comparison of spectra, it was necessary to establish conditions whereby different forms of a substance, should they exist, might be converted to a single form thus eliminating differences in solid-state infrared spectra.

Polymorphism occurs frequently in complex compounds, particularly with those molecules in which hydrogen bond formation is possible, and in general the polymorphic variations of a substance give rise to different infrared spectra. Means of overcoming this difficulty have already been proposed for a number of steroids (Mesley & Johnson, 1965) and barbiturates (Cleverley, 1960). Amongst sulphonamides, polymorphism has been reported with sulphanilamide (Watanabe, 1942) and sulphathiazole (Grove & Keenan, 1941; Miyazaki, 1947), and infrared spectra of two forms of sulphanilamide have been published by Barnes, Liddel & Williams (1942).

Experimental

MATERIALS

The samples of sulphadiazine sodium, sulphadimethoxine, sulphaguanidine, sulphanilamide, sulphaphenazole and sulphathiazole were the Authentic Specimens of the British Pharmaceutical Codex. Samples of the remaining substances were supplied by Mr. C. A. Johnson of the British Pharmacopoeia Commission. Solvents used were of B.P. or A.R. quality.

SOLVENT TREATMENTS

To obtain as many forms of each substance as possible, the following treatments were used: evaporation to dryness of solutions in water,

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ethanol, methanol, acetone and chloroform, normally on a water-bath but in some instances also at room temperature; precipitation from alkaline solution by addition of acid, or vice versa; heating at temperatures up to 140°. The solubility of the substances in the different solvents varied widely and not all of these treatments could be used with each substance. Where the existence of further forms was suspected, or where these treatments failed to give crystalline products, other treatments were tried, including the use of mixed solvents and true recrystallization by allowing saturated solutions to cool.

INFRARED ABSORPTION SPECTRA*

Samples were prepared for infrared examination both as mulls in liquid paraffin (Nujol) and as pressed alkali halide discs using the technique previously described (Mesley & Johnson, 1965). The low solubility of the sulphonamides precluded the use of solutions for obtaining spectra.

Spectra of all the forms were recorded using a Grubb Parsons GS 2 or Spectromaster grating spectrometer. The recommended solvent treatments were repeated independently using spectra recorded on a Unicam SP 200 spectrometer with sodium chloride prism or a Perkin-Elmer 237 grating spectrometer.

X-ray powder diffraction patterns of five forms of sulphadimidine sodium were obtained (by Mr. K. Goodhead) using a Philips generator with a Unicam 9 cm camera and copper Kα radiation.

Results and discussion

Table 1 lists the substances examined and the number of solid forms of each substance found. These are not necessarily all crystalline, as in many instances an amorphous form was obtained from certain treatments. From the point of view of obtaining consistent spectra, this was as troublesome as the true polymorphic variations. The list also includes some forms detected in alkali halide discs, though they were not always isolated in the free state.

TABLE 1. INCIDENCE OF POLYMORPHISM IN THE 18 SULPHONAMIDES EXAMINED

Substance	No. of solid forms encountered	Substance	No. of solid forms encountered
Phthalylsulphathiazole	2	Sulphaguanidine	5
Succinylsulphathiazole	5	Sulphamethizole	1
Sulphacetamide sodium	3	Sulphamethoxydiazine	4
Sulphadiazine	1	Sulphamethoxypyridazine	3
Sulphadiazine sodium	1	Sulphanilamide	3
Sulphadimethoxine	1	Sulphaphenazole	1
Sulphadimidine	2	Sulphapyridine	7
Sulphadimidine sodium	7	Sulphasomidine	2
Sulphafurazole	1	Sulphathiazole	4

* Throughout the paper the use of the word "spectrum" refers to the infrared absorption spectrum.

The purpose of using Authentic Specimens is to avoid dependence on published spectra, particularly where different spectra may be obtained from the same substance. Even in those instances where no evidence of polymorphism was found, the existence of other forms cannot be ruled out, and no spectra are therefore included in this report. In fact spectra of most of the substances in this category have already been published elsewhere. A survey of all the forms encountered and a review of published spectra are given below.

Phthalylsulphathiazole. Only one crystalline form was obtained as a result of the various solvent treatments used, but the original sample showed certain additional absorptions which may have been due to a second crystalline form. The spectrum obtained from a potassium bromide disc was distinctly different from that of a liquid paraffin mull, presumably due to the production of an amorphous form. The Sadtler Standard collection includes spectra of both potassium bromide disc and Nujol mull, which agree with those obtained in this work.

Succinylsulphathiazole. The original form (A) was a monohydrate, and was not recovered from any of the solvent treatments used. On heating it gave form D. Evaporation at room temperature of an acetone solution prepared from either of these forms gave form B. Evaporation of similar solutions on a water-bath gave variously forms C and D and the amorphous form, or mixtures of any two of these. Alcoholic solvents tended to give only the amorphous form. Form B was consistently recovered when the material was dissolved in dilute sodium hydroxide solution and precipitated by addition of dilute hydrochloric acid. This form was not stable on grinding with potassium bromide, the resulting spectrum being predominantly that of the amorphous form. The Sadtler Standard collection includes a Nujol mull spectrum of form A and a potassium bromide disc spectrum which is mainly that of the amorphous form.

Sulphacetamide sodium. The original form (A) was a monohydrate, and was recovered by recrystallization from aqueous solution. Evaporation of solutions in ethanol and methanol normally gave the amorphous form, but on occasions this crystallized to give the anhydrous form B. A potassium bromide disc prepared from form B showed the presence of some form A, presumably due to traces of moisture in the potassium bromide. A spectrum of this substance in the Sadtler Pharmaceutical collection, recorded as a potassium bromide disc, shows a mixture of form A with the amorphous form.

Sulphadiazine. No evidence of polymorphism was found. The spectrum agreed with those previously published by Hayden, Sammul, Selzer & Carol (1962), Sheinker & Kuznetsova (1957) and in the Sadtler Standard and Pharmaceutical collections.

Sulphadiazine sodium. No evidence of polymorphism was found. The spectrum agreed with that in the Sadtler Pharmaceutical collection.

Sulphadimethoxine. No evidence of polymorphism was found. The

spectrum agreed with those published by Bellomonte, Calo & Cardini (1959) and by Chouteau, Davidovics & Defretin (1963). A spectrum in the Sadtler Pharmaceutical collection shows only broad bands of low intensity and is not recognizable as sulphadimethoxine.

Sulphadimidine. Only one crystalline form was detected. Prolonged grinding with potassium bromide gives an amorphous form with a different spectrum, and partial conversion to this was observed when the material was ground alone in a mechanical mill for 15 min and then examined as a Nujol mull. Spectra of the two forms have been published: in the Sadtler Standard collection (Nujol mull) and by Hayden & others (1962) (potassium bromide disc). A potassium bromide disc spectrum in the Sadtler Pharmaceutical collection shows a mixture of the two forms.

Sulphadimidine sodium. Only solutions in water, ethanol and methanol were investigated, but under various conditions these yielded one amorphous and at least six crystalline forms. The infrared spectra of some of these forms were very similar, though they could be distinguished by their X-ray diffraction patterns, and at least two of the crystalline forms contained residual solvent. All were converted to the same form on heating, provided that no amorphous material was present, though temperatures in excess of 140° were sometimes necessary. No spectrum of this substance has been published.

Sulphafurazole. No evidence of polymorphism was found. The spectrum agreed with those published by Hayden & others (1962) and in the Sadtler Pharmaceutical collection.

Sulphaguanidine. One amorphous and four crystalline forms were identified. The original form (A) was a hydrate, and was recovered by recrystallization from aqueous solution. Form B was sometimes obtained from form A by evaporation of a methanol solution on a water-bath, though on some occasions form A was recovered. Form C was sometimes obtained from form A by evaporation of acetone solution on a water-bath, though more often a mixture of B and C was obtained. On the other hand form D (obtained from B by evaporation of ethanol solution on a water-bath), when treated in the same way, gave a mixture of form D and the amorphous form. The leaflet issued with the B.P.C. Authentic Specimen recommends evaporation of an acetone solution, without specifying any temperature. Under varying conditions this has yielded all five forms, so this is obviously not a good choice of solvent. A potassium bromide disc prepared from form B gave the spectrum of form A (presumably due to moisture in the potassium bromide). After heating this disc the spectrum was intermediate between those of form D and the amorphous form. A spectrum in the Sadtler Pharmaceutical collection is of form A.

Sulphamethizole. No evidence of polymorphism was found in this work, and the spectrum obtained agreed with spectra of two samples published in the Sadtler Pharmaceutical collection. It differed, however, in several respects from that published by Sammul, Brannon & Hayden

(1964), said to be of a potassium bromide disc prepared from material recrystallized from a mixture of ethanol and iso-octane. This solvent treatment was tried but produced no change in spectrum. Another spectrum which differs from both of these has been published by Sheinker, Postovskii, Voronina & Kushkin (1957).

Sulphamethoxydiazine. One amorphous and three crystalline forms were obtained. The original form (A) could usually be recovered by recrystallization from aqueous ethanol. Recrystallization from aqueous solution gave form B, which was converted to form A by heating. Precipitation from acetone solution by addition of water, or from alkaline solution by addition of acid, gave form C. Evaporation of a methanol solution gave the amorphous form, and this was also present in potassium bromide discs prepared from all three crystalline forms. A spectrum of such a disc, corresponding to that prepared from form A, has been published by Chouteau & others (1963).

Sulphamethoxypyridazine. One amorphous and two crystalline forms were identified. The original form (A) was not recovered from any of the solvent treatments used. A second crystalline form (B) was normally obtained by recrystallization from a mixture of ethanol and iso-octane. Most other treatments gave the glassy form, which was also present in potassium bromide discs prepared from forms A and B. Spectra of potassium bromide discs published by Hayden & others (1962) and by Chouteau & others (1963) show mixtures of form B and the amorphous form. A Nujol mull spectrum of form A has been published by Bellomonte & others (1959). The potassium bromide disc spectrum in the Sadtler Pharmaceutical collection shows the presence of all three forms.

Sulphanilamide. The existence of three forms of sulphanilamide was reported by Watanabe (1942) and methods of preparation were described by Yakowitz (1948). Spectra of two forms described as B and C were published by Barnes & others (1943). The Authentic Specimen as received gave the spectrum of form B, and was converted to form C by heating or by evaporation of ethanol solution on a water-bath. A third form (presumably A) was obtained together with form B when a methanol solution was evaporated to dryness in a stream of air at room temperature. This form was not obtained in a pure state. The leaflet issued with the B.P.C. Authentic Specimen recommends evaporation of an acetone solution. This normally gives a mixture of forms B and C. Apart from the spectra of Barnes & others (1943), spectra of form B have been published by Chouteau & others (1963) and in the Sadtler Standard and Pharmaceutical collections. A spectrum published by Hayden & others (1962) corresponds to a mixture in which form C predominates.

Sulphaphenazole. No evidence of polymorphism was found. The spectrum agreed with those of Bellomonte & others (1959) and of Sammul & others (1964).

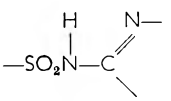
Sulphapyridine. One amorphous and six crystalline forms were identified. The first sample examined consisted mainly of form A, together with a small amount of another form, designated F, which was not otherwise encountered; a second sample was entirely form A. Pure form A was obtained by heating forms B, D and F to 140°. Form C was only partially converted to A and the effect of heat on form E was not investigated. Form B was obtained from A by recrystallization from a mixture of ethanol and iso-octane, but other forms gave either form C or mixtures. Form C was obtained on one occasion when a chloroform solution of form A was allowed to evaporate overnight. Evaporation of a similar chloroform solution in a current of air at room temperature gave form D. Form E was obtained when a solution of form C in acetone was evaporated in a similar manner; when form A was treated in this way a mixture of forms A and B was obtained. Forms A, B and C were not affected by grinding with potassium bromide, but a potassium bromide disc prepared from form D gave the spectrum of the amorphous form. Spectra published by Sheinker & Kuznetsova (1957) and in the Sadtler Pharmaceutical collection are of form A.

Sulphasomidine. Only one crystalline form was encountered, and this could be recovered by recrystallization from aqueous solution. Evaporation of solutions in ethanol and methanol sometimes gave an amorphous form, and other treatments were liable to produce mixtures. No spectrum of this substance has been published.

Sulphathiazole. Two crystalline forms of sulphathiazole were reported by Grove & Keenan (1941) and three by Miyazaki (1947). In the present work an amorphous form and three crystalline forms were encountered, and from their manner of preparation the forms now designated A, B and C appear to correspond to Miyazaki's α, β and α'. Carless & Foster (1966) have recently shown by differential thermal calorimetry that the material distributed by the Pharmaceutical Society of Great Britain, i.e. the B.P.C. Authentic Specimen, contains two or more forms. The presence of forms A and C has been confirmed, though their infrared spectra are in fact very similar. From this mixture, form A was recovered by recrystallization from a mixture of acetone and chloroform and form C was obtained by recrystallization from dilute ammonia solution. Recrystallization from ethanol, n-propanol or isobutanol gave substantially form B. Evaporation of an ethanol solution to dryness, recommended in the leaflet accompanying the B.P.C. Authentic Specimen, gave the amorphous form, which on standing was liable to crystallize spontaneously as either form B or form C. Evaporation of a methanol solution of the original mixture gave form C, but the same treatment with a solution of form B gave form B unchanged. Spectra published in the Sadtler Standard and Pharmaceutical collections are both of form A; that given by Sheinker & others (1957) appears to be substantially form C.

General discussion

The incidence of polymorphism amongst these sulphonamides appears to be even more prevalent than with the steroids (Mesley & Johnson, 1965) or barbiturates (Cleverley & Williams, 1959). Moreover, reproducible interconversion between different forms is more difficult to achieve than in the instance of the steroids. It has been shown (Mesley, 1966) that the polymorphic forms of the steroids frequently differ in the type of hydrogen bonding between the molecules in the lattice, and similar differences might be expected with the sulphonamides. However, in many of the sulphonamides tautomerism can also occur, between the amide form (I) and the imide form (II), and both forms are known to



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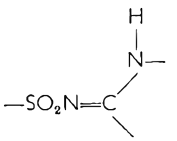

II

occur in solution. On the basis of their infrared spectra, Sheinker & others (1957) postulated that sulphamethizole and sulphathiazole both exist in the solid state in the imide form, and Sheinker & Kuznetsova (1957) suggested that sulphadiazine and sulphapyridine were also imides, whereas sulphacetamide has the amide structure. These infrared assignments were disputed by Uno, Machida, Hanai & others (1963), who used deuteration to show that sulphadiazine was in fact an amide, although they agreed with the imide structure for sulphapyridine and sulphathaziole. Schwenker (1962) has shown that sulphaguanidine also has the imide structure in the solid phase.

In the light of these findings it might be anticipated that where polymorphism occurs some forms might be amides whilst others are imides. However, no spectral differences have been observed between polymorphic forms of the same substance which could be ascribed to amide–imide tautomerism, and it must therefore be assumed that the high incidence of polymorphism amongst the sulphonamides is due mainly to the variety of hydrogen bonding possibilities.

With a number of these substances it was found that individual forms responded differently to the same solvent treatment. This is difficult to explain without assuming that particular types of association between molecules can persist in solution. In fact, association of sulphonamide molecules in solution has been demonstrated by Chaplin & Hunter (1937) and by Baxter, Cymerman-Craig & Willis (1955), though in very dilute solution the proportion of associated molecules is usually small. Nevertheless these associated molecules probably form nuclei round which crystals are formed and thus dictate the form in which the substance crystallizes. When different crystalline forms are dissolved in the same solvent it may be possible for the two types of association to persist in solution, giving rise to different solid forms (usually, but not necessarily, those with which one started) when the solid is evaporated. This behavi-

our was noticed with prednisolone (Mesley & Johnson, 1965) and has now been observed with succinylsulphathiazole, sulphadimidine sodium, sulphaguanidine, sulphamethoxydiazine, sulphanilamide, sulphapyridine and sulphathiazole. Although in most of these cases the solutions were evaporated to dryness, in some instances this effect was also found when saturated solutions were allowed to crystallize.

Difficulty was also encountered in obtaining reproducible results when solutions of the same form were evaporated to dryness. In some instances this was due to the solid initially appearing as a glass which subsequently crystallized spontaneously in one of two or more forms. In others the temperature at which crystals were produced or to which they were subsequently heated may have been critical. This trouble may be overcome by recrystallizing from a saturated solution rather than evaporating the solution to dryness.

RECOMMENDED PROCEDURES

In establishing a procedure for obtaining a consistent solid form, it is desirable that the whole of the material should be recovered, so that impurities, if present in the sample, will still be present when the infrared spectrum is recorded. Owing to the behaviour mentioned above this is not always possible, and recrystallization may be necessary, with a possible loss of impurities. In a few instances particular forms are so stable that they can only be converted to a common form by chemically altering the molecule (e.g. by forming the sodium derivative) and then recovering the substance by precipitation.

When two samples of the same substance are to be compared, in most instances it will be found that they give the same infrared spectrum without any recourse to solvent treatment. If two samples give different spectra but are thought to be of the same substance, then both should be subjected to the treatment suggested below for that substance. These treatments were found to be effective for all the forms obtained in this work; there is a possibility that other forms may be encountered, and whilst these treatments would probably still be effective, this cannot be guaranteed.

Phthalylsulphathiazole. Dissolve in minimum of sodium hydroxide solution, neutralize with dilute hydrochloric acid, filter, wash precipitate with water and dry. Examine as Nujol mull.

Succinylsulphathiazole. Dissolve in minimum of sodium hydroxide solution, neutralize with dilute hydrochloric acid, filter, wash precipitate with water and dry without heating above 100°. Examine as Nujol mull.

Sulphacetamide sodium. Recrystallize from water. Dry without heating above 100°. Examine as Nujol mull or halide disc.

Sulphadimidine. Dissolve in ethanol, acetone or chloroform and evaporate solution to dryness on water-bath. Examine as Nujol mull.

Sulphadimidine sodium. Dissolve in water and evaporate solution to dryness. If spectra still differ, convert to sulphadimidine by addition of

dilute hydrochloric acid to aqueous solution, filter, wash precipitate with water and dry. Examine as Nujol mull.

Sulphaguanidine. Recrystallize from water, filter and dry crystals at room temperature in current of air or under vacuum. Examine as Nujol mull or halide disc.

Sulphamethoxydiazine. Recrystallize from 50% aqueous ethanol. If spectra still differ, heat to 140° for 15 min. Examine as Nujol mull.

Sulphamethoxypyridazine. Dissolve in minimum amount of hot ethanol, add an equal volume of iso-octane (2,2,4-trimethylpentane) and evaporate to dryness on water-bath. The product should be white (a yellow colour indicates the presence of amorphous material, in which case the treatment should be repeated). Examine as Nujol mull.

Sulphanilamide. Dissolve in ethanol, evaporate solution to dryness on water-bath. Examine as Nujol mull.

Sulphapyridine. Heat to 140° for 15 min. If spectra still differ, dissolve in minimum of sodium hydroxide solution, add dilute hydrochloric acid dropwise until neutral to litmus paper (material is soluble in excess acid), filter, wash precipitate with a little cold water and dry. Examine as Nujol mull.

Sulphasomidine. Recrystallize from water. Examine as Nujol mull or halide disc.

Sulphathiazole. Recrystallize from n-propanol. Examine as Nujol mull or halide disc.

It will be noted that in many of these suggested treatments it is recommended that the substance be examined as a mull. This is due to the instability of many of the crystalline forms when ground and pressed with potassium bromide. Indeed, of the twelve substances listed above, ten showed changes in the spectrum of at least one form when examined as potassium bromide discs.

Acknowledgements. Thanks are due to Mr. C. A. Johnson of the British Pharmacopoeia Commission for confirming many of the results.

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J. Pharm. Pharmac., 1967, **19**, 305–312 Received November 23, 1966

Histidine decarboxylase in the stomach of the rat

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Two enzymes capable of decarboxylating L-histidine *in vitro* have been identified in rat and mouse stomach; one, located in the fundic portion, shows maximal activity at a pH value of 5·6, whilst the other, in the pyloric portion, requires a pH of 7·6 for optimal activity. The enzyme in rat fundus is stable when stored at low temperatures and is inhibited only slightly by benzene, α-methyldopa and α-methylhistidine; the pyloric enzyme, on the other hand, is rapidly destroyed on storage at low temperatures, and is slightly stimulated by benzene but much inhibited by α-methyldopa and α-methylhistidine. Dopa and 5-hydroxytryptophan compete with the substrate for the pyloric histidine decarboxylase but have no effect on the fundic enzyme. Starvation inhibits the activity of the fundic enzyme but has only a slight effect on the pyloric enzyme. It is concluded that the two enzymes in the stomach capable of forming histamine differ from the specific and non-specific histidine decarboxylases found in other tissues.

At least two enzymes capable of forming histamine *in vitro* have been described in recent years; one is specific for L-histidine whereas the other is non-specific and decarboxylates other aromatic amino-acids as well as L-histidine (Lovenberg, Weissbach & Udenfriend, 1962). It is probable that the specific enzyme is the more important for the *in vivo* formation of histamine (Dawson, Maudsley & West, 1965). The properties of the histidine decarboxylase in rat stomach have not been fully established although it has generally been considered to be a specific enzyme for L-histidine (Waton, 1956; Schayer, 1957; Telford & West, 1961a). Recently, Håkanson & Owman (1966) showed that the enzyme in rat stomach is also capable of decarboxylating dihydroxyphenylalanine (dopa) and 5-hydroxytryptophan (5-HTP).

The present work was designed to determine the nature of histidine decarboxylase in rat stomach responsible for the formation of histamine *in vitro*. Code (1965) concluded that histamine is the final common local chemostimulator of the parietal cells of the gastric mucosa, and so an attempt has been made to study the relationship between histidine decarboxylase activity and gastric function.

Experimental

Male Sprague-Dawley rats weighing 150–170 g were used in all experiments. They were fed on 41B cube diet, allowed drinking water *ad libitum*, and housed at 70° ± 1° F (21° C). At different times, groups of at least seven animals were killed by a blow on the head and rapidly decapitated.

Preparation of stomach extracts. The stomachs were removed, opened along the line of the lesser curvature, washed with isotonic saline (0·9% w/v sodium chloride solution), blotted dry on filter paper, weighed, cut into small pieces with scissors, and homogenized with isotonic saline

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(5 ml/g) in a glass homogenizer. After centrifugation at 5,000 × *g* in a refrigerated centrifuge for 15 min, aliquots of the supernatant (equivalent to 50 or 100 mg tissue) were removed for the incubation experiments. On other occasions, the stomachs were divided into two portions; the thin fundic part was separated from the thick pyloric part, an intermediate area about 2 mm wide on either side of the demarcation line being discarded.

Histidine decarboxylase activity of the extracts. This was estimated using the method developed by Kobayashi (1963), in which the tissue extract is incubated with carboxyl-labelled histidine under standard conditions and the $^{14}CO_2$ formed is estimated in a counter. The incubation mixture consisted of [*carboxyl*-^{14}C]DL-histidine (0·1 μc in 0·1 ml distilled water containing 10 μg L-histidine), stomach extract equivalent to 50 or 100 mg tissue, streptomycin (25 mg in 0·1 ml water), and phosphate buffer (M/15) sufficient to produce a final volume of 3 ml. Streptomycin was used to inhibit bacterial decarboxylation of histidine occurring during the incubation (Callingham, Kobayashi, Maudsley & West, 1965). When no tissue was present, the results obtained were similar to those found when using boiled tissue; they represent the blank values. Incubation was allowed to proceed for 2 hr at 37° in a shaking incubator, during which time the evolved $^{14}CO_2$ was absorbed on to filter paper impregnated with hyamine hydroxide. The reaction was then stopped by the addition of 0·3 ml M citric acid, but shaking continued for a further hour to allow the hyamine to absorb completely the $^{14}CO_2$. The radioactivity of the filter paper was determined in a Packard Tricarb liquid scintillation counter (at an efficiency of counting of 70%); a coefficient of variation of reproducibility of about 1% was obtained by allowing time for 10,000 counts to accumulate. Each value in the figures is the mean of at least two separate estimations and has been corrected for the blank value.

Histamine content of the tissues. The method used was that described by Parratt & West (1957). Briefly, the tissues were extracted with 10% w/v trichloroacetic acid (5 ml/g), the excess acid was removed by ether, and the solutions were assayed on the isolated atropinized guinea-pig ileum. The specificity of the responses was confirmed by mepyramine maleate. The values of histamine refer to the base.

Starvation. Food was withheld from a group of rats for 24 hr and the histidine decarboxylase activities of their stomachs were then estimated and compared with those of freely-fed animals.

Results

Effect of pH on the enzyme activity of rat stomach. The histidine decarboxylase activity shows two peaks (Fig. 1); the higher peak has an optimal pH value of about 5·6 whilst the lower peak occurs at about pH 7·6.

Only one peak of activity is present in each of the extracts made after dividing the stomach into two portions (Fig. 2). The optimal pH value of the fundic portion is about 5·6 whilst that of the pyloric portion is

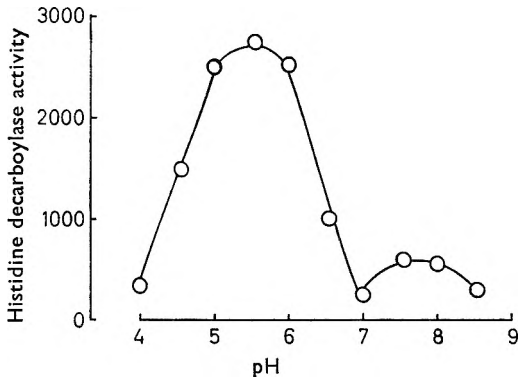

FIG. 1. The effect of pH on the histidine decarboxylase activity of rat stomach, expressed as counts/min/100 mg tissue. Note the two peaks of activity at pH values of 5·6 and 7·6.

about 7·6. These optimal pH values have been used in all subsequent incubation experiments. The results in Fig. 2 also show that the maximal enzyme activity in the fundic portion is about 10 times that in the pyloric part.

FACTORS AFFECTING THE REACTION RATE OF THE TWO ENZYMES

Substrate concentration. When the radioactive histidine is diluted with known amounts of non-radioactive L-histidine (10–1,000 μg) before incubation, and the $^{14}CO_2$ evolved is measured after incubation, estimates can be made of the amounts of histamine formed. For the pyloric enzyme, there is a linear relationship at pH 7·6 between the amount of substrate and the enzyme activity but the graph of activity against substrate concentration for the fundic enzyme at pH 5·6 is not linear but hyperbolic

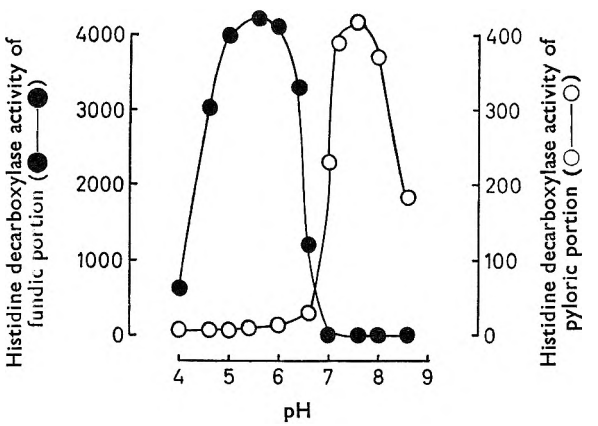

FIG. 2. The effect of pH on the histidine decarboxylase activities of the fundic (●——●) and pyloric (○——○) portions of rat stomach, expressed as counts/min/50 mg tissue. Note the different scales.

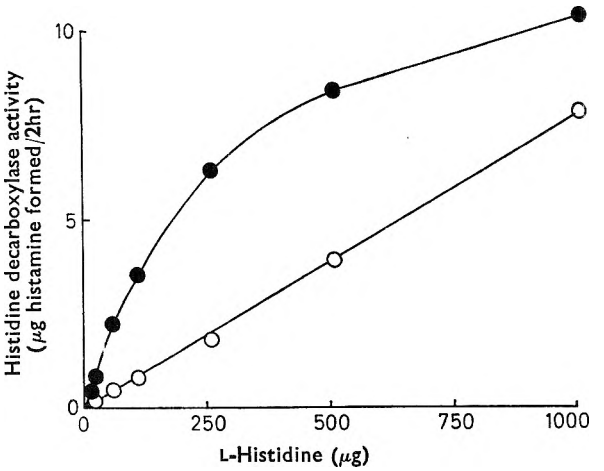

FIG. 3. The effect of substrate concentration on the histidine decarboxylase activities of the fundic (●——●) and pyloric (○——○) portions of rat stomach, expressed as μg histamine formed by 50 mg tissue in 2 hr.

(see Fig. 3). The substrate affinity of the enzyme in the fundic portion ($K_m = 1{\cdot}07 \times 10^{-4}$ g/ml, about 7×10^{-4} M) is nearly three times that of the pyloric portion ($K_m = 2{\cdot}67 \times 10^{-4}$ g/ml, about $1{\cdot}7 \times 10^{-3}$ M).

Enzyme concentration. When the enzyme concentration in the incubation mixture is increased by using more tissue extract, there are similar increases in the rates of the reaction of both preparations when measured at their optimal pH values (see Fig. 4).

Incubation time. There are linear relationships between times of incubation and enzyme activity of the two portions of stomach when estimates are made at the optimal pH values (Fig. 5).

Storage. The pyloric enzyme activity rapidly disappears when extracts are kept at −15° and only 10% of the activity remains after

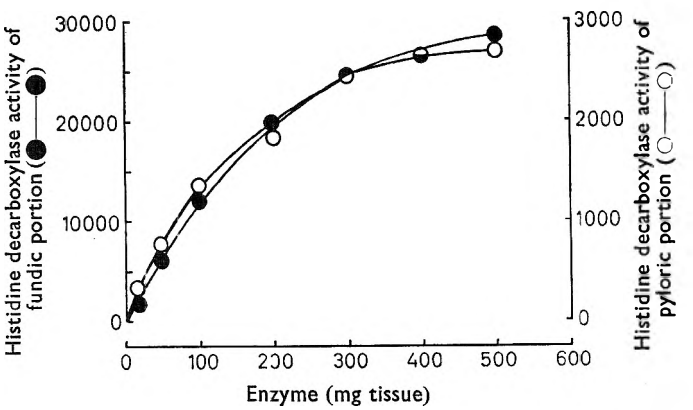

FIG. 4. The effect of enzyme concentration on the histidine decarboxylase activities of the fundic (●——●) and pyloric (○——○) portions of rat stomach, expressed as counts/min. Note the different scales.

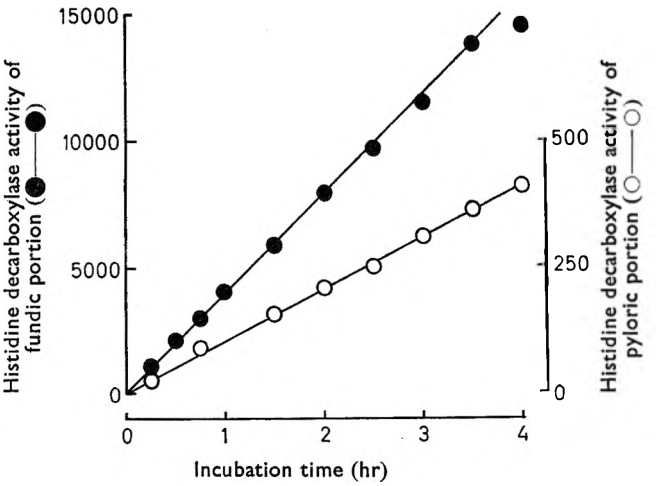

FIG. 5. The effect of incubation time on the histidine decarboxylase activities of the fundic (●——●) and pyloric (○——○) portions of rat stomach, expressed as counts/min/50 mg tissue. Note the different scales.

14 days. On the other hand, the fundic enzyme is little affected by this treatment (Fig. 6).

Effect of benzene. Benzene (20 mg) potentiates the histidine decarboxylase activity of the pyloric portion by about 20% but has little or no effect on the enzyme in the fundic portion.

STUDIES ON ENZYME SPECIFICITY

Effect of α-methylhistidine and L-*α-methyldopa.* Both substances inhibit the histidine decarboxylase activity of the pyloric portion but have

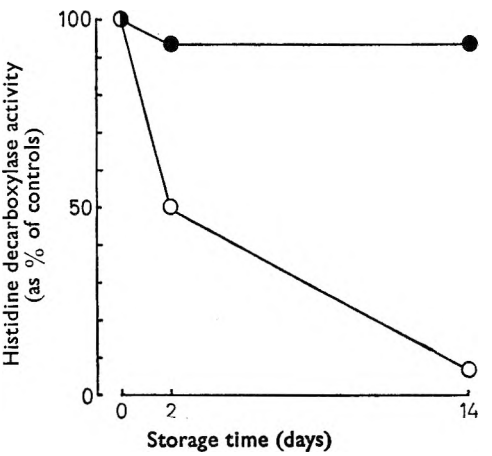

FIG. 6. The effect of storage at −15° C on the histidine decarboxylase activities of the fundic (●——●) and pyloric (○——○) portions of rat stomach, expressed as percentages of control values.

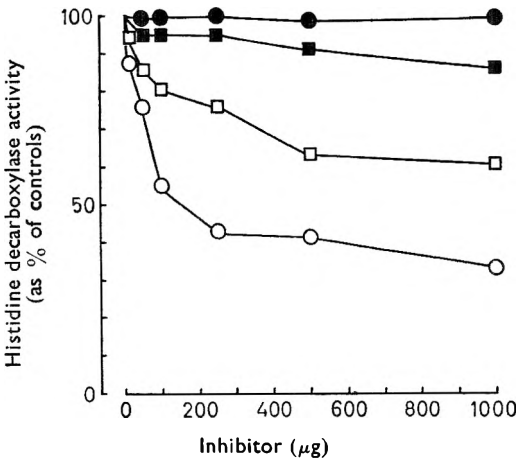

FIG. 7. The effect of L-α-methyldopa (circles) and α-methylhistidine (squares) on the histidine decarboxylase activities of the fundic (solid symbols) and pyloric (open symbols) portions of rat stomach, expressed as percentages of control values.

little effect on that in the fundic portion, even when used in concentrations up to 1,000 μg in the incubation mixture (see Fig. 7).

Effect of dopa and 5-hydroxytryptophan. Both substances have no effect on the fundic histidine decarboxylase but compete with the substrate, L-histidine, for the pyloric enzyme, markedly reducing the output of $^{14}CO_2$ (Fig. 8).

Stereospecificity. Non-radioactive L-histidine added to the incubation mixture of each enzyme reduces the output of $^{14}CO_2$ due to reduction of the specific activity of the original mixture. On the other hand, D-

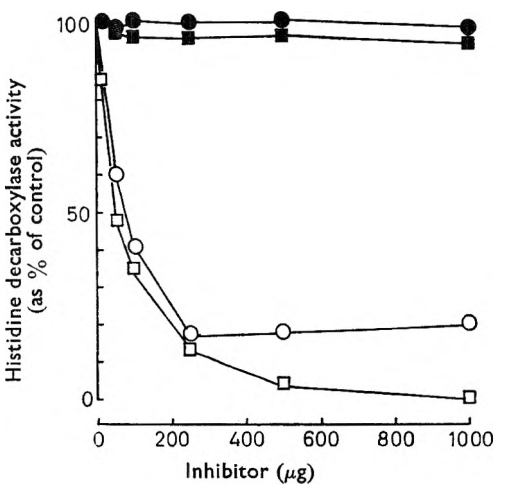

FIG. 8. The effect of DL-dopa (squares) and DL-5-hydroxytryptophan (circles) on the histidine decarboxylase activities of the fundic (solid symbols) and pyloric (open symbols) portions of rat stomach, expressed as percentages of control values.

histidine in concentrations up to 1,000 μg in the incubation mixture of 3 ml has no effect on either enzyme.

EFFECT OF STARVATION

The enzyme activity of the fundic portion is markedly inhibited by starvation, only 3% of the total activity remaining; in the pyloric portion, however, activity is only reduced to 79% of the control value. Whereas the histamine content of the fundic portion is unaffected by starvation (control value, 4 μg/g), that in the pyloric portion is reduced by 33% (control value, 36 μg/g). It is thus possible that the histamine in the fundic portion is normally kept at a low basal level and the histamine formed by the high enzyme activity in this portion of the stomach is catabolized at a fast rate or transported to other parts of the body.

Histidine decarboxylase in mouse stomach. As in rat stomach, two enzymes capable of forming histamine have been identified in mouse stomach. One resides in the fundic portion and has an optimal pH value for activity of 5·6; the other in the pyloric part has an optimal pH value between 7·6 and 8·0 (see Fig. 9).

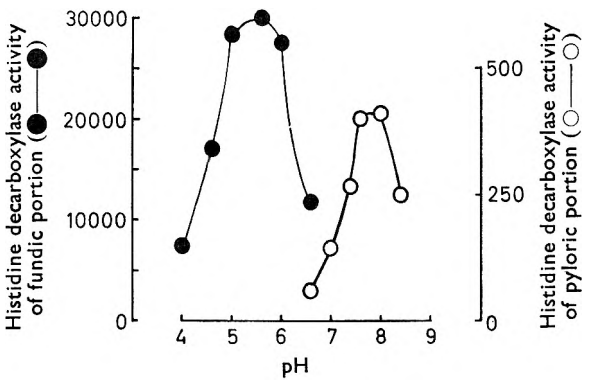

FIG. 9. The effect of pH on the histidine decarboxylase activities of the fundic (●——●) and pyloric (○——○) portions of mouse stomach, expressed as counts/min/50 mg tissue. Note the different scales.

Discussion

The results of the present work show that an enzyme capable of forming histamine *in vitro* resides in the thin fundic portion of rat stomach and that it differs in reactivity from the enzyme located in the thick muscular pyloric portion. The failure to find enzyme activity in the fundic portion (Håkanson & Owman, 1966) may be the result of making incubation experiments either at pH values other than the optimal (pH 5·6) or after fractionation with ammonium sulphate which markedly reduces activity.

The fundic enzyme has many of the characteristics of the specific histidine decarboxylase found in rat foetal liver (Telford & West, 1961b) and in rat hepatoma (Mackay, Riley & Shepherd, 1961). It has a high

affinity for the substrate (K_m of 7×10^{-4} M), and requires an acid pH value (pH of 5·6) for optimal activity; it is only slightly inhibited by α-methylhistidine which is considered to be one of the more potent and specific inhibitors of the specific enzyme (Robinson & Shepherd, 1962).

The pyloric enzyme has many of the characteristics of the non-specific histidine decarboxylase; it requires an alkaline pH value (pH of 7·6) for optimal activity, it is inhibited by α-methyldopa, it is able to use dopa and 5-HTP as substrates, and it is potentiated by benzene (Telford & West, 1961a; Schayer, 1963). However, it is inhibited by α-methylhistidine and has a higher affinity for the substrate (K_m of $1{\cdot}7 \times 10^{-3}$ M) than does the non-specific enzyme (Mackay & others, 1961).

The fundic enzyme is greatly reduced in activity by a physiological stress such as starvation whereas this procedure has little effect on the pyloric enzyme. As the optimal pH value for activity of the fundic enzyme is near to that of rat stomach secretion (pH 4·5; Lane, Ivy & Ivy, 1957) and it is more than 10 times as active as the pyloric enzyme, the fundic enzyme has to be considered as a major source of gastric histamine. Further work is, however, needed to show that these results obtained with *in vitro* tests apply in the intact animal.

Levine (1965) found that oesophageal ligation (thereby preventing food from entering the stomach) lowers gastric acid secretion in the rat by 97%. Starvation in the present work reduced the histidine decarboxylase activity of the fundic portion of the stomach by about the same amount. This indicates a relationship between the enzyme activity in the fundic portion and the rate of acid secretion, and supports the view of Code (1965).

Acknowledgements. We wish to thank Dr. R. W. Schayer for a gift of α-methylhistidine, Dr. Y. Kobayashi and Dr. D. V. Maudsley for helpful discussions, and Miss Elizabeth Hide for her technical assistance.

A. G. Radwan is a U.A.R. scholar.

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J. Pharm. Pharmac., 1967, **19**, 313–317 Received November 18, 1966

Noradrenaline-phospholipid interactions

A. W. CUTHBERT, B. A. CALLINGHAM, SYLVIA WARREN AND ELISABETH PAINTER

The uptake of (±)-[^{3}H]noradrenaline from an aqueous phase to an ether phase containing dissolved lecithin has been measured. No differences between the behaviour of (+)- or (−)-noradrenaline in this system could be detected. The biological implications of this finding are discussed.

ASSOCIATION of noradrenaline with phospholipids has received sporadic attention. It was found that phospholipids were able to remove catecholamines from the aqueous to the hydrophobic phase in biphasic systems containing chloroform (Kendall, 1942) or ether (Euler, 1946a, b; Norlander, 1950). Others (Mass & Colburn, 1965) found that noradrenaline interacted with phospholipids and metal ions to give ether-soluble complexes.

More recently membrane lipids have been implicated in the interactions of catecholamines with their receptors. For instance, Dikstein & Sulman (1965) found that when rabbit aortic strips were treated with dibenamine the drug was bound to a cephalin fraction. Adrenaline was able to protect the cephalin from interactions with dibenamine. Most recently, Naylor (1966) found that catecholamines facilitated the transfer of calcium ions to the chloroform phase in a biphasic system containing heart lipids. The opposite effect was observed with the β-blocking drugs, propranolol and pronethalol.

Thus, various authors have concluded that lipids are involved in catecholamine uptake, storage and receptor interaction. One simple way to test this hypothesis is to examine whether the two stereoisomers of noradrenaline show unequal properties in model systems. The interaction between catecholamines and their receptors is certainly stereospecific, as is the uptake of catecholamines into sympathetic nerve terminals at physiological concentrations (Iversen, 1963; Beaven & Maickel, 1964).

Experimental

Two types of experiments were made. First, the uptake of [^{3}H]-noradrenaline by lecithin in ether at a constant catecholamine concentration, but with a varying ratio of labelled to unlabelled material, was measured. Second, the uptake by lecithin of [^{3}H]noradrenaline at increasing concentrations of unlabelled noradrenaline was determined, the concentration of the labelled material remaining constant.

In all instances the biphasic system consisted of 4 ml lecithin-ether solution (10 mg/ml) with 10 ml of aqueous phase. The aqueous phase consisted of 5 ml 0·2M phosphate buffer (pH 6·5) and 5 ml 0·01N hydrochloric acid containing the dissolved noradrenaline. After preparation the systems were shaken gently for 15 min and then allowed to separate into two phases. One ml samples of the ether phase were added to 3 ml ethanol and 10 ml phosphor [toluene with 4 g/litre 2,5-diphenyl-

From Department of Pharmacology, University of Cambridge.

oxazole and 100 mg/litre 1,4-bis-2(5-phenyloxazolyl)-benzene] and the radioactivity measured in a liquid scintillation counter (Nuclear Chicago). Controls for uptake of radioactivity by ether alone, inherent radioactivity of ether and lecithin and for variations in counting efficiency were made.

The lecithin used was highly purified egg lecithin supplied by Mann Research Laboratories Inc. The [^{3}H]noradrenaline was supplied by the Radiochemical Centre, Amersham, and was the racemic form.

Results

UPTAKE OF [^{3}H]NORADRENALINE BY LECITHIN AT CONSTANT TOTAL NORADRENALINE CONCENTRATION

In these experiments the total noradrenaline concentration was 200 ng/ml in the aqueous phase of which the labelled material constituted 0·395 to 12·6 ng/ml (2·5 to 80 mμc/ml). In separate experiments the unlabelled noradrenaline was either the (+)- or (−)-isomer. In Fig. 1 the uptake of [^{3}H]noradrenaline in the presence of (+)- and (−)-noradrenaline is shown, and is compared with that obtained in the absence of unlabelled noradrenaline.

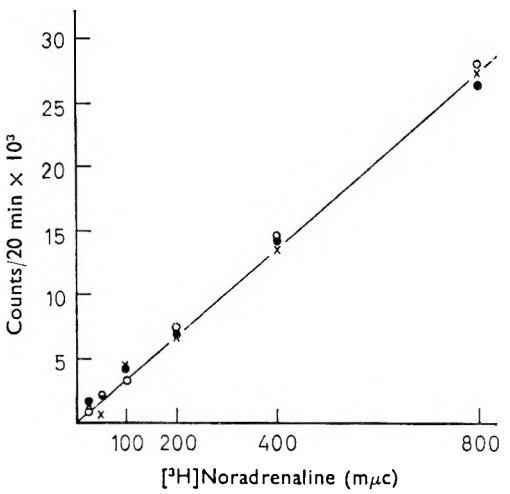

FIG. 1. Uptake of [^{3}H]noradrenaline by 1 ml of ether-lecithin phase with varying concentrations of [^{3}H]noradrenaline in the aqueous phase. ● [^{3}H]noradrenaline alone, ○ in the presence of (−)-noradrenaline and × in the presence of (+)-noradrenaline.

It is obvious that the uptake of [^{3}H]noradrenaline by lecithin is the same in the presence of (+)- or (−)-noradrenaline, or in their absence. From this certain conclusions can be drawn. First, from the linear uptake curve obtained in the absence of unlabelled noradrenaline it is clear that,

$$\text{Uptake} = K \times [[^3\text{H}]\text{noradrenaline}]$$

Provided the concentration of noradrenaline is much smaller than the concentration required to produce saturation then uptake in the presence of unlabelled noradrenaline will be,

$$\text{Uptake} = K \times [[^3\text{H}]\text{noradrenaline}] + K' \times [(-)\text{-noradrenaline}] + K'' \times [(+)\text{-noradrenaline}]$$

that is, the uptake of radioactivity will be unaltered. As the total amount of catecholamines remained constant in these experiments, Fig. 1 may be interpreted to mean that (*a*) (+)- and (−)-noradrenaline show an equal affinity for lecithin, or (*b*) either (+)- or (−)-noradrenaline show no affinity for lecithin, that is the curve obtained is identical with that for [^{3}H]noradrenaline alone.

Although the latter possibility is unlikely, the data of Fig. 1 must be analysed further. Conversion of counts/min to disintegrations/min allows the uptake of [^{3}H]noradrenaline to be calculated as a percentage of the total catecholamine present. The results are presented in Fig. 2.

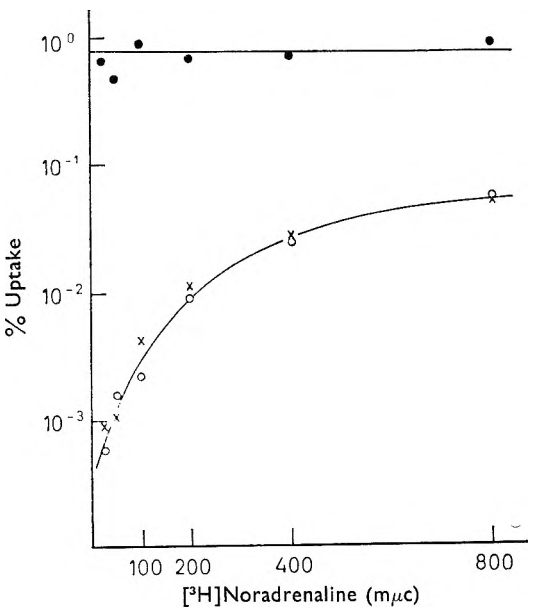

FIG. 2. Uptake of [^{3}H]noradrenaline, expressed as a percentage of the total amount of catecholamine present, with varying concentrations of [^{3}H]noradrenaline. ● [^{3}H]noradrenaline alone, ○ in the presence of (−)-noradrenaline, and × in the presence of (+)-noradrenaline.

In the absence of unlabelled noradrenaline the percentage uptake of tritiated material remained constant, 1 ml of the ether-lecithin phase taking up 0·8% of the total catecholamine present in the aqueous phase. In the presence of (+)- or (−)-noradrenaline the percentage uptake of labelled material was reduced by equal amounts. From this it must be concluded that both (+)- and (−)-noradrenaline show an equal affinity for lecithin.

UPTAKE OF [^{3}H]NORADRENALINE AT CONSTANT CONCENTRATION BY LECITHIN WITH INCREASING CONCENTRATIONS OF UNLABELLED (+)- OR (−)-NORADRENALINE

In these experiments the concentration of [^{3}H]noradrenaline in the aqueous phase remained constant at 10 mμc/ml. Noradrenaline in the aqueous phase is able to react reversibly with lecithin dissolved in

the ether phase thus,

$$L + N \rightleftharpoons LN$$

Control experiments showed that ether alone was only able to take up minute amounts of noradrenaline from the aqueous phase at pH 6·5, so that almost all the noradrenaline taken up by the ether phase is associated with lecithin. From mass action considerations it is known that the linear relation between uptake and concentration will no longer hold as saturation is approached. The decrease of the proportionality constant is related to the reduced uptake of [^{3}H]noradrenaline in the presence of increasing concentrations of (−)-noradrenaline. The reduction in uptake of [^{3}H]noradrenaline in the presence of increasing concentrations of (−)-noradrenaline is shown in Fig. 3. Assuming an initial proportionality constant of 0·8% (from Fig. 2) the amount of (−)-noradrenaline taken up per ml of ether-lecithin solution can be calculated for various concentrations of (−)-noradrenaline. The calculated values are shown in Table 1 and have been plotted as a second curve in Fig. 3.

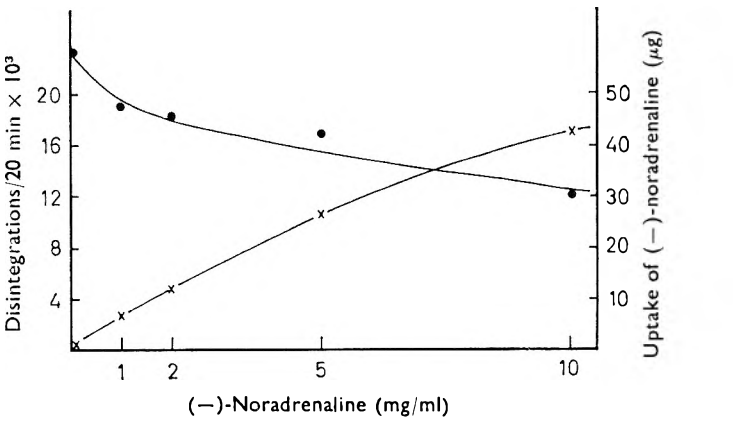

FIG. 3. Uptake of [^{3}H]noradrenaline with varying (−)-noradrenaline concentrations (●). Calculated uptake of (−)-noradrenaline per ml of ether-lecithin phase from 1 ml aqueous phase with varying (−)-noradrenaline concentrations (×).

From the uptake curve of Fig. 3 it is clear that the system is not fully saturated even when the concentration of (−)-noradrenaline in the aqueous phase was 10 mg/ml. From these results it is concluded that the concentration of (−)-noradrenaline producing 50% saturation of lecithin is at least 5 mg/ml.

TABLE 1.

Concentration of (−)-noradrenaline in aqueous phase mg/ml	[^{3}H]noradrenaline uptake % compared with value obtained in absence of (−)-noradrenaline	Calculated uptake of (−)-noradrenaline (μg) per ml ether-lecithin from 1 ml aqueous phase
0	100·0	0
1	83·5	6·7
2	76·2	12·2
5	66·7	26·7
10	53·3	42·6

Discussion

These experiments have shown that (+)- and (−)-noradrenaline have an equal ability to associate with lecithin. If this is indeed the case for other compound lipids then little biological significance can be attached to these interactions if the biological process itself shows stereospecificity.

Calculation of the equilibrium constant for the reaction

$$\text{lecithin} + \text{noradrenaline} \rightleftharpoons \text{lecithin-noradrenaline complex}$$

shows this to be around 33, which makes the standard free energy of formation of the complex (ΔF°) −2·0 kcal. Free energies of this order imply that only weak physical forces, such as van der Waals' forces, are involved; also it is unlikely that the formation of such a complex would be stereospecific.

The uptake of catecholamines into sympathetic nerve terminals when the former are in high concentration shows no stereospecificity (Iversen, 1965). It is possible that complex formation between phospholipids and noradrenaline play a part in this process.

It can be argued that the uptake of noradrenaline by our system is due to the solubilization of the aqueous phase by lecithin micelles, such a process would not be expected to show stereospecificity. We can make a reasonable estimate of the extent of solubilization in the following way. Elworthy & McIntosh (1964) showed that lecithin micelles in benzene could solubilize water to the extent of 0·33 g/g lecithin. If we assume this figure for our results then the percentage of uptake due to solubilization varies from 5 to 8%. Consider for instance the uptake of noradrenaline by 1 ml of lecithin-ether solution from 10 ml of noradrenaline solution (10 mg/ml). This has been estimated as 426 μg. One ml of lecithin-ether solution solubilizes 3·3 mg of aqueous phase, that is, 33 μg noradrenaline or 7·7% of the total uptake. Even if this percentage was much higher, the amount of noradrenaline not taken up in bulk water would be large enough to detect differences between the binding of the two isomers. We conclude therefore, that uptake by solubilization does not alter the interpretation of the results.

Finally, it is interesting to consider the stoichiometry of the lecithin-noradrenaline interaction. If we assume a molecular weight of 750 for lecithin, then at a noradrenaline concentration of 10 mg/ml the complex has a formula of 5·7 lecithin molecules to each noradrenaline molecule.

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J. Pharm. Pharmac., 1967, **19**, 318–321 Received November 16, 1966

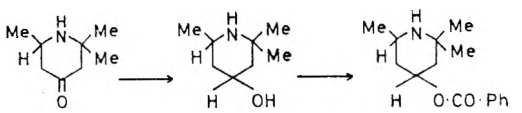
The configuration of β-eucaine and β-isoeucaine

F. PERKS AND P. J. RUSSELL

The configurations of the two isomers, β-eucaine (benzamine hydrochloride B.P.C. 1954) and β-isoeucaine have been deduced. As a result of a study of the nuclear magnetic resonance spectra and of catalytic hydrogenation and oxidation, the α-form has been assigned an equatorial hydroxyl group on C-4 and the β-form an axial hydroxyl group in this position.

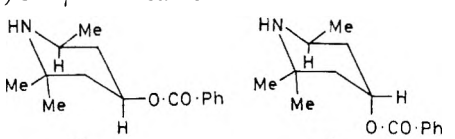
THE compound, β-eucaine (benzamine hydrochloride, B.P.C. 1954; 4-benzoyloxy-2,2,6-trimethylpiperidine hydrochloride) was prepared as a cocaine substitute by reduction of 2,2,6-trimethylpiperid-4-one (I) with sodium amalgam to yield a mixture of two isomers of 2,2,6-trimethyl-4-hydroxypiperidine (II) of melting points 137–138° and 160–163°.

The labile isomer of higher melting point was converted to the stable isomer by refluxing with sodium amylate (Harries, 1897), and this was then *O*-benzoylated to give β-eucaine (III).



I II III

King (1924) prepared and examined β-eucaine and its isomer, β-isoeucaine, in the four optically active and two racemic forms and Stenlake (1954) attempted to deduce the configurations of both β-eucaine and β-isoeucaine by conformational analysis, ascribing to them the structures (IV) for β-eucaine and (V) for β-isoeucaine.



IV V

No experimental evidence was provided to support this view, but it was deduced that the presence in both cocaine and β-isoeucaine of mydriatic activity was due to a similar configuration of the benzoyloxy-group in both molecules. Unfortunately the configuration of cocaine accepted at the time was that of Fodor (Fodor & Nador, 1953), where the benzoyloxy-group was assigned to the axial position. It has since been shown (Fodor, Kovacs & Weisz, 1954) that this group in cocaine occupies the equatorial position. The foregoing conclusions would therefore appear to be invalid and the present work outlines an attempt to deduce the configurations of both isomers of β-eucaine from experimental evidence.

The two isomers of II have melting points of 137–138° and 160–163° and are usually referred to in the literature as the α- and β-forms respectively. This practice is followed in the present report.

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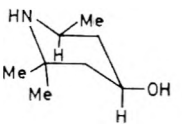
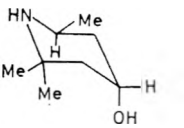
The usual conformational arguments would indicate that in each case the piperidine ring possesses the chair form and that, of the three methyl groups in positions 2 and 6, two will occupy equatorial positions for reasons of steric repulsion and 1,3 interactions. Hence the two possible conformations of 2,2,6-trimethyl-4-hydroxypiperidine will be as shown in VI and VII, i.e. the hydroxyl group in position 4 may be either axial or



VI

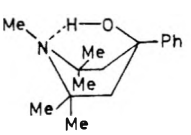


VII

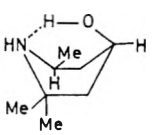
equatorial. The following techniques were employed in attempts to assign a definite configuration to each isomer.

Measurement of pK_a. These were made on a Cambridge pH meter using a glass electrode and calomel reference electrode. α-Form of II, $pK_a = 10{\cdot}0$; β form of II, $pK_a = 10{\cdot}6$, α-*O*-benzoyl derivative, $pK_a = 9{\cdot}4$; β-*O*-benzoyl derivative, $pK_a = 9{\cdot}8$. It might have been expected that the form possessing an equatorial hydroxyl group, i.e. the chair conformer corresponding to VI, would exhibit some degree of hydrogen bonding with the nitrogen atom which would enhance the base strength, but this is not indicated in the pK_a values of the two forms of II which therefore do not contribute any positive evidence to the configuration.

Infrared spectra. These were measured on a Unicam SP.100 I.R. Spectrometer. Lyle (1957) has shown that in 1,2,2,6,6-pentamethyl-4-phenyl-4-hydroxypiperidine (VIII) a band at 3350 cm^{-1} is due to an intramolecular bonded O–H stretch. Zenitz, Martini, Priznar & Nachod (1952) reported a similar band at 3390 cm^{-1} in pseudotropine.



VIII



IX

The fact that the infrared spectra of both forms of II show only a non-bonded –OH frequency at 3630–3640 cm^{-1} is taken to indicate that a stabilized boat structure IX is absent in each case, probably because the secondary amine is a weaker base than the tertiary amino-group of VIII.

N ⇌ O acyl migrations. The migration of acyl groups such as acetyl and benzoyl from nitrogen to oxygen and vice-versa has been employed especially by Fodor & Kovacs (1952) in studies in the tropine series. Accordingly the α- and β-*O*-benzoyl derivatives and the β-*N*-benzoyl derivative of II were prepared (King, 1924) and acyl migrations were attempted (Nickon & Fieser, 1952). No evidence of migration of acyl group was found, the starting material being recovered unchanged.

Oxazine formation. Attempts to form an oxazine by refluxing either the α- or β-form of II with *p*-nitrobenzaldehyde in chlorobenzene (Hardegger & Ott, 1953) were unsuccessful.

Epimerization of II. The α- and β-forms of II were separately refluxed with (*a*) amyl alcohol and (*b*) sodium in amyl alcohol, and the results examined by thin-layer chromatography. With both forms the use of amyl alcohol alone produced no change, but refluxing the β-form with sodium in amyl alcohol produced a virtually quantitative conversion to the α-form. This was confirmed by its nmr and infrared spectra, and by melting point and mixed melting point determination.

Catalytic hydrogenation of 2,2,6-*trimethylpiperidine*-4-*one* (*I*). Catalytic hydrogenation of the ketone (I) in methanol using Adams catalyst gave the β-form of II as shown by thin-layer chromatography, melting point and mixed melting points, and by nmr and infrared spectra. Since the reacting hydrogen atom will attack from the non-hindered side, this is interpreted as indicating that the β-form has an axial hydroxyl group and an equatorial hydrogen atom as in VII.

Oxidation of α- and β-forms of II. It has already been reported (Harries, 1918) that for the *N*-methyl derivatives of II, the α-form is very resistant to oxidation by chromate to the *N*-methyl derivative of the ketone I, whereas the β-form is very readily oxidized. In sterically hindered secondary alcohols it is usual that those isomers having axial hydroxyl groups are more readily oxidized by chromate (Barton, 1964) and this again, indicates that the β-form has the axial hydroxyl group.

Catalytic oxidation. The α- and β-forms of II were catalytically oxidized using platinum catalyst prepared by reduction of Adams platinum dioxide. The β-form absorbed the calculated amount of oxygen in 4 hr, whereas the α-form had only taken up 10% of this after 24 hr. This indicates that the β-form has the more readily oxidizable axial hydroxyl group, which is supported by the observations of Angyal (1963) and Anderson & Post (1963), for the inositols.

Nuclear magnetic resonance. This was measured in pyridine and D_2O solutions on a Perkin Elmer R.10 nmr spectrometer.

Assuming that the structures VI and VII represent the preferred conformations of the α- and β-isomers, the hydrogen atom on C-4 should be readily distinguishable in the nmr spectrum, because of the presence of the hydroxyl group and might be expected to occur at $\tau = 5$ to 6.

On the assumption that a first order approach can be made we would expect VII to show splitting of the hydrogen atom at C-4 by the four H atoms on C-3 and C-5, all at a 60° dihedral angle and hence all being identically coupled. This would be expected to result in a quintuplet of relative magnitudes 1, 4, 6, 4, 1 at regular intervals ($J = 3$ to 5) and for the *O*-benzoyl ester, structure V, a similar prediction is made. Structure VI would be expected to show more complex splitting of the hydrogen atom on C-4 due to: (*a*) coupling with the axial hydrogen atoms on C-3 and C-5 at a dihedral angle of 180° ($J' = 10$ to 12), (*b*) coupling with the equatorial hydrogen atoms on C-3 and C-5, dihedral angle of 60° ($J'' = 3$ to 5), resulting in a nonet of relative magnitudes 1, 2, 1, 2, 4, 2, 1, 2, 1

with intervals of J″, J″, J′ − 2J″, J″, J″, J′ − 2J″, J″, J″. Again, the corresponding *O*-benzoyl ester, structure IV, will show a similar pattern.

Results

The α-form of II gave a nonet centred at 5·85 τ and an expanded trace showed relative magnitudes of 1, 2, 1, 2, 4, 2, 1, 2, 1 at intervals of 4·75, 4·5, 2·1, 4·75, 4·8, 2·25, 4·3 and 5·0 cycles/sec at 60 Mcycles/sec giving J″ = 4·5 to 5·0 cycles/sec and J′ = 11·35 cycles/sec. The *O*-benzoyl derivative of the α-form of II (benzamine) also gave a similar pattern of relative magnitudes, 1, 2, 1, 2, 4, 2, 1·3, 2·3, 1 centred at 4·7 τ, at intervals of 4·5, 4·75, 2, 4·5, 4·5, 1·75, 4·75, 4·5 cycles/sec giving J″ = 4·5–4·75 cycles/sec and J′ = 11–11·5 cycles/sec.

The β-form of II was much less soluble in pyridine and D_2O than the α-form and hence gave a spectrum of rather poorer resolution which showed a quintuplet of relative magnitudes 1, 2·5, 3·25, 2·5, 1 at intervals of 3, 3·1, 3·1, 3 cycles/sec, J = 3 cycles/sec. The *O*-benzoyl ester of the β-form of II (β-isoeucaine) also gave a quintuplet centred at 4·5 τ, of relative magnitudes 1, 4, 6, 4, 1 at intervals of 3, 3, 3, 3 cycles/sec, giving J = 3 cycles/sec. Whilst this work was in progress Chen & LeFevre (1965) published the results of an nmr study of 2,2,6,6-tetramethylpiperidin-4-ol which are in agreement with the foregoing observations.

Discussion

The methods of pK_a measurements, infrared spectra, acyl migration and oxazine formation are inconclusive, the clearest evidence being that obtained by a study of the nmr spectra of the α- and β-forms of II, and of their *O*-benzoyl derivatives. These clearly indicate that the α-form has configuration VI (equatorial hydroxyl) and the β-form that of VII (axial hydroxyl) and that β-eucaine and β-isoeucaine therefore have configurations IV and V respectively.

Acknowledgement. Our grateful thanks are due to Messrs. Burroughs, Welcome Ltd. for the generous gift of materials.

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J. Pharm. Pharmac., 1967, **19**, 322–328 Received December 5, 1966

Influence of nicotine on the coronary circulation of the isolated heart of the cat

T. ROMERO AND J. TALESNIK

Nicotine administered to the isolated perfused heart of the cat produces initial bradycardia followed by long-lasting tachycardia and enhancement of the strength of contractions. The coronary flow after a reduction shows a prolonged increase that continues after the heart hyperactivity has ceased. When nicotine was given after hexamethonium or after reserpine pretreatment, the cardio-stimulat on disappeared and only reduction of the coronary flow was obtained. It is concluded that the complexity of the pharmacological effect of nicotine on the coronary flow of the isolated heart preparation depends on an indirect vasodilator action mediated by the heart stimulation due to liberation of intracardiac catecholamines and also on vasoconstriction due to a direct effect of the drug on the coronary vasculature.

IT has been postulated that the vasodilator effect of catecholamines on the coronary circulation is secondary to the myocardial stimulating action and that the arteriolar smooth muscle responses are due to the increased myocardial metabolism (Berne, 1958; Douglas, Armengol & Talesnik, 1960). The vasodilatation is preceded by a transient phase of diminution of the coronary flow that has been ascribed to a direct action of the catecholamines on the coronary vessels (Berne, 1958; Hardin, Scott & Haddy, 1961).

According to Ahlquist (1948), the effect of catecholamines depends on two types of adrenaline receptors: (1) α-receptors, responsible for the vasoconstriction, and (2) β-receptors, responsible for the myocardial stimulation (tachycardia and increase in the force of contraction) as well as for the coronary vasodilatation; but Ahlquist did not comment on whether the coronary vasodilatation was a primary or a secondary effect. However it has been demonstrated on the isolated dog heart, arrested with potassium chloride, that isoprenaline produces coronary vasodilatation in the absence of cardiac stimulation. This response can be abolished by blocking the β-receptors with pronethalol (Klocke, Kaiser, Ross & Braunwald, 1965). These results indicate that the vasodilatation may be a primary action of catecholamines.

Nicotine produces myocardial stimulation similar to that obtained with adrenaline or noradrenaline (Hoffmann, Hoffmann, Middleton & Talesnik, 1945; Burn, 1960) and apparently this action depends on the intracardiac liberation of catecholamines (Hoffmann & others, 1945). The changes in the coronary flow observed with nicotine have been very divergent. Thus it has been reported that nicotine increases the coronary flow (Travell, Rinzler & Karp, 1960; Bellet, West, Müller & Manzoli, 1962), that it reduces it (Leaders & Long, 1962; Kareva, 1963) or that it does not alter it (Regan, Frank & others, 1961). The complexity of the pharmacological action of nicotine (Burn, 1960; Comroe, 1960), as well as the difficulty in obtaining comparable data from investigations made under different experimental conditions, are some of the factors involved in this problem.

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Nevertheless the effects of nicotine on coronary flow have been ascribed to the liberation of catecholamines (Travell & others, 1960).

The present study has been undertaken on the isolated cat heart in an attempt to elucidate the precise nature of the mode of action of nicotine on the coronary vasomotor tone.

Methods

Sixteen experiments were made on the isolated cat heart preparation perfused according to Langendorff's method as described by Douglas & others (1960). The perfusion fluid used was Tyrode solution oxygenated with a mixture of oxygen 95% and carbon dioxide 5%, at 38° and a constant perfusion pressure of approximately 40 mm Hg. The frequency and amplitude of the ventricular contractions and the coronary flow were registered continuously on smoked paper. The flowmeters were of the rheograph type described by Douglas & others (1960). Since the records of the inflow and the outflow from the coronary vessels were very similar the latter have been omitted in the figures shown.

Single doses of nicotine (sulphate) and noradrenaline (Levofed, Winthrop) were administered through the aortic cannula in a constant volume of 0·1 ml. Prolonged administration of hexamethonium (C_6), nicotine and atropine (Bellafolina, Sandoz) was carried out with a slow injection pump (0·8 ml/min).

Four cats were pretreated 24 hr before the experiment with reserpine (Serpasil, Ciba) by subcutaneous injection of 4 mg/kg body weight.

Results

EFFECT OF SINGLE INJECTIONS OF NICOTINE

The administration of 5 μg or more of nicotine produced, after 1 to 5 sec, bradycardia lasting from 10 to 15 sec; with the larger doses there was an initial cardiac arrest. After the period of bradycardia a long lasting

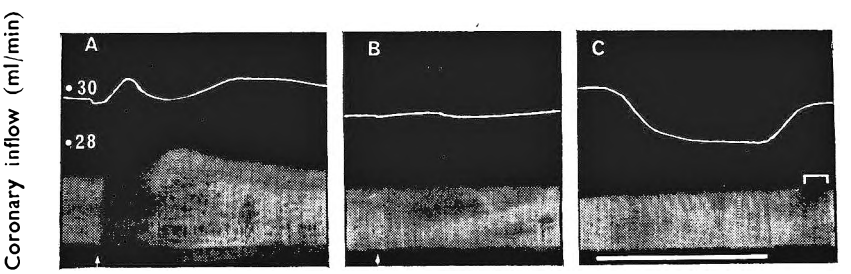

FIG. 1. A. Effect of single injections of nicotine (20 μg at arrow). Early cardio-inhibition is followed by prolonged cardio-stimulation. Between (A) and (B), perfusion with hexamethonium (1 mg/min). B. Effect of single injection of nicotine (20 μg at arrow) after hexamethonium. Both cardio-inhibition and cardio-stimulation are suppressed. The effect on the coronary inflow, observed in (A), does not occur. C. Prolonged administration of nicotine after hexamethonium. Although the ventricular contraction remains unaltered, a sustained reduction of the coronary inflow can be observed during nicotine infusion. Time scale: 10 sec.

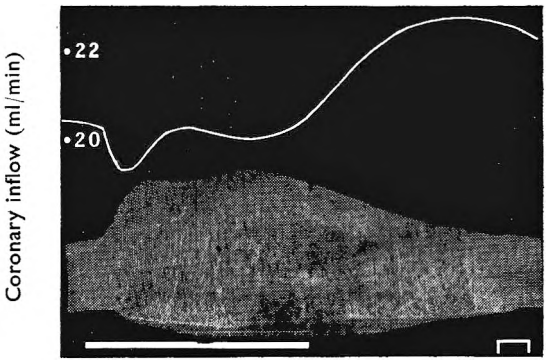

FIG. 2. Effect of the prolonged injection of nicotine (200 μg/70 sec at bar). The early cardio-inhibitor effect was abolished by atropine. The coronary inflow increases at the end of the nicotine infusion. Time scale: 10 sec.

tachycardia and increase in the amplitude of the contractions occurred. This phase of the nicotine effect could be observed for 60 to 120 sec (Fig. 1A). During these changes in heart activity (an expression of the product of heart rate and amplitude of the ventricular contractions), changes in the coronary flow were also observed. During the phase of bradycardia there was an increase in the coronary flow. When the period of myocardial stimulation (tachycardia and increased force of contraction) began, the coronary flow diminished, but later, when the myocardial stimulation had become maximal, the coronary flow increased again and remained raised long after myocardial stimulation was over (Fig. 1A).

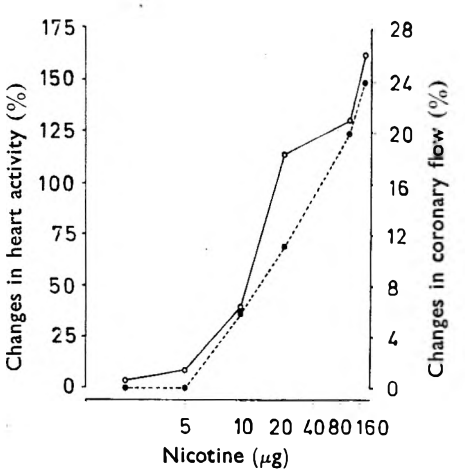

FIG. 3. Relationship between nicotine dosage, heart activity (○) and coronary flow (●). Heart activity has been defined as the product of heart rate and amplitude of the ventricular contractions.

Nicotine (50 to 200 μg) given over 45 to 70 sec (Fig. 2) produced tachycardia and increase in the amplitude of the contractions by 80 to 160% of the initial values. The early phase of bradycardia was abolished by previous administration of atropine (0·5 mg slowly injected in 15 min). If nicotine was then given, the coronary flow was slightly reduced while myocardial stimulation reached its maximal level. When the nicotine injection was stopped, a rapid increase of the coronary flow was observed. This long-lasting effect persisted for 1 to 2 min after the myocardial activity had returned to normal.

The total coronary flow increased by 6 to 24% after single injections or prolonged administration of nicotine, depending on the degree of myocardial stimulation evoked by the different doses (Fig. 3).

Perfusion with hexamethonium (1 mg/min) abolished both the initial bradycardia and the myocardial stimulation induced by single injections of 10 to 200 μg of nicotine (Fig. 1B). The coronary flow usually did not change or was slightly reduced (by not more than 5%) for a short time (Fig. 1 B).

Slow infusions over 40–70 sec of nicotine in amounts of 200 to 1,000 μg produced a reduction of the coronary flow by 5 to 25% depending on the dose used. The diminution of the coronary flow appeared 1 to 5 sec after the nicotine infusion began (Fig. 1C). When nicotine administration ceased, the coronary flow recovered quickly, reaching its initial level in about 30 to 40 sec. When slow injections of higher doses of nicotine (total dose of 500 μg or more) were used, a slight increase in the amplitude of the contractions sometimes appeared but the coronary flow showed the same pattern of reduction.

It seemed possible that increased myocardial activity, by increasing the extravascular support to the coronary vessels, might itself increase the impedance to coronary flow and thus mask any direct action of nicotine on the vessels. We therefore repeated these experiments on hearts treated with hexamethonium and caused to fibrillate by electrical stimulation (Figs 4A and B). Under these conditions nicotine (1 mg infused

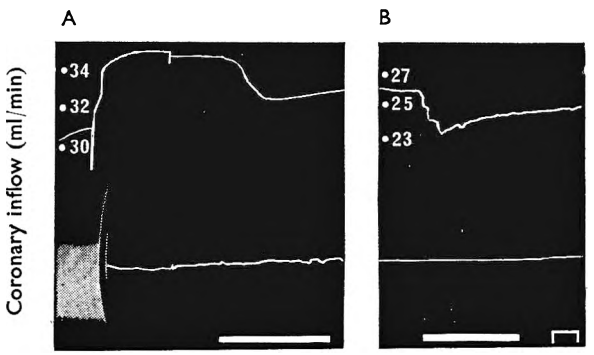

FIG. 4. Effect of nicotine after hexamethonium in electrically induced ventricular fibrillation. A. When fibrillation starts there is an increase in the coronary inflow. Prolonged injection of nicotine reduces the coronary inflow. B. The same response at a lower level of coronary inflow. In (A) nicotine 1 mg/40 sec at bar. In (B) nicotine 1 mg/35 sec at bar. Time scale: 10 sec.

over 35–40 sec) induced a similar but slightly smaller diminution of coronary flow.

EFFECT OF NICOTINE AFTER PRE-TREATMENT WITH RESERPINE

In the hearts from 4 animals previously treated with reserpine, both single and prolonged injections of nicotine produced initial bradycardia which was abolished by atropine (Fig. 5A and B). With single doses of

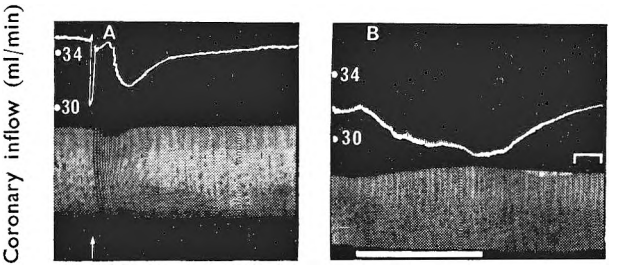

FIG. 5. Effect of nicotine after reserpine pre-treatment. A. Cardio-inhibition is produced by a single injection of nicotine (50 μg at arrow) without cardio-stimulation. Reduction of the coronary flow occurs. Between (A) and (B) atropine was administered. B. Prolonged injection of nicotine (1 mg/45 sec at bar) after atropine produces only a reduction of the coronary inflow during the nicotine infusion. Time scale: 10 sec.

50 μg of nicotine it was possible to observe a reduction of the coronary flow in the absence of any myocardial stimulation (Fig. 5A). Prolonged injections of a total dose of 100 to 1,000 μg of nicotine during 45 to 70 sec produced a reduction of the coronary flow of 10 to 25%; very high doses also caused a slight increase in the amplitude of the ventricular contractions (10%) (Fig. 5B).

Discussion

The action of nicotine in stimulating the synapses of both sympathetic and parasympathetic ganglia is well known. In the isolated heart, stimulation of the vagal synapses is most probably responsible for the initial bradycardia that follows the administration of nicotine (Perry & Talesnik, 1953). There is strong evidence that nicotine also causes a release of catecholamines when administered to the isolated heart (Hoffmann & others, 1945; Burn & Rand, 1958). The site of action of the catecholamines is by no means clear, although the existence of sympathetic ganglia or chromaffin tissue in the heart has been postulated (Hoffmann & others, 1945; Burn, 1960) and is supported by experiments claiming that stimulation of pre-ganglionic sympathetic fibres with synapses situated in the heart was possible (Nagy & Szentivangi, 1961). Furthermore acetylcholine can induce catecholamine liberation from the heart.

As shown in Fig. 3 the degree of coronary vasodilatation produced by nicotine is related to the degree of myocardial stimulation evoked. This confirms the results of previous workers (Travell & others, 1960; Bellet & others, 1960).

It has been suggested however that nicotine has direct coronary vasoconstrictor properties also mediated by an adrenergic mechanism (Leaders & Long, 1962; Kareva, 1963). The problem is still more difficult to analyse if one considers the recently demonstrated fact that the response of isolated and perfused strips of coronary smooth muscles and arteriolar segments to catecholamines is variable according to the diameter of the vessel. The smaller arterioles are relaxed by noradrenaline while the larger vessels (over 1·7 mm in diameter) are constricted with lower doses. These differences have been attributed to a quantitative difference in distribution of the α- and β-receptors (Zuberbuhler & Bohr, 1965). Therefore, considering only the isolated heart, the gross effect of nicotine on the total coronary flow would be the resultant of its main action in addition to a number of others of varying predominance, all of which might interact.

The use of hexamethonium allows the reaction of the coronary vessels to nicotine to be studied in the absence of bradycardia and of subsequent myocardial stimulation. The bradycardia is almost certainly suppressed by the blocking action of hexamethonium at the vagal intracardiac synapses (Perry & Talesnik, 1953). The doses of hexamethonium used were large enough to ensure that the vagal ganglionic cells were adequately blocked (Perry & Talesnik, 1953; Middleton, Oberti, Prager & Middleton, 1956; Leaders & Long, 1962; Kareva, 1963). The myocardial stimulation is reduced or abolished by an inhibition of the discharge of catecholamines by some mechanism not yet well understood (Burn, 1960; Burn & Gibbons, 1964).

There is a significant difference in the action of high doses of nicotine on the coronary flow before and after the administration of hexamethonium. A high dose of nicotine produced a substantial reduction in the total coronary flow in heart treated with hexamethonium (Fig. 1C) despite the lack of any myocardial stimulation, suggesting that the changes in coronary flow are not due to gross modifications of the extravascular support. Thus it is quite possible that the reduction of the coronary flow under these conditions is due to a direct vasoconstrictor action. Furthermore, after pre-treatment of the animals with reserpine, which depletes the catecholamine stores (Burn & Rand, 1958), nicotine acts on the coronary bed by producing direct vasoconstriction as it does in the experiments with hexamethonium.

Accordingly it is clear that the overall effect of nicotine on coronary tone in the isolated heart depends on a combination of several different mechanisms of action. When nicotine produces increased heart activity and an associated liberation of catecholamines the main effect is a vasodilatation, described as a servo-mechanism of coronary adaptation by Douglas & others (1960) and Douglas & Talesnik (1960). This secondary vasodilatation competes with a direct vasoconstrictor effect that can be unmasked when the influence of the catecholamines is suppressed. It is relevant to emphasize the importance of the servo-mechanism of coronary adaptation since in the rabbit with experimental atherosclerosis, which physically impairs the vasodilatation, nicotine elicits only vasoconstriction

(Travell & others, 1960). Furthermore, in the fibrillating heart (Fig. 4A and B) treated with hexamethonium, nicotine still elicits vasoconstriction, and it seems clear that this effect must be a direct one upon the coronary vessels. The vasoconstrictor effect of nicotine is smaller when the heart is fibrillating and this may well be due to the fact that fibrillation alone is known to cause an increase in coronary flow (Douglas & others, 1960).

Apart from these major effects of nicotine, there remain to be explained the two transient initial effects observed. The first is the initial increase in coronary flow seen after nicotine is given in hearts not perfused with hexamethonium or atropine (Fig. 1A). This seems certain to be related to the bradycardia evoked by nicotine since the reduction of the extravascular coronary impedance (extravascular "support"; Katz, Jochim & Bohning, 1938) under bradycardia or cardiac arrest has already been pointed out as responsible for this effect (Douglas & others, 1960; Leaders & Long, 1962).

The second is the diminution of the coronary flow that follows the short initial increase (Figs 1A and 2). This also seems to depend on a change in the extravascular impedance (extravascular "support"), in this case an increase due to the myocardial stimulation (Douglas & others, 1960; Travell & others, 1960; Leaders & Long, 1962).

Acknowledgement. We are indebted to Professor W. L. M. Perry for his criticism and revision of the typescript.

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J. Pharm. Pharmac., 1967, **19**, 329–331 Received November 9, 1966

Apparatus for studying the disintegration of tablet coatings

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A new apparatus has been designed to assess rates of disintegration of tablet coatings and the availability of coated tablet contents. Random movement of the coated tablet in a known volume of mildly turbulent disintegrating fluid at a standard temperature is achieved in a perforated Perspex drum made to revolve slowly in the fluid which moves up and down in its container at the same time. The procedure eliminates the tendency for coatings to stick together or to the grid during a test, which is seen with present pharmacopoeial designs and which causes large variability in the results.

SYSTEMATIC examination of the disintegration or dissolution rates (herein called disintegration rates) of tablet coatings, with the possible exception of the "enteric" coating, has received scant attention.

The test of the British Pharmacopoeia (1963) for the coated tablet is the test for the uncoated tablet with extended time. The apparatus is such that the coating of a tablet can be unevenly abraded by falling many times onto the grid with the same side downmost. Also, with the five coated tablets which are used in this test, the chance of the wetted coatings sticking to each other or to the grid is much greater than is likely with the uncoated tablet. Sticking is due to the presence of gelatin, acacia and similar substances in the coating. The presence of five coated (or uncoated) tablets in the stomach or in one small section of the intestine at any point in time is unlikely, hence the sticking which frequently occurs in the disintegration apparatus is unlikely to occur in the gut.

Drugs may be incorporated in tablet coatings, and different layers of the coating may contain different drugs or different concentrations of the same drug, necessitating sequential release from the layers of coating in the gastrointestinal tract. There is, therefore, a case for considering the disintegration of coated tablets singly in an apparatus where factors causing uneven disintegration rate of the coat or a part of the coat are absent and in which disintegration occurs layer by layer.

During the development of a spray-pan coating method (Anderson & Sakr, 1966) we had occasion to study disintegration rates of the tablet coatings produced by this method and to compare them with the tablet coatings produced using the traditional method. We concluded that the B.P. apparatus was unsatisfactory for this purpose.

THE APPARATUS

The new apparatus (Fig. 1) consists of a cylindrical disintegration drum of Perspex, 3 cm diameter, 3·2 cm long, perforated with 140 equally spaced holes each 0·24 cm diameter, and fitted internally with a trip bar to minimize contact with the sides of the drum and to ensure that the coated tablets tumble in the fluid and do not slide round the inside. The tablet is placed inside the drum (the cap can be removed) which is then made to revolve by a pulley drive 10 times/min within a disintegration tube of 300 ml capacity, 5·5 cm diameter, 14 cm in height, containing the

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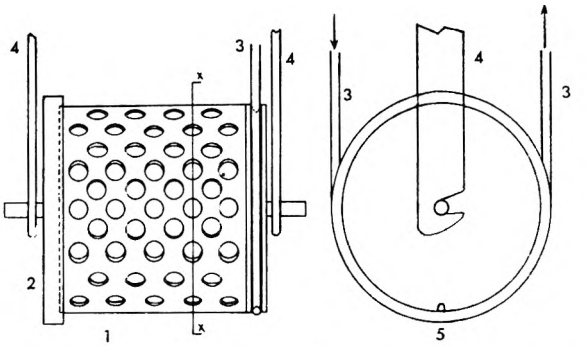

FIG. 1. Disintegration drum. 1. Drum; 2. Cap; 3. Drive; 4. Supporting shafts; 5. Trip bar. The drum revolves in a disintegration tube which moves up and down and contains fluid.

desired disintegration fluid. The disintegration tube is made to move up and down 25 times/min so that the revolving drum just fails to break the surface of the fluid at the bottom of the stroke of the tube, and just fails to touch the bottom of the tube at the top of the stroke. The revolving of the perforated drum, and the vertical up-and-down movement of the fluid in the tube, ensure that the tablet tumbles in a mildly turbulent fluid; contact with the inner surface of the drum is minimal and, because of the tumbling, random. A tap may be fitted to the disintegration tube to allow sampling of the fluid. Temperature control can be obtained by using a jacketed disintegration tube, or by having a thermostatically controlled heater in the tube, or by housing the apparatus in a cabinet with a thermostatically controlled atmosphere.

Blue coatings containing Sky Blue 1900 [Williams (Hounslow) Ltd.] were deposited on white core tablets by the spray-pan method which has been shown to yield evenly deposited layers of coating (Anderson & Sakr, 1966). The contrast in colour between core and coat allowed easy visual determination of the end-point which was complete removal of blue coating. The dye liberated as the coating disintegrated was determined spectrophotometrically at 635 mμ.

DISINTEGRATION STUDY

For disintegration study, one, two and five tablets were used and 20 determinations were done in each case. The new apparatus and the B.P. apparatus were both used at 37°; different samples of the same batch of tablets were used. Release rate of the dye from the coating using single tablets in the test is shown in Fig. 2. The use of up to five tablets in a test did not significantly affect the rate. Results for release rate in the B.P. test are not included because of the uneven disintegration rate of the coating. It frequently happened that the core on one side had begun to disintegrate before all the coating on the other side had been removed.

This uneven disintegration is not allowed for in the B.P. test which considers only the total disintegration time of the whole tablet, and it renders the B.P. test of little value when information about the disintegration rate of the coating is required. A uniform release rate and a

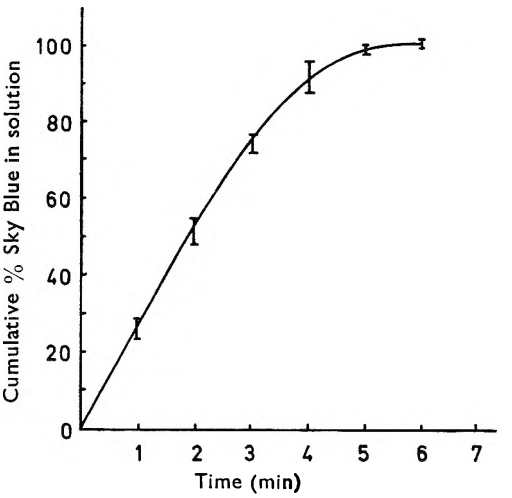

FIG. 2. Rate of release of Sky Blue 1900 from tablet coating. Each point is the mean of five determinations, using single tablets.

disintegration time with small error are necessary for the determination of total availability, with time, of the contents of the coating.

TABLE 1. VARIABILITY OF DISINTEGRATION OF TABLET COATINGS IN THE TABLET DISINTEGRATION APPARATUS OF THE BRITISH PHARMACOPOEIA AND IN A NEW APPARATUS

	Mean disintegration time (min)		Coefficient of variation (%)	
Number of tablets per test	B.P. apparatus	New apparatus	B.P. apparatus	New apparatus
1	3·4	2·4	15·6	2·4
2	3·2	2·5	10·5	1·6
5	2·9	2·8	9·8	1·4

Each mean includes 20 determinations with the relevant number of tablets.

Results of the disintegration tests (Table 1) using the new apparatus show less variability than those obtained with the B.P. type of apparatus. This is due to two factors: elimination of the sticking together of the coatings during the test, and of uneven disintegration of the coatings caused by failure of the tablets to tumble and present both sides with equal frequency to the grid in the B.P. apparatus.

The new apparatus is suitable for release rate studies involving whole coatings, and, as a result of uniform disintegration, different, distinguishable layers of one coating.

Acknowledgement. We thank Mr. I. Aird for help in construction of the disintegration apparatus.

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J. Pharm. Phamac., 1967, **19**, 332–334 Received December 22, 1966

A note on a micro and a semimicro method for the assay of ephedrine

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Two colorimetric methods for the assay of ephedrine are proposed. One is a semimicro method in which the intensity of red colour produced by the oxidation of ephedrine with hydrogen peroxide is measured. The other is a micro method in which measurement is made of the intensity of the brown colour of the respective copper dithiocarbamate, produced by interaction of ephedrine, carbon disulphide and ammoniacal copper sulphate, the limit of sensitivity being 100 μg. Both methods gave reproducible results when used to assay ephedrine in pharmaceutical preparations. Results were compared with those from the Egyptian pharmacopoeial method.

WITH the aim of devising a simple and precise colorimetric estimation for ephedrine, the colour tests for ephedrine with hydrogen peroxide (Snell & Snell, 1954) and with copper dithiocarbamate (Fiegel, 1960) were found to be a suitable starting point.

The hydrogen peroxide method uses as a basis the oxidation by hydrogen peroxide of ephedrine in alkaline medium to give a red colour. This colour, we found to obey Beer's law in the range 2·5–15 mg.

The dithiocarbamate method depends upon the reaction of ephedrine with carbon disulphide and ammoniacal copper sulphate solution. A water-insoluble brown copper salt of the dithiocarbamic acid is produced which, when dissolved in benzene or chloroform, gives a brown solution obeying Beer's law in the range of 100–900 μg.

Experimental and results

HYDROGEN PEROXIDE METHOD

Reagents. Ephedrine hydrochloride, hydrogen peroxide (30 vol.), sodium hydroxide (0·1N), sodium chloride 16%, ether, all of analytical grade.

Procedure. A standard curve is plotted, using varying concentrations of ephedrine hydrochloride solution.

To 1·5 ml of ephedrine hydrochloride solution containing 2·5–15 mg/ml, add 16% sodium chloride (4 ml), 0·1N sodium hydroxide (0·5 ml) and hydrogen peroxide (6 drops). Mix thoroughly, heat on a water bath for 5 min and cool. Measure the colour developed in a Unicam S.P. 1300 colorimeter using filter No. 1 (370–515 mμ). The colour developed obeys Beer's law when ephedrine hydrochloride is present in amounts ranging from 2·5–15 mg. The colour is stable for over 3 hr.

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DITHIOCARBAMATE METHOD

Reagents. Ephedrine hydrochloride, concentrated ammonium hydroxide, benzene, copper sulphate 5% solution, carbon disulphide, all of analytical grade.

Procedure. A standard curve is plotted using varying concentrations of ephedrine hydrochloride solution.

To 1 ml of an aqueous solution of ephdrine hydrochloride containing 100–900 μg/ml add 5% copper sulphate solution (1 ml), concentrated ammonium hydroxide (3 drops) and carbon disulphide–benzene (1 : 3) (5 ml). Shake the mixture thoroughly for 5 min. Separate the organic layer and measure the colour with a Beckman DU spectrophotometer at a wavelength of 440 mμ. The colour obeys Beer's law over a concentration range of 100–900 μg/5 ml of organic layer. The colour reaches its maximum in the organic layer after 10 min and remains stable for a further 15 min.

Experiments made on solutions containing different added amounts of ephedrine hydrochloride show the reproducibility of the two methods (Table 1). From Table 1 it is obvious that the amounts of ephedrine recovered by both methods were almost quantitative.

TABLE 1. ESTIMATION OF EPHEDRINE HYDROCHLORIDE BY THE HYDROGEN PEROXIDE METHOD AND THE COPPER DITHIOCARBAMATE METHOD

Hydrogen peroxide method				Copper dithiocarbamate method			
Ephedrine HCl (mg)		Recovery (%)	Error (%)	Ephedrine HCl (μg)		Recovery (%)	Error (%)
Added	Found			Added	Found		
2·5	2·425	97	−3	100	100	100	0·0
				200	198	99	−1
5	4·91	98·2	−1·8	300	303	101	+1
				400	396	99	−1
7·5	7·80	104	+4	500	511·5	102·3	+2·3
				600	607·2	101·2	+1·2
10	9·84	98·4	−1·6	700	700	100	0·0
				800	793·6	99·2	−0·8
12·5	12·5	100	0·0	900	911·7	101·3	+1·3
15	14·81	98·7	−1·3	1000	965	96·5	−3·5

ASSAY OF EPHEDRINE IN SOME PHARMACEUTICAL PREPARATIONS

Some preparations of ephedrine (official in the Egyptian Pharmacopoeia and British Pharmaceutical Codex) were assayed by the proposed methods and the results compared with those from the Egyptian Pharmacopoeia method of analysis.

Preliminary treatment. A. Tablets of ephedrine hydrochloride, 30 mg (Memphis Co.). A weighed quantity of the powdered tablets equivalent to 15 mg of ephedrine salt was transferred to a separator with water, made alkaline with strong ammonium hydroxide and extracted successively with ether (4 × 20 ml quantities), the final volume being adjusted to a concentration of 2·5–15 mg/ml for the hydrogen peroxide method or of 100–90 μg/ml for the copper dithiocarbamate method.

B. Ampoules of ephedrine sulphate 50 mg/ml (Misr Co.). The sample was diluted with distilled water to give the optimum concentration for either method.

C. Elixir of ephedrine B.P.C. 1963. A measured volume of the elixir was transferred to a separator, made alkaline with ammonium hydroxide and extracted successively with ether. The final volume was adjusted to give a suitable concentration for each method.

The ephedrine content of the tablet and elixir was also determined by the copper dithiocarbamate method directly without extraction.

The data are in Table 2. These show that the proposed methods are comparable with the E.P. method of assay. Moreover, the copper dithiocarbamate method is applicable directly to the filtrate of the tablet and diluted elixir without previous treatment.

TABLE 2. COMPARATIVE ANALYSIS OF SOME OFFICIAL PREPARATIONS OF EPHEDRINE OFFICIAL IN THE EGYPTIAN PHARMACOPOEIA AND BRITISH PHARMACEUTICAL CODEX

Preparations and its ephedrine content	E.P. Method		Hydrogen peroxide method		Copper dithiocarbamate method			
	Ephedrine content (%)	devn (%)	Ephedrine content (%)	devn (%)	Ephedrine content by extraction (%)	devn (%)	Ephedrine content without extraction (%)	devn (%)
Tablet 0·5 gr	97·2	−2·8	99·52	−0·48	98·5	−1·5	98·82	−1·18
Ampoule 50 mg/ml ..	101·95	+1·95	104·2	+4·2			103·57	+3·57
Elixir 0·46 g/100 ml ..	100·4	+0·4	99·1	−0·9	99·0	−1·0	98·3	−1·7

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Localization of 5-hydroxytryptamine uptake in rat brain after intraventricular injection

SIR,—With the help of the histochemical fluorescence method for the demonstration of catecholamines and 5-hydroxytryptamine (5-HT) (see review by Hillarp, Fuxe & Dahlström, 1966; Corrodi & Jonsson, 1967), it has recently been possible to show that intraventricularly administered catecholamines are specifically taken up into those parts of the central catecholamine neurons lying in a zone around the ventricles and the ventral part of the subarachnoid space (Fuxe & Ungerstedt, 1966, 1967a). The facts support the view that there exists in the central catecholamine neurons a reserpine-resistant uptake-concentration mechanism for catecholamines probably localized at the level of the nerve cell membrane (Hamberger, 1967). Whether there exist similar uptake mechanisms in the central 5-HT neurons is the object of this report (Dahlström & Fuxe, 1964; Fuxe, 1965). Light microscopic autoradiography of intraventricularly administered tritiated 5-HT suggested an accumulation of amine in nerve terminals (Aghajanian, Bloom & others, 1966). To avoid the blood-brain barrier the intraventricular route was used.

Female Sprague-Dawley rats, 150 g, were given intraventricular injections (20 μl) stereotaxically into the left lateral ventricle. The rats were operated on in fluothane-nitrous oxide-oxygen anaesthesia which enabled the rats to wake up within 15 min after the injection. Body temperature was maintained within normal limits during the experiment. The intraventricular injections were made on normal rats, on reserpine pretreated rats (10 mg/kg, i.p., 12 hr before death) and also on rats pretreated with reserpine (10 mg/kg, i.p., 12 hr before death) and nialamide (500 mg/kg, i.p., 1 hr before death). 5-HT was injected in doses of 1 to 5 μg and the rats were killed 30 min afterwards. Some rats in this last group were also pretreated with desipramine (25 mg/kg, i.p., 1 hr before the 5-HT), (+)-amphetamine (10 mg/kg, i.p., 1 hr before the 5-HT), or tryptamine (50–100 mg/kg, i.p., 20 min before the 5-HT). All doses given refer to the base. The rats were killed by decapitation under light chloroform anaesthesia. The telencephalon, the diencephalon, the mesencephalon, the pons, the medulla oblongata and parts of the spinal cord were rapidly dissected, quickly frozen in liquid propane cooled by liquid nitrogen, freeze-dried, embedded in paraffin, sectioned, and mounted as previously described (Dahlström & Fuxe, 1964, Hamberger, Malmfors & Sachs, 1965) although modified to provide optimum reaction conditions for 5-HT (Fuxe & Jonsson, 1967).

5-Hydroxytryptamine. When 5-HT was given to untreated rats there was a clear accumulation of amine in very fine varicose fibres and nerve cell bodies lying close to the ventricles and the ventral part of the subarachnoid space. This was seen as an increased yellow fluorescence in nerve terminals, for example in the nucleus suprachiasmaticus, and in nerve cell bodies, for example in the nucleus raphe dorsalis. These neuronal structures were in all probability identical with the 5-HT nerve terminals and cell bodies, since they had the same appearance and distribution as the weakly fluorescent 5-HT neurons of the untreated control animals. The catecholamine neurons present in this zone did not seem to accumulate 5-HT.

Reserpine–5-HT. When 5-HT (1–5 μg) was given to reserpine pretreated rats there was no clear accumulation of amine within the 5-HT neurons, except in the area close to the injection site (5 μg), where the exogenous 5-HT concentration was probably high. Medium to strongly yellow-fluorescent nerve terminals could for example be observed in the septal area and a moderate diffuse yellow fluorescence was present in the part of the nucleus caudatus and putamen lying

close to the lateral ventricle on the side of injection. We could not say with certainty whether this diffuse yellow fluorescence represented an accumulation of amine in very fine, densely packed 5-HT or dopamine nerve terminals.

Reserpine–nialamide–5-HT. When 5-HT (1–5 μg) was given to reserpine-nialamide pretreated rats there was a clear to marked accumulation of amine within 5-HT cell bodies, non-terminal axons, for example, those in the spinal cord, and nerve terminals in the zones mentioned above. There was no certain accumulation of 5-HT within those parts of the central catecholamine neurons lying in the above mentioned zones with the possible exception of the yellow fluorescence observed in the nucleus caudatus and putamen close to the injection site. With the highest doses there was also observed a yellow fluorescence in the cells of the capillary walls.

It was not possible to obtain any observable blockade of this accumulation with desipramine or amphetamine which, however, in the doses used, clearly blocked the accumulation of intraventricularly administered noradrenaline in the central catecholamine neurons (Fuxe & Ungerstedt, 1967b). Tryptamine, on the other hand, partly prevented the accumulation of 5-HT within the 5-HT neurons. Thus the zone in which yellow fluorescent 5-HT nerve terminals were observed was clearly decreased in thickness, and the intensity of the yellow fluorescence that appeared was reduced compared to reserpine-nialamide-5-HT treated rats.

These experiments support the view that there exists a reserpine-sensitive uptake-storage mechanism in the 5-HT neurons (see Dahlström, Fuxe & Hillarp, 1965; Carlsson, 1966), since 5-HT did not accumulate in the 5-HT neurons of reserpine-pretreated rats unless the exogenous 5-HT concentrations were very high. Furthermore, there seems to exist a reserpine-resistant uptake-concentration mechanism for 5-HT in all parts of the 5-HT neurons since 5-HT accumulated in such neurons in rats pretreated with reserpine and a monoamine oxidase inhibitor. This mechanism does not seem to be related to the reserpine-sensitive uptake-storage mechanism because it appears as efficient in the non-terminal axons as in the terminals. Thus, it is probably localized to the level of the nerve cell membrane as was suggested earlier for the catecholamine neurons (for references, see Hamberger, 1967). The 5-HT accumulation occurred selectively in the 5-HT neurons and not in the catecholamine neurons. Finally the reserpine resistant uptake-concentration mechanism for 5-HT is not blocked to any observable degree by desipramine or amphetamine in the doses used. The experiments also indicate that the hallucinogenic agent, tryptamine (Sai-Halasz, Brunnecker & Szara, 1958) may have indirect actions via the 5-HT neurons either by blocking the reserpine-resistant uptake-concentrating mechanism or by releasing accumulated amines.

Acknowledgements. This work has been supported by the Swedish State Medical Research Council (12x–715–02), and by M. Bergwall's Foundation and Stiftelsen Therese and Johan Anderssons Minne. For a generous gift of reserpine we thank CIBA Ltd (Stockholm).

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Fenfluramine and critical flicker frequency

SIR,—Fenfluramine hydrochloride (Ponderax) is a recently marketed appetite-suppressant agent which, although having chemical resemblances to amphetamine, does not appear clinically to produce central stimulation (Traherne, 1965; Munro, Seaton & Duncan, 1966). It has been claimed to possess sedative acitivity and has been used for this reason in patients with anxiety states (Raich, Richels & Raab, 1966).

Amphetamine, phenmetrazine and diethylpropion are appetite suppressant agents possessing central stimulant properties, and they have been shown to increase the critical flicker frequency in normal subjects (Smart & Turner, 1966; Turner, 1967). This method is a valuable test of central function which has been shown to be sensitive in assessing the action of several centrally-acting drugs when administered in modest therapeutic doses (Turner, 1967). In a double-blind experiment, identical tablets of fenfluramine 20 and 40 mg and a placebo were administered in random order in a latin square design and at intervals of not less than 3 days to 6 young adult subjects of either sex. The critical flicker frequency was measured before dosing and at $1\frac{1}{2}$ and 3 hr thereafter by a technique (Turner, 1965a; Smart & Turner, 1966) which involved exposing the subjects in random order to intermittent light at 25 and 50 c/sec for 1 min before measuring the ascending and descending thresholds of critical flicker frequency.

The results were submitted to an analysis of dispersion (Rao, 1952) which is the multivariate analogue of the analysis of variance. This permits a more accurate evaluation than does an analysis of variance of the responses to drug and placebo over time.

No significant difference was demonstrated between the effects of fenfluramine 20 and 40 mg and placebo on mean critical flicker frequency at either $1\frac{1}{2}$ or 3 hr. There was a significant difference between ascending and descending thresholds ($P < 0.001$), and between thresholds after adaptation to light at 25 and 50 c/sec ($P < 0.05$), but these were not influenced by either dose of drug or placebo. This is consistent with the stability of these factors which has been previously demonstrated (Turner, 1965b; Turner, Patterson & Smart, 1966).

These findings indicate that fenfluramine in therapeutic doses does not influence the critical flicker frequency, and this is in keeping with the clinical absence of central stimulation associated with its use.

Acknowledgements. R. C. Hill is in receipt of a research grant from Roche Products Ltd., and Paul Turner of a Wellcome Senior Research Fellowship in

Clinical Science. Tablets of fenfluramine hydrochloride (Ponderax) and placebo were supplied by Selpharm Laboratories Ltd. We thank Mr. J. V. Smart for help with the statistics.

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Isolation, aggressiveness and brain 5-hydroxytryptamine turnover

SIR,—Male albino mice submitted to prolonged isolation showed a smaller increase in brain 5-hydroxytryptamine (5-HT) compared with normal animals, when treated with monoamine oxidase inhibitors (Valzelli, 1966). The present report supplies additional quantitative evidence using the method of Tozer, Neff & Brodie (1966) to calculate the turnover of brain 5-HT.

Male Swiss albino mice, 20 ± 2 g, were isolated (1 animal/cage) or grouped (10 animals/cage) for 4 weeks under the conditions previously described (Consolo, Garattini & Valzelli, 1965). At the end of 4 weeks, isolated and grouped animals received an intraperitoneal injection of tranylcypromine (20 mg/kg). Animals were killed at various times after tranylcypromine injection and their brains analysed for 5-HT (Shore, 1959) and for 5-hydroxyindoleacetic acid (5-HIAA) (Giacalone & Valzelli, 1966).

It is evident that while the level of brain 5-HT is comparable in the two experimental conditions (Table 1), there is always a small but significant decrease of brain 5-HIAA in isolated compared with grouped mice.

The administration of tranylcypromine induces an increase of brain 5-HT and a decrease of brain 5-HIAA, which are respectively linear on a normal or on a logarithmic scale (see Fig. 1) in grouped or isolated animals. However the slope of the curves was different, which indicated an increase in the turn-

TABLE 1. LEVELS OF BRAIN 5-HT AND 5-HIAA IN ISOLATED AND GROUPED MICE

Experiment No	Isolated mice		Grouped mice	
	5-HT	5-HIAA	5-HT	5-HIAA
1	0·65 ± 0·02	0·32 ± 0·01*	0·65 ± 0·03	0·41 ± 0·01
2	0·81 ± 0·03	0·42 ± 0·02**	0·80 ± 0·02	0·51 ± 0·01
3	0·75 ± 0·01	0·41 ± 0·01*	0·76 ± 0·01	0·49 ± 0·01
4	0·70 ± 0·02	0·34 ± 0·01**	0·71 ± 0·02	0·38 ± 0·01

* = $P < 0.01$.
** = $P < 0.05$.
Figures represent μg/g ± s.e.

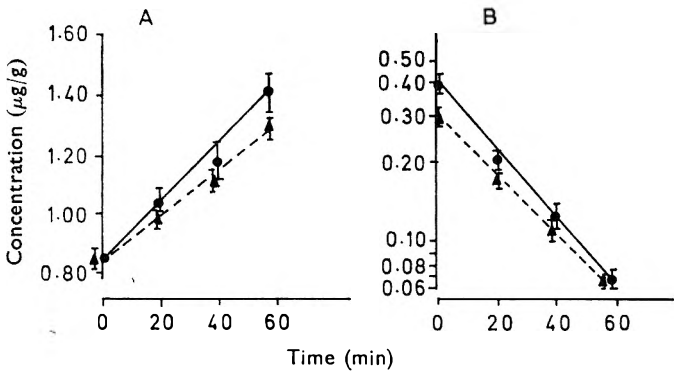

FIG. 1. Increase of brain 5-HT (A) and decrease of brain 5-HIAA (B) after tranylcypromine (20 mg/kg i.p.) in normal (●——●) and in isolated aggressive (▲ - - - ▲) mice. On the ordinates the levels of 5-HT or 5 HIAA in μg/g of brain; on the abscissae the time (min) after tranylcypromine administration. The vertical bars represent the standard error of the mean. Each point is based on 8 determinations.

over time of brain 5-HT of about 57% in isolated compared with grouped mice (see Table 2). The reason for this change is probably not related to a different level of tranylcypromine in the brain of isolated or grouped mice (Valzelli, 1966).

TABLE 2. DYNAMIC ASPECTS OF 5-HT METABOLISM IN BRAIN OF ISOLATED AND GROUPED MICE AFTER TRANYLCYPROMINE ADMINISTRATION

	Isolated mice	Grouped mice
$[5\text{-HT}]_0$ μg/g	0·84 ± 0·02	0·84 ± 0·04
$[5\text{-HIAA}]_0$ μg/g	0·29 ± 0·01*	0·38 ± 0·01
Rate constant of 5-HIAA loss	1·42 ± 0·08	1·69 ± 0·12
5-HT turnover rate, μg/g/hr	0·38	0·59
5-HT turnover time, min	122	79

* = $P < 0{\cdot}01$.
The calculations were made according to Tozer & others (1966).

Since the mice invariably became aggressive after 4 weeks of isolation, the changes observed in brain 5-HT turnover suggest possible correlations between behavioural changes and central metabolism of 5-HT. The data also show that changes in 5-HT turnover are not necessarily reflected in the level of brain 5-HT.

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March 7, 1967

Acknowledgements.

This work was supported by the U.S. Dept. of Army, through its European Research Office (Contract N. DA–91–591 EUC–4058). We are grateful to Mr. Guiseppe Peci for his helpful technical assistance.

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The concentration of adrenaline and noradrenaline in the brain and the heart of the goldfish, *Carassius auratus*

SIR,—It was previously found by Östlund, Bloom & others (1960) and Euler & Fänge (1961) that catecholamines were present in the heart of some cyclostomata. The heart of *Mixine glutinosa* contains a high concentration of adrenaline and noradrenaline and is stimulated after the administration of noradrenaline (Bloom, Östlund & others, 1961). The heart of *M. glutinosa* has no innervation (Augustinsson, Fänge & others, 1956); however, fibre-like connections were observed between the chromaffin cells (Bloom & others, 1961). Adrenaline and noradrenaline were also found in the heart of some elasmobranchs and teleosts (Östlund, 1954; Euler & Fänge, 1961; Brodie & Bogdanski, 1964). Both amines produced dilatation of the gill vessels in teleosts (Fänge, 1962).

Neither catecholamine-containing cells nor adrenergic fibres were detected in the hearts of some teleosts (cod, trout, pike and plaice) by Falk, Mecklenburg & others (1966) but Bogdanski, Bonomi & Brodie (1963) found catecholamines to be present in the heart of *Carassius auratus*—the goldfish. We now report the concentration of noradrenaline and adrenaline in the heart and encephalon of *Carassius auratus* (var. *Brasiliensis*).

The experiments were done on adult fish of either sex, of body weight about 4·5 g. The encephalon and the heart were dissected, put on a filter paper, weighed, and kept at −30°. The estimations of catecholamines were made on the same day. The hearts and encephalons of two animals were separately pooled for each estimation. Recoveries of adrenaline and noradrenaline in pure solutions ranged from 75 to 92%. This investigation was made during September, 1966. Adrenaline and noradrenaline were extracted as proposed by Euler & Lishajko (1961), and estimated spectrophotofluorimetrically (Aminco Bowman) (Bertler, Carlsson & Rosengren, 1958).

TABLE 1. CONCENTRATION OF NORADRENALINE AND ADRENALINE IN THE ENCEPHALON AND THE HEART OF THE GOLDFISH *Carassius auratus* (Var. *Brasiliensis*)

	Adrenaline	Noradrenaline
Encephalon		
Females	0·45 ± 0·04	0·12 ± 0·02
Males	0·41 ± 0·03	0·11 ± 0·02
Heart		
Females	0·23 ± 0·02	0·28 ± 0·02
Males	0·20 ± 0·02	0·27 ± 0·02

Each value is a mean (± standard error) from 6 estimations. The results are not corrected for recovery. The approximate weight of encephalon was of 60 mg and that of the heart of 110 mg.

The experiments showed that in the encephalon of both sexes the concentration of adrenaline was higher than that of noradrenaline, with a ratio of 3·4 to 1 (Table 1), while in the heart, the concentrations are about the same.

A higher concentration of adrenaline was found in the brain and the heart of the toad (Östlund, 1954; Falck, Häggendal & Owman, 1963; Jofre, 1967). It is supposed that adrenaline is a neurotransmitter in this species (Lœwi, 1937; Falck & others, 1963; Azuma, Binia & Vischer, 1965). Perhaps this might also be true of the brain of the fish, but not of the heart where there is a substantial amount of noradrenaline present.

Acknowledgement. We would like to thank Prof. Dr. J. M. Gallardo for his assistance with the dissection.

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Disulfiram and some effects of amphetamine in mice and rats

SIR,—The recent findings, that amphetamine releases noradrenaline in a physiologically active form (Glowinski & Axelrod, 1965, 1966) and that its central effects are blocked by α-methyl-*p*-tyrosine, an inhibitor of tyrosine hydroxylase (Weissman & Koe, 1965; Hanson, 1966; Weissman, Koe & Tenen, 1966; Randrup & Munkvad, 1966), seem to support the view that amphetamine acts indirectly and that the action is exerted through the catecholamine mediator.

Disulfiram has been found to inhibit the β-hydroxylation of dopamine to noradrenaline (Musacchio, Goldstein & others, 1966). It seemed interesting to examine the influence of this substance on some amphetamine effects.

Mice of two strains, R_3 and $C_{57}BL$, and also Wistar rats were used. Disulfiram was administered intraperitoneally 2 hr before an experiment. Spontaneous motor activity in single mice or rats was registered during one to two hr with a photoelectric meter. (±)-Amphetamine sulphate (5 mg/kg s.c.)

TABLE 1. THE EFFECT OF DISULFIRAM ON THE AMPHETAMINE-INDUCED MOTOR HYPERACTIVITY IN MICE

Strain	Disulfiram i.p. mg/kg	Activity counts	Inhibition %	P
R_3	—	911 (±107·8)	—	—
"	100	403 (± 97·1)	55·8	<0·01
"	200	281 (± 65·4)	69·2	<0·001
"	400	60 (± 11·2)	93·4	<0·001
$C_{57}BL$	—	1013 (± 53·2)	—	—
"	50	466 (± 60·4)	54·0	<0·001
"	100	376 (± 52·3)	62·9	<0·001

Disulfiram was injected 2 hr, (±)-amphetamine sulphate (5 mg/kg s.c.) ½ hr before the experiment. The activity was recorded in single mice during ½ hr sessions. Figures represent the means of 10 mice.

was given 0·5 hr earlier. The amphetamine-induced stereotyped behaviour in rats was tested by a rating scale (Weissman & others, 1966) every ½ hr for 4 hr starting immediately after the injection of (±)-amphetamine sulphate (10 mg/kg s.c.). The toxicity in aggregated mice was recorded 1, 2, 4, 8 and 24 hr after the injection of (±)-amphetamine sulphate (30–35 mg/kg i.p.). All the tests were made in groups of at least 10 animals.

The results in Table 1 show that pretreatment with disulfiram (50–400 mg/kg) reduced significantly amphetamine-induced hyperactivity in mice. This antagonistic effect was seen in rats too. The activity count in rats injected with amphetamine was 747 (±85·2), and in those pretreated with disulfiram (100 mg/kg) it was 269 (±46·2). This represents 64% inhibition, ($P < 0.001$).

The amphetamine stereotyped behaviour in rats was not blocked by disulfiram, (100 mg/kg) given in single or repeated doses—two or three every 2 hr. The higher doses (200 mg/kg) produced little blockade but after this treatment some of the rats died.

The previous or simultaneous treatment with disulfiram (50–100 mg/kg) did not reduce the toxicity of amphetamine in aggregated mice. The protective action of disulfiram was also not seen after injections on two or three occasions. On the contrary, some enhancement of lethality was observed during the 4 hr after amphetamine, especially in mice given three doses of disulfiram.

In the experiments presented here we did not determine the brain catecholamines. Nevertheless the doses of disulfiram injected are known from the literature to decrease the noradrenaline level and to increase the dopamine level (Hashimoto, Ohi & Imaizumi, 1965; Goldstein & Nakajima, 1966; Musacchio & others, 1966; Symchowicz, Korduba & others, 1966). α-Methyltyrosine, which lowers the brain content of both catecholamines, has been found to block either the motor hyperactivity or the stereotyped behaviour induced by amphetamine (Weissman & others, 1966; Randrup & Munkvad, 1966). Our findings seem to indicate that the motility effect of amphetamine is associated with noradrenaline, whereas dopamine may be involved in the stereotyped behaviour.

These conclusions are in agreement with the results obtained with diethyldithiocarbamate (Randrup & Scheel-Krüger, 1966) recognized as an active metabolite of disulfiram.

The failure of disulfiram to reduce the amphetamine toxicity in aggregated mice may support the view that this latter effect is a consequence of a more complex mechanism than the one mediated by catecholamines (Mennear & Rudzik, 1966; George & Wolf, 1966).

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Tracing the changes in capillary permeability during rat anaphylaxis

SIR,—When rats die after anaphylactic shock, there is always haemoconcentration and gross haemorrhage in the small intestine with occasional damage to the lungs and heart (Dawson, Starr & West, 1966). It was of interest therefore to examine the distribution of the specific antigen used for challenge after it has been suitably labelled with radioactive iodine, and to trace its localization in target organs in the rat.

Groups of male Sprague-Dawley rats, 120–150 g, were sensitized by an intraperitoneal injection of horse serum (0·5 ml) mixed with *Bordetella pertussis* vaccine (0·25 ml of 80,000 $\times$ 10^6 organisms per ml). Twelve days later, they were injected intravenously, under light ether anaesthesia, with 2 ml of the solution of labelled horse serum (equivalent to 1 ml original serum) and killed 3 hr later. The peritoneal cavity of each animal was washed for 2 min with 0·5 ml 0·9% saline and the fluid was then removed. Different tissues were dissected, cleaned and weighed. Radioactivity in the saline washing and in the tissues was counted in a Packard Tricarb liquid scintillation counter. The phosphor consisted of naphthalene, PPO, dimethyl POPOP, xylene, 1,4-dioxane, and ethanol. Counting efficiency was 38·0 $\pm$ 0·23%. The degree of diffusion of the labelled serum from the circulation into the peritoneal cavity was taken as a measure of the change in capillary permeability occurring in anaphylaxis.

To prepare the iodine-labelled horse serum, carrier-free sodium iodide solution in 0·9% saline (29 ml containing 9·47 μc ^{131}I) was added slowly, with continuous stirring, to an equal volume of horse serum at pH 7·5. Hydrogen peroxide (1·0 ml, 100 vols) was then added to release nascent iodine, and the reaction was continued in a shaking incubator at 37° for 1·5 hr (McFarlane, 1956). The mixture was dialysed against 2 litre quantities of ice-cold distilled water for up to 72 hr until 0·5 ml aliquots of the dialysate showed no radioactivity. With this method, iodination of the serum protein is minimized, and the physical and chemical characters of the horse serum are retained.

The results show that diffusion of ^{131}I-labelled horse serum into the peritoneal cavity of rats after anaphylactic shock is about 4 times greater in sensitized animals (average net activity of peritoneal washings, 75 $\pm$ 10 counts/min) than in control non-sensitized animals (activity, 18 $\pm$ 6 counts/min) given the same dose (2 ml) of labelled antigen. Thus, capillary permeability is greatly increased in animals undergoing anaphylactic shock and radioactive antigen passes through the intestinal vasculature into the cavity of the peritoneum. However, this was the only difference found as the radioactivity in the heart, small intestine, thymus, liver, spleen, lung, kidney and brain of sensitized rats after challenge was not significantly different from that of non-sensitized animals similarly challenged.

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Antagonistic action of vitamin D_2 on noradrenaline-induced lipolysis *in vitro*

SIR,—Results obtained with prostaglandin E_1 and digitoxin suggested that a possible role was played by calcium in the lipolysis induced by noradrenaline (Fassina & Contessa, 1966, 1967). The variations of the calcium concentration in the incubation medium of epididymal fat modified the stimulating action of noradrenaline on lipolysis. We therefore investigated the influence on the same lipolytic process of vitamin D_2, a drug known to have a specific effect on calcium metabolism.

Vitamin D_2 acts as an inhibitor of lipolysis *in vitro* (Table 1). The drug antagonizes the noradrenaline-induced lipolysis starting from a concentration of 10^{-5}M, as indicated by the decreased concentration of free fatty acids as well as of glycerol in the incubation medium of rat epididymal fat. The basal lipolysis was not significantly affected.

TABLE 1. INHIBITION BY VITAMIN D_2 OF NORADRENALINE-INDUCED LIPOLYSIS *in vitro*

Drugs in the incubation medium Molar conc.	△ FFA* μ-equiv./g/150 min	△ Glycerol* μmoles/g/150 min
Noradrenaline $0{\cdot}2 \times 10^{-5}$	$20{\cdot}86 \pm 0{\cdot}77$	$9{\cdot}31 \pm 0{\cdot}70$
Noradrenaline ,, + Vit. $D_2 10^{-5}$	$15{\cdot}03 \pm 1{\cdot}19$	$5{\cdot}25 \pm 0{\cdot}88$
Noradrenaline ,, + Vit. $D_2 10^{-4}$	$10{\cdot}54 \pm 1{\cdot}31$	$3{\cdot}34 \pm 0{\cdot}40$
Noradrenaline ,, + Vit. $D_2 10^{-3}$	$5{\cdot}25 \pm 0{\cdot}35$	$2{\cdot}10 \pm 0{\cdot}20$

Rat epididymal fat (100 ± 10 mg) was incubated in 2 ml of Krebs–Ringer bicarbonate buffer containing 2·5% bovine albumin, at 37° for 150 min, in a metabolic shaker. Free fatty acids (FFA) (Dole, 1956) and glycerol (Korn, 1955) were titrated in the incubation medium. Drugs were added to the medium before introducing the fat. Vitamin D_2 was dissolved in ethanol. The same volume of ethanol (0·05 ml) was added in all assays.

* FFA and glycerol absolute increase over control (fat incubated without drugs). Each value represents the mean ± s.e. of six experiments.

Vitamin D_2 dissolved in ethanol was inactivated by an exposure to ultraviolet light for 16 hr. As indicated by Cima, Levorato & Mantovan (1967), after this period the chromatographic spot of vitamin D_2 almost completely disappeared, but the inactivated drug still inhibited noradrenaline-induced lipolysis. Consequently, the effect of vitamin D_2 on lipolysis does not seem to be connected with the specific vitamin action.

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