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'You don't take a hammer to a Rotapress'

In 1843 William Brockedon, a North Country Chemist, was granted a patent for the novel process of "Shaping Pills, Lozenge and Black Lead by pressure in Dies." At the time his pre-occupation was the manufacture of pencils and it is thought that he applied the pressure by means of a *hammer*. No doubt Mr. Brockedon would be not a little startled if he could see the far-reaching consequences both social and scientific that his ingenious idea has unleashed to-day.

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The *in vitro* and *in vivo* metabolism of optically active methylcyclohexanols and methylcyclohexanones

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The *in vitro* reduction and oxidation of the isomeric methylcyclohexanones and methylcyclohexanols by horse liver alcohol dehydrogenase, in the presence of $NADH_2$ or NAD has been studied. The enzymic reactions closely resemble the corresponding *in vivo* metabolic conversions and support the view that the oxido-reductase in the animal body is similar to liver alcohol dehydrogenase. The findings are discussed in terms of the conformations of the alcohols and ketones and a correlation of the observed rates, K_m values, and the steric specificity supports the view that the *in vivo* inversion of the thermodynamically less stable alcohols (±)-*cis*-2-, (±)-*trans*-3-, and *cis*-4-methylcyclohexanol could occur through a ketone intermediate.

From the studies of Prelog (1959), and Graves, Clark & Ringold (1965) it appears that an oxido-reductase system is responsible for the biological oxidation and reduction of alicyclic alcohols and ketones. The identity of the enzyme, however, has not yet been clearly established, although Tao & Elliott (1962) suggested that LADH might be responsible, though Prelog (1964) considered that this could not be the enzyme as it would not reduce the *trans*-decalin-1,4-diones.

Views vary about the stereochemical course of the reaction. Prelog (1959 & 1964) proposed a "head-to-tail" orientation for the substrate relative to the coenzyme in the transition state, Tao & Elliott (1962) suggested a "face-to-face" orientation, Graves & others (1965) have proposed two orientations—"flat and upright"—and Cheo, Elliott & Tao (1967) have also suggested that there are two possible orientations which they describe as "face-to-face" and "perpendicular".

The present investigation attempts to clarify these two problems through a systematic comparison of the biological and enzymic transformations of the enantiomeric methylcyclohexanones and methylcyclohexanols. In addition the opportunity has been taken to present a simple stereochemical model of the reaction mechanism which appears to have a reasonably good predictive value.

MATERIALS

Liver alcohol dehydrogenase (LADH—Alcohol: NAD oxido-reductase [E.C. No. I.I.I.I.]), twice recrystallized and prepared by the method of Bonnichsen & Brink (1955) was purchased from Worthington Biochemical Corporation, Freehold, N.J. Nicotinamide adenine dinucleotide (NAD) and reduced nicotinamide adenine dinucleotide ($NADH_2$) were obtained from Sigma Chemical Company, St. Louis.

Tritiated nicitonamide adenine dinucleotide NAD(T) was prepared according to San Pietro (1955). All derivatives of methylcyclohexane were purified by preparative

gas-liquid chromatography and distillation under reduced pressure and were characterized by thin-layer and gas-liquid chromatography; their retention time, Rf values, refractive indices and optical rotations at 589 and 305 or 309 nm are given in Table 1.

(±)-2-, (±)-3-, and 4-Methylcyclohexanone were obtained commercially (Eastman Kodak Ltd., Rochester). (+)-2-, (−)-2-, and (−)-3-Methylcyclohexanone were obtained by chromic acid oxidation of (+)-*trans*-2, (+)-*cis*-2-, and (+)-*cis*-3-methylcyclohexanol respectively (Ohloff, Osiecki & Djerassi, 1962). (+)-3-Methylcyclohexanone was obtained from (+)-pulegone by refluxing with 2·5N HCl.

The optically inactive isomeric methylcyclohexanols (±)-*cis*-, and (±)-*trans*-2-methylcyclohexanol (±)-*cis*,- and (±)-*trans*-3-methylcyclohexanol, and *cis*-, and *trans*-4-methylcyclohexanol were separated from commercial (Eastman Kodak Ltd) *cis*/*trans* mixture of the 2-, 3-, and 4-methylcyclohexanols respectively, by preparative gas-liquid chromatography. The optically active isomeric methylcyclohexanols were prepared biologically by feeding the appropriate ketone to rabbits and isolating the corresponding glucuronides from the 24 h urine by the basic lead acetate method of Kamil, Smith & Williams (1951). The glucuronide or its triacetylmethyl ester was then hydrolysed with N HCl and the aglycone was recovered by steam distillation and extraction of the distillate with ether. For the preparation of (+)-*cis*-, and (+)-*trans*-2-methylcyclohexanol, (±)-2-methyl-cyclohexanone was fed. (−)-*cis*, and (−)-*trans*-3-Methylcyclohexanol were obtained by feeding (+)-3-methylcyclohexanone. (+)-*cis*-3-Methylcyclohexanol was prepared by diastereomeric resolution and hydrolysis of the triacetylmethyl ester of the glucuronide obtained after feeding (±)-3-methylcyclohexanone.

METHODS AND RESULTS

Thin-layer chromatography

Plates were prepared as described by Elliott, Robertson & Williams (1966). For the separation of the 3-, and 4-methylcyclohexanones and methylcyclohexanols, solvent system 1 (light petroleum b.p. 50–70°–ethyl acetate, 9 : 1 v/v) was used. For the separation of the 2-methylcyclohexanones and methylcyclohexanols, solvent system 2 (light petroleum b.p. 50–70°–ethyl acetate, 8 : 2 v/v) was used. Alcohols were located with the phosphomolybdic acid spray of Kritchevsky & Kirk (1952), and ketones with Brady's reagent.

Gas-liquid chromatography, infrared spectroscopy and optical rotatory dispersion studies.

The instrumentation and conditions employed were as described by Cheo & others (1967), except where specifically indicated in the Tables.

Face specificity studies

A modification of the method of Krakow, Ludowieg & others (1963) was used. For alcohols, a control system comprising ethanol (0·1 ml), yeast alcohol dehydrogenase (YADH), (0·1 mg), NAD(T), (0·5 μmol) and sodium phosphate buffer (400 μmol, pH 9·6) in a total volume of 3 ml was used. After the first stage of the reaction was complete the mixture was boiled for 2 min, centrifuged and adjusted to pH 7 with NaH_2PO_4 (200 μmol) and sulphuric acid. Sodium pyruvate (12 μmol) and lactic dehydrogenase were then added, and the reaction followed spectrophotometrically at 340 nm.

The test system had the same composition and was treated in the same way as the control system except that 90 μmol of the appropriate methylcyclohexanol was used in place of ethanol and the YADH was replaced by LADH (200 mg). In both control and test systems, the nicotinamide was isolated according to Marcus, Vennesland & Stern (1958), dissolved in Panax TPP/3 solution and counted in a scintillation counter (Panax SC-LP). For ketones, the control system was prepared and treated as specified for alcohols. In the test system, NADT(H) was generated as described above for the alcohol control system except that, after centrifugation, acetaldehyde, which would interfere in the subsequent reaction, was removed by passing nitrogen through the mixture. In the second stage the appropriate ketone (90 μmol) and LADH (200 μg) were added and the subsequent procedure was as described for alcohols. Table 2 compares the tritium content of the nicotinamide obtained from the test and control systems for the various substrates, and indicates the stereospecificity of the transfer.

Kinetic studies

For the determination of Michaelis constants, reactions were followed spectrophotometrically on a Beckman DK-2A spectrophotometer at 340 nm in quartz cuvettes. Changes in absorbance were recorded continuously from within 10 s of the initiation of the reaction. Initial rates of change were measured and a steady state was assumed since the reactions were linear over the first 3-min. Ketones were dissolved in 0·003M NaH_2PO_4 buffer at pH 7·0, alcohols were dissolved in 0·1M

Table 1. *Physical properties, Michaelis constant and relative rates of oxidation or reduction of alcohols and ketones*

	*Retention time (min)	Rf	$[n]_D^{23}$	$[\alpha]_D^{23}$	$[\alpha]^{MeOH}$ †‡	K_m (molar)¶	Relative rates§
(±)-2-Methylcyclohexanone	3·1	0·85	1·4559	0	0	$1·79 \times 10^{-2}$	1·1
(+)-2-Methylcyclohexanone	3·1	0·85	1·4563	+14·5	+519	$1·03 \times 10^{-2}$	3·6
(−)-2-Methylcyclohexanone	3·1	0·85	1·4544	−13·9	−512	$2·12 \times 10^{-2}$	0·4
(±)-3-Methylcyclohexanone	4·1	0·46	1·4430	0	0	$1·44 \times 10^{-3}$	57·6
(+)-3-Methylcyclohexanone	4·1	0·46	1·4409	+14·3	+983	$1·19 \times 10^{-2}$	1·1
(−)-3-Methylcyclohexanone	4·1	0·46	1·4416	−13·6	−958	$1·48 \times 10^{-3}$	68·1
4-Methylcyclohexanone	4·1	0·50	1·4429	0	0	$1·88 \times 10^{-3}$	40·3
(±)-*cis*-2-Methylcyclohexanol	7·1	0·75	1·4621	0	0	$1·10 \times 10^{-2}$	12·7
(+)-*cis*-2-Methylcyclohexanol	7·1	0·75	1·4627	+8·9	−520	$1·55 \times 10^{-2}$	7·3
(±)-*trans*-2-Methylcyclohexanol	9·1	0·66	1·4580	0	0	$1·60 \times 10^{-2}$	15·5
(+)-*trans*-2-Methylcyclohexanol	9·1	0·66	1·4590	+22·4	+508	$1·21 \times 10^{-2}$	18·2
(±)-*cis*-3-Methylcyclohexanol	13·1	0·21	1·4550	0	0	$2·70 \times 10^{-3}$	35·4
(+)-*cis*-3-Methylcyclohexanol	13·1	0·21	1·4549	+6·0	−990	$1·75 \times 10^{-3}$	64·4
(−)-*cis*-3-Methylcyclohexanol	13·1	0·21	1·4559	−5·7	+970	$6·36 \times 10^{-3}$	22·5
(±)-*trans*-3-Methylcyclohexanol	9·8	0·27	1·4531	0	0	$1·56 \times 10^{-3}$	17·0
(−)-*trans*-3-Methylcyclohexanol	9·8	0·27	1·4540	−6·1	+968	$8·47 \times 10^{-3}$	12·4
cis-4-Methylcyclohexanol	9·6	0·30	—	0	0	$4·10 \times 10^{-3}$	36·9
trans-4-Methylcyclonexanol	12·9	0·21	—	0	0	$1·3 \times 10^{-3}$	52·1
Cyclohexanol						$5·52 \times 10^{-3}$	100·0
Cyclohexanone						$2·3 \times 10^{-3}$	100·0

* A 10% diglycerol/celite column was used at 80°C, flow rate 120 ml/min. Sample size was 0·1–0·5 μl of a 10% ethereal solution.

† 305 nm for the 2-ketone and 309 nm for the 3-ketone.

‡ Alcohols were oxidized by CrO_3 to the corresponding ketones before measurement.

¶ K_m values were obtained from Lineweaver and Burke plots.

§ Oxidation rates of ketones were compared with cyclohexanone at the same concentration, while rates of alcohol were compared with cyclohexanol.

Table 2. *Stereospecificity of hydrogen transfer*

Compound	Activities (counts 10^{-6}/100 s mg^{-1}) Test system (T)	Control system (C)	T/C × 100	Stereo specificity
(±)-2-Methylcyclohexanone	9·0	9·0	100	A
(±)-3-Methylcyclohexanone	1·1	1·1	100	A
4-Methylcyclohexanone	8·0	8·3	96	A
(±)-*cis*-2-Methylcyclohexanol	8·5	8·3	102	A
(±)-*trans*-2-Methylcyclohexanol	8·7	8·6	101	A
(±)-*cis*-3-Methylcyclohexanol	9·1	9·2	99	A
(±)-*trans*-3-Methylcyclohexanol	8·9	9·1	98	A
cis-4-Methylcyclohexanol	9·1	9·7	94	A
trans-4-Methylcyclohexanol	9·2	9·5	97	A

$Na_4P_2O_7$ buffer at pH 9·8. The substrate concentration was varied between 3·7 × 10^{-4}M and 7·8 × 10^{-3}M. Coenzyme concentrations were 1 × 10^{-4}M for $NADH_2$ and 0·95 × 10^{-4}M for NAD. The reactions were initiated by the addition of LADH, 35 μg in 0·2 ml of water. For the determination of relative rates of reduction the reactions were made as described for the K_m determinations except that the substrates were studied at a final concentration of 2 × 10^{-3}M. Table 1 lists the K_m values and relative rates of oxidation of the isomeric methylcyclohexanones and methylcyclohexanols.

Product formation from ketones

All reactions were studied at 24°. The ketone (16 or 50 mg) was dissolved in 0·003M NaH_2PO_4 buffer (200 ml), pH 7·0 to which was added $NADH_2$ (150 or 200 mg)

Table 3. *Product formation from ketones* in vitro

Compound	Amount of each reactant (mg) coenzyme	substrate	Unreacted ketone	$[\alpha]_D^{23}$	$[\alpha]^{MeOH}$*	Ratio *cis*/*trans*
(+)-2-Methylcyclohexanone	150	50	present	+22·4	+521	0/100
(−)-2-Methylcyclohexanone	150	50	present	+8·9	−512	100/0
(±)-2-Methylcyclohexanone	150	50	present	+8·9 +22·4	−522 +520	28/72
(±)-2-Methylcyclohexanone	150	16	trace	+8·9 +22·4	— —	45/55
(±)-2-Methylcyclohexanone	200	16	undetected	+8·9 +22·4	— —	50/50
(+)-3-Methylcyclohexanone	150	50	present	−6·1 —	+983 +968	65/35
(−)-3-Methylcyclohexanone	150	50	present	+7·0 —	−990 −958	65/35
(±)-3-Methylcyclohexanone	150	50	present	— —	−600 −580	65/35
(±)-3-Methylcyclohexanone	150	16	trace	— —	0 0	70/30
(±)-3-Methylcyclohexanone	150	25	present	—	—	65/35
4-Methylcyclohexanone	150	50	present	—	—	30/70

* These values were obtained from the ketones derived by chromic acid oxidation of the alcohol products, $[\alpha]_{305}^{MeOH}$ for the 2-position and $[\alpha]_{309}^{MeOH}$ for the 3-position.

† The unattacked ketone had $[\alpha]_{309}^{MeOH}$ +790.

and crystalline LADH (8 mg). The course of the reaction was determined at frequent intervals by spectrophotometric estimation of the amount of $NADH_2$ present. Reactions were allowed to proceed until all the coenzyme was consumed (10–15 h), the enzyme was then denatured by boiling for 5 min, after which the products were removed by continuous extraction with ether. The etheral extract was dried with $MgSO_4$. The components in the residue after removal of the solvent were separated by preparative gas chromatography, and purified by distillation at 30° under reduced pressure. Each fraction was identified by thin-layer chromatography, gas chromatography and optical rotation measurements. Alcohols were converted to ketones using the oxidizing agent of Curtis, Heilbron & others (1953) and their optical rotatory dispersion curves were traced. Where both the *cis*- and *trans*-isomers were produced the ratios were determined by measuring the areas under the respective curves of samples separated by analytical gas chromatography. In one experiment with (±)-3-methylcyclohexanone only 25 mg of the substrate was used and the reaction was terminated after 6 h. Table 3 lists the ketones studied, the amount of coenzyme used, the amount of unreacted ketone, the physical properties and identities of the products, and the ratio of *cis*- and *trans*-isomers formed.

Product formation from alcohols

The reactions were as described for the ketones except that in each case either 15, or 50 mg of the appropriate alcohol was oxidized with NAD (150 or 200 mg). The buffer used was 0·1M $Na_4P_2O_7$ at pH 9·8. Table 4 lists the compounds studied, the amount of enzyme used, the physical properties and identities of the products.

Metabolic experiments

These were as described by Elliott, Tao & Williams (1965) and the 24 h urine was hydrolysed by refluxing with 1N HCl for 1 h. The hydrolysate was then steam distilled, the distillate extracted with ether, dried with $MgSO_4$ and the ether removed.

Table 4. *Product formation from alcohol* in vitro

Compound	Amount of each reactant (mg)		Unreacted alcohol	$[\alpha]^{MeOH}$*	Ketone formed
	coenzyme	substrate			
(±)-*cis*-2-Methylcyclohexanol	150	50	present	+260	(+) & (−)-2-,
	150	16	trace	0	(±)-2-,
(+)-*cis*-2-Methylcyclohexanol	150	50	present	−513	(−)-2-,
(±)-*trans*-2-Methylcyclohexanol	150	50	present	+150	(+) & (−)-2-,
	150	16	trace	0	(±)-2-,
(+)-*trans*-2-Methylcyclohexanol	150	50	present	+526	(+)-2-,
(±)-*cis*-3-Methylcyclohexanol	150	50	present	−470	(+) & (−)-3-,
	150	16	trace	−20	(+) & (−)-3-,
	200	16	undetected	0	(±)-3-,
(+)-*cis*-3-Methylcyclohexanol	150	50	present	−987	(−)-3-,
(−)-*cis*-3-Methylcyclohexanol	150	50	present	+960	(+)-3-,
(±)-*trans*-3-Methylcyclohexanol	150	50	present	−780	mainly (−)-3-,
	150	16	trace	−60	(+) & (−)-3-,
	200	16	undetected	0	(±)-3-,
(−)-*trans*-3-Methylcyclohexanol	150	50	present	+990	(+)-3-,
cis-4-Methylcyclohexanol	150	50	present	—	4-,
trans-4-Methylcyclohexanol	150	50	present	—	4-,

* 305 nm for the 2-ketone and 309 nm for the 3-ketone.

Table 5. *The metabolic products of the* 2-, 3-, *and* 4- *methylcyclohexanones and methylcyclohexanols* in vivo

Compound	Alcohol isolated	$[\alpha]_D$	$[\alpha]^{MeOH}$ *	Ratio *cis*/*trans*
(±)-2-Methylcyclohexanone	(+)-*cis*-2	+8·9	−510	27/73
	(+)-*trans*-2	+22·4	+520	
(+)-2-Methylcyclohexanone	(+)-*trans*-2	+22·9	+512	0/100
(−)-2-Methylcyclohexanone	(+)-*cis*-2	+8·9	−508	100/0
(±)-3-Methylcyclohexanone	(±)-*cis*-3	0	0	70/30
	(±)-*trans*-3	0	0	
(+)-3-Methylcyclohexanone	((−)-*cis*-3	−5·9	+960	70/30
	(−)-*trans*-3	−8·2	+980	
4-Methylcyclohexanone	*cis*-4	—	—	30/70
	trans-4	—	—	
(±)-*cis*-2-Methylcyclohexanol	(+)-*cis*-2	+8·9	−524	20/80
	(+)-*trans*-2	+22·9	+520	
(±)-*trans*-2-Methylcyclohexanol	(±)-*trans*-2	0	0	0/100
(±)-*cis*-3-Methylcyclohexanol	(±)-*cis*-3	0	0	100/0
(±)-*trans*-3-Methylcyclohexanol	(±)-*cis*-3	0	0	70/30
	(±)-*trans*-3	0	0	
cis-4-Methylcyclohexanol	*cis*-4	—	—	30/70
	trans-4	—	—	
trans-4-Methylcyclohexanol	*trans*-4	—	—	0/100

* These values were obtained from the ketones derived by chromic acid oxidation of the alcoho products $[\alpha]_{305}^{MeOH}$ for 2-position and $[\alpha]_{309}^{MeOH}$ for the 3-position.

The residue was separated by preparative gas-liquid chromatography and the individual alcohols purified by distillation under reduced pressure. The characterization of the aglycones, and the determination of *cis*- and *trans*-ratios was as described for the products formed *in vitro* from ketones. A list of the compounds studied, the physical characterization of the aglycones, and the ratios of *cis*-alcohol to *trans*-alcohol formed is given in Table 5.

DISCUSSION

From an examination of Catalin molecular models representing possible coenzyme substrate interactions in the transition complex it can be seen that, of the theoretically infinite number of possible orientations for the substrate and coenzyme, if the enzyme is "A" stereospecific (Table 2) only six are effectively different. These are (i) the normal "face-to-face" orientation, in which the axial 3- and 5-hydrogen atoms of the substrate are directed towards the "A" face of the coenzyme, and C-1 of the substrate lies directly above C-4 of the coenzyme; (ii) the normal "head-to-tail" orientation in which the substrate, by rotation through 180° about a fixed point located at the carbonyl group lies in the same plane as the coenzyme, but with C-1 of the substrate and C-4 of the coenzyme facing each other; (iii) the normal "perpendicular" approach—an intermediate disposition between (i) and (ii)—arrived at by rotating the substrate molecule through 90° from the original face-to-face position. The remaining three dispositions are those in which the orientation of the substrate molecule relative to the coenzyme is reversed—that is the face bearing the 2,4,6-axial hydrogen atoms is presented to the coenzyme. To distinguish these from the normal orientations, they are termed "reverse face-to-face", "reverse head-to-tail" and "reverse perpendicular" orientations, and each substrate in reverse orientation subtends, relative to the coenzyme, exactly the same angle as the corresponding substrate in its normal orientation. Cheo & others (1967) have shown that the substrates react biologically

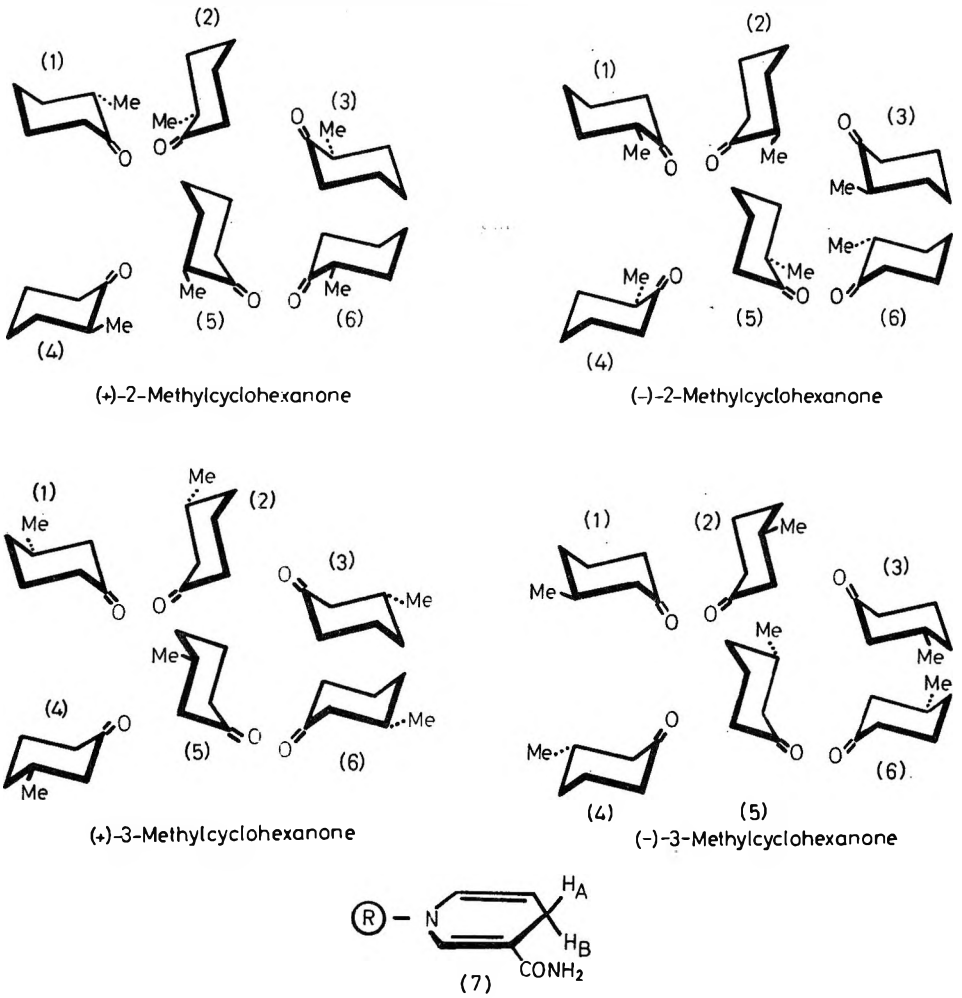

FIG. 1. Orientation of the 2-, and 3-methylcyclohexanones. (1) Face-to-face orientation, (2) Perpendicular orientation, (3) Head-to-tail orientation, (4) Reverse face-to-face orientation, (5) Reverse perpendicular orientation, (6) Reverse head-to-tail orientation. All orientations are relative to NADH (7) in the disposition shown.

in their most stable conformations and Fig. 1 demonstrates the various possibilities for the 2-, and 3-methylcyclohexanones. In these models it is considered that the function of the enzyme is to bind the coenzyme and substrate in the activated complex with the properties assigned to it by Vennesland (1958); the coenzyme is considered to be the asymmetric reagent.

Further examination of the models reveals that if there is to be a fruitful transfer of hydrogen, no steric interaction between the carboxyamide group of the nicotinamide moiety of the coenzyme and the alkyl substituent of the substrate should occur, and C-1 of the substrate should be accessible to the reactive "A" face of the coenzyme. Models indicate that, for ketones, the most important influence on hydrogen transfer is exercised by the steric interaction occurring between substituents on C-2 of the substrate and the carboxyamide group of the coenzyme, and it is this group that is decisive in determining the stereochemical course of the reaction. With alcohols,

because of their different configuration, carboxyamide interactions are demonstrably less significant, so that hydrogen transfers can occur that are impossible with ketones. However it is important to observe that if all experimentally found reaction products are to be accounted for correctly, the oxygen atom of the substrate must be directed away from the nitrogen atom of the nicotinamide ring as observed by McKinley-McKee (1964). Non-bonded hydrogen interactions are important only if they limit the accessibility of C-1. If these requirements are satisfied it is possible to predict not only that a reaction will occur, but also the identity of the product, if the stereochemical relation of the precursor to its product and the orientation of the substrate in the transition complex are known. In practice, the presence or absence of a carboxyamide interaction determines whether the (+) or (−)-stereoisomer, or both, will react, and the orientation in the substrate coenzyme complex determines whether a hydrogen atom is transferred to or from the "normal" or "reverse" side of the substrate. Transfer to the normal side of a ketone results in the formation of an equatorially orientated hydroxyl group, and transfer to the reverse side results in an axial hydroxyl group. Removal of hydrogen from an alcohol will result in the corresponding stereochemically related ketone.

Table 6 gives, for the 2- and 3-methylcyclohexanones and methylcyclohexanols, the only fruitful orientations of the various substrates that are without inhibitory interactions and in which the C-1 atom is accessible for hydrogen transfer. It also tabulates the predicted products in these orientations, the actual products obtained in enzymic experiments, and the experimental reversibility of each reaction. As shown in the Table, substrates react in only two of the six possible orientations—namely face-to-face

Table 6. *Orientations and interactions of the* 2 *and* 3-*methylcyclohexanones and methylcyclohexanols*

Substrate	Orientations without interaction	Predicted alcohol or ketone	Actual alcohol or ketone	Reversibility of reaction
(+)-2-Methyl-cyclohexanone	face-to-face only	(+)-*trans*-2-	(+)-*trans*-2-	complete
(−)-2-Methyl-cyclohexanone	reverse perp. only	(+)-*cis*-2-	(+)-*cis*-2-	complete
(+)-*trans*-2-Methylcyclo-hexanol	face-to-face only	(+)-2-methyl	(+)-2-methyl-	complete
(−)-*cis*-2-Methylcyclo-hexanol	reverse perp. only	(+)-2-methyl-	(+)-2-methyl-	irreversible
(−)-*trans*-2-Methyl hexanol	face-to-face only	(−)-2-methyl-	(−)-2-methyl-	irreversible
(+)-*cis*-2-Methyl-cyclohexanol	reverse perp.	(−)-2-methyl-	(−)-2-methyl	complete
(+)-3-Methyl cyclohexanone	face-to-face	(−)-*cis*-3-	(−)-*cis*-3-	complete
	reverse perp.	(−)-*trans*-3-	(−)-*trans*-3-	complete
(−)-3-Methyl-cyclohexanone	face-to-face	(+)-*cis*-3-	(+)-*cis*-3-	complete
	reverse perp.	(+)-*trans*-3-	(+)-*trans*-3-	complete
(−)-*cis*-3-Methyl-cyclohexanol	face-to-face	(+)-3-methyl-	(+)-3-methyl-	complete
(−)-*trans*-3-Methyl-cyclohexanol	reverse perp.	(+)-3-methyl-	(+)-3-methyl-	complete
(+)-*cis*-3-Methyl-cyclohexanol	face-to-face	(−)-3-methyl-	(−)-3-methyl-	complete
(+)-*trans*-3-Methyl cyclohexanol	reverse perp.	(−)-3-methyl-	(−)-3-methyl-	complete

and reverse perpendicular. For example with (+)-2-methylcyclohexanone the only orientation without interactions is face-to-face, in which orientation the predicted alcohol would be (+)-*trans*-2-methylcyclohexanol, and this is the alcohol that was isolated experimentally. Similarly (−)-2-methylcyclohexanone yields (+)-*cis*-2-methylcyclohexanol only, in a reverse perpendicular orientation as predicted. In the reverse reactions (+)-2-methylcyclohexanone is produced either as a result of a face-to-face reaction of (+)-*trans*-2-methylcyclohexanol, or by a reverse perpendicular reaction of (−)-*cis*-2-methylcyclohexanol, whilst (−)-2-methylcyclohexanone is produced as a result of either a face-to-face reaction of (−)-*trans*-2-methylcyclohexanol or a reverse perpendicular reaction of (+)-*cis*-2-methylcyclohexanol, and in each case the predicted and experimentally obtained products were the same. Where the reactions are completely reversible, [(+)-2-methylcyclohexanone ⇌ (+)-*trans*-2-methylcyclohexanol and (−)-2-methylcyclohexanone ⇌ (+)-*cis*-2-methylcyclohexanol] the substrates have the same orientation relative to the coenzyme in both the oxidized and reduced forms, whereas where the reactions are not reversible [(−)-*cis*-2-methylcyclohexanol → (+)-2-methylcyclohexanone and (−)-*trans*-2-methylcyclohexanol → (−)-2-methylcyclohexanone], an approach to the transition state from the ketone side is not possible because carboxyamide interactions prevent the ketone from participating. For a reaction to be reversible, the reacting species must have a common transition state. Further, since the two alcohols (−)-*cis*-2-, and (−)-*trans*-2-methylcyclohexanol produce ketones irreversibly, the geometry of the transition state, as Prelog has suggested (1964), must resemble that of the alcohol more closely than that of the ketone.

The most notable difference between the reactions of the 2-, and 3-methylcyclohexanones and methylcyclohexanols, is an apparent lack of stereospecificity in the reactions of the latter, due to the complete absence of carboxyamide interactions with all of these isomers in all possible orientations. Since the particular isomer produced is determined only by the accessibility of C-1, and the orientation of the substrate relative to the coenzyme, reactions with these ketones are possible in both normal and reverse orientations. It is predictable that both (+)- and (−)-3-methylcyclohexanone will react either in a face-to-face or a reverse perpendicular orientation, and in practice (+)-3-methylcyclohexanone yields (−)-*cis*-3-, and (−)-*trans*-3-methylcyclohexanol in a ratio of 65 : 35 whilst, (−)-3-methylcyclohexanone yields (+)-*cis*-3-, and (+)-*trans*-3-methylcyclohexanol also in a ratio of 65 : 35. Hence if the reaction with the racemic ketone (±)-3-methylcyclohexanone is allowed to go to completion, the two alcohols produced—*cis*-3-, and *trans*-3-methylcyclohexanol—will be racemic. If the reaction does not go to completion, as for example in a system where there is either excess of substrate, or a limited amount of coenzyme, the product will be partially optically active (Graves & others, 1965) and if the reaction proceeds for a short time, only one product may be isolated because the rates of the two reactions are not the same. The results in Table 3 demonstrate this point. Since the products obtained *in vivo* were invariably racemic, there must be adequate amounts of the coenzyme available to effect the biological transformation. Further, the finding that the *cis*-/*trans*-ratio (in the presence of excess of coenzyme) is 65 : 35 indicates that the face-to-face reaction occurs with greater ease than the reverse perpendicular reaction, the actual ratio observed being a direct comparative measure of the facility of the two reactions.

The present findings also support the earlier suggestion of Tao & Elliott (1962) that in the biological conversion of (±)-*cis*-2-methylcyclohexanol to the glucuronides of

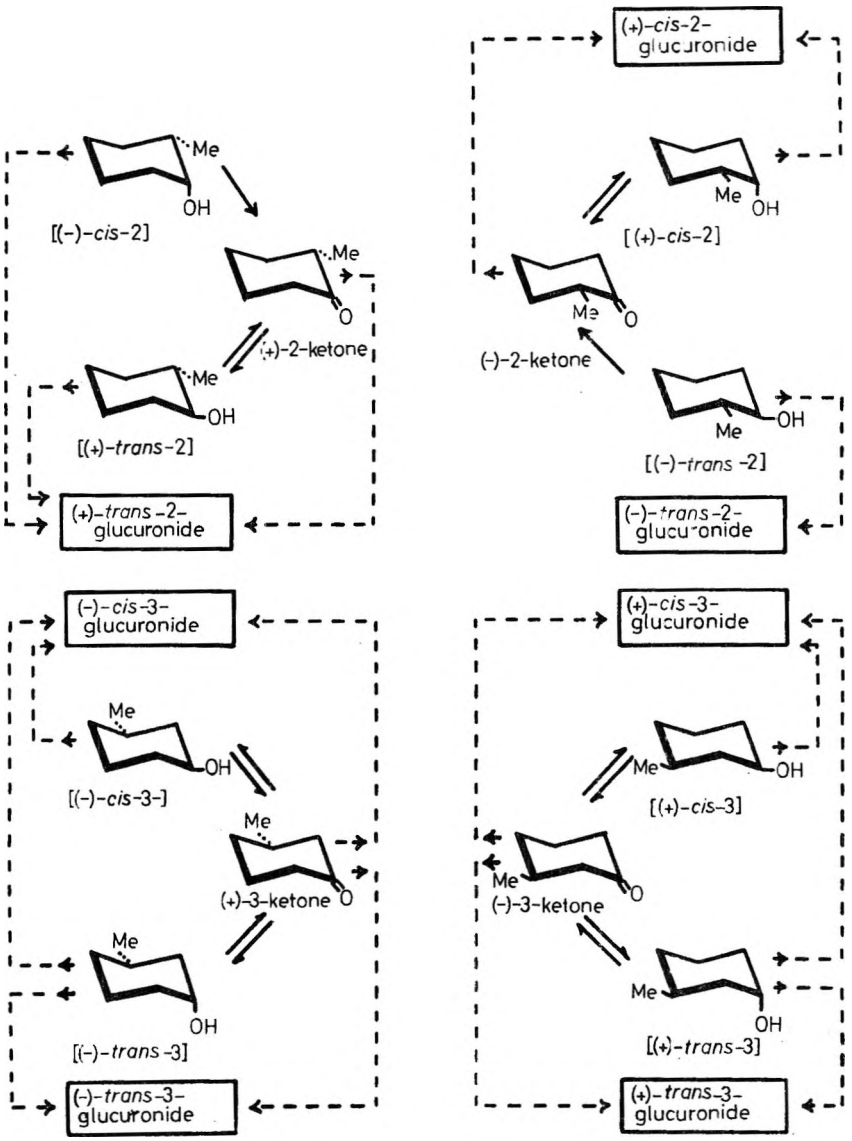

FIG. 2. *In vitro* (———) and *in vivo* (– – –) reactions of the 2- and 3-methylcyclohexanols and methylcyclohexanones.

(+)-*trans*-2- and (+)-*cis*-2-methylcyclohexanol and the similar conversions of (±)-*trans*-3-, and *cis*-4-methylcyclohexanols to the corresponding, thermodynamically more stable epimers, the inversions occur through a ketone intermediate. As shown in Table 5 when racemic *cis*-2-methylcyclohexanol is incubated with excess NAD and LADH the product is racemic 2-methylcyclohexanone and this, when incubated with liver alcohol dehydrogenase and excess $NADH_2$ (Table 3), is converted to (+)-*cis*-2-, and (+)-*trans*-2-methylcyclohexanol in a ratio of 28:72. Further confirmation is provided in the tritium transfer experiments in which NAD(T) was first reduced by (±)-*cis*-2-methylcyclohexanol to NADH(T) and in another experiment NADH(T) was used to reduce the ketone to the corresponding alcohols. Since radioactivity was

retained in the NADH(T) the hydrogen on C-1 of the (+)- and (−)-*cis*-2-methylcyclohexanols must have been successively transferred from the alcohols to the ketone and then to the (+)-*cis*- and (+)-*trans*-2-alcohols that were formed.

The *in vitro* results also afford an explanation of the particular metabolites found *in vivo*. Fig. 2 outlines the *in vitro* and *in vivo* reactions of the 2- and 3-methylcyclohexanols and methylcyclohexanones. The scheme indicates that whilst the (−)-*cis*-2-alcohol is rapidly converted *in vitro* to the (+)-2-ketone by an irreversible reaction (the Michaelis constant is particularly favourable) and thence by reduction to the (+)-*trans*-alcohol, finally to be conjugated with glucuronic acid, such a route is not possible for the (+)-*cis*-alcohol which can only be reversibly oxidized to (−)-2-methylcyclohexanone by a reaction having a relatively low affinity and low velocity in the reverse direction. Since the formation of the (−)-*trans*-alcohol from the (−)-ketone by an enzymic reaction has been shown not to occur; the only means of disposing of the (+)-*cis*-alcohol is by conjugation with glucuronic acid. Since the ratio of (+)-*cis*-2-, and (+)-*trans*-2-alcohols isolated from the urine was 27 : 73 it appears that the (+)-*cis*-2-alcohol accumulates in the body and is only slowly excreted. The intermediate conversion of the *trans*-2-alcohols to the 2-ketone *in vivo* is excluded because, had this occurred, the products would have been (+)-*trans*-2-, and (+)-*cis*-2-methylcyclohexanol (see Fig. 2). Not all of the enzymically possible reactions occur in the rabbit. For example the conversion of (−)-*trans*-2-methylcyclohexanol to (+)-*cis*-2-methylcyclohexanol, and the conversion of the *cis*-3-methylcyclohexanols, to the corresponding *trans*-3-methylcyclohexanols predictable on the basis of enzyme experiments, do not occur *in vivo*, the apparent reason being that the thermodynamically more stable alcohols are conjugated directly. The ketones, similarly, before conjugation are converted mainly into the more stable alcohol. These differences in the biological transformations indicate that the conjugation of equatorial alcohols with glucuronic acid occurs preferentially, and this is in accord with chemical conformational expectations.

The metabolic pattern of the 3-methylcyclohexanols is similar to that of the 2-methylcyclohexanols in that the (±)-*cis*-3-alcohols are conjugated in unchanged form, whilst the *trans*-3-alcohols are inverted presumably through a ketone intermediate, and the *in vitro* experiments duplicate the previously postulated pathway, viz. (±)-*trans*-3-methylcyclohexanol → (±)-3-methylcyclohexanone → (±)-*cis*-, and (±)-*trans*-3-methylcyclohexanol. Fig. 2 shows that all of the *in vitro* reactions unlike those of the 2-methylcyclohexanols, are completely reversible. Consequently, there is an equal possibility of all the alcohols being formed when racemic 3-methylcyclohexanone is fed, and this in fact occurs, (±)-*cis*-, and (±)-*trans*-3-methylcyclohexanols being formed in a ratio of 70 : 30.

Similar considerations apply in accounting for the inversions observed with the 4-methylcyclohexanols, and again the only assumption that needs to be made is that the inversions occur as a result of the formation of a ketone intermediate.

Acknowledgement

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The effects of 1-[di(2-chloroethyl)aminomethyl]benzimidazole and related compounds on the growth of experimental tumours

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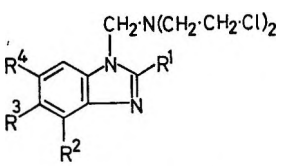
The inhibitory activity of some benzimidazole Mannich-base nitrogen mustards on the growth of experimental tumours, viz. mouse fibrosarcoma in mice and Yoshida ascites sarcoma in rats has been examined. Amongst the compounds tested 5,6-dichloro-1-[di(2-chloroethyl)aminomethyl]benzimidazole and 1-[di(2-chloroethyl)-aminomethyl]-2-phenylbenzimidazole showed inhibitory effect on mouse fibrosarcoma; while 4-bromo-1-[di(2-chloroethyl)amino-methyl]benzimidazole, 4-chloro-1-[di(2-chloroethyl)aminomethyl]-benzimidazole and 1-[di(2-chloroethyl)aminomethyl]-5-methoxybenzi-midazole were active against Yoshida ascites sarcoma.

The concept of incorporating an alkylating function e.g. the 2,2-dichloroethylamino-group, into a molecule known to play an important role in the biogenesis of a biologically active molecule, has received much attention (Benitez, Ross, & others, 1960). The phenylalanine mustards have been studied exhaustively in animals and in man as anticancer drugs (Greene, Baker & Greenberg, 1960). Their encouraging biological properties have created an increasing interest in the synthesis of allied hetero-aromatic compounds in a search for potential anticancer agents of high activity with minimum toxicity. In view of the biological importance of the benzimidazole moiety, a variety of benzimidazole mustards, i.e. 2-[di(2-chloroethyl)aminomethyl]benzimidazoles have been synthesized (Herschberg, Gellhorn & Gump, 1957, Gump & Nikawitz, 1959). Revankar & Siddappa (1967), employed a Mannich-type reaction, condensed benzimidazole and substituted benzimidazoles containing a labile N–H bond with formaldehyde and di(2-chloroethyl)amine to obtain a number of benzimidazole Mannich-base nitrogen mustards (I) as potential anticancer agents. Their inhibitory activities have been examined on experimental tumors in rats and mice and the results are here reported.



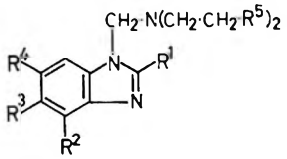
I

EXPERIMENTAL

Materials and methods

Mouse fibrosarcoma (*MFS*). This was induced by subcutaneous injection of 6,12-dimethylbenzo[1,2-*b*,4,5-*b'*]dithianaphthene in an inbred SWR mouse (Waravdekar & Ranadive, 1957), obtained from Indian Cancer Research Centre (ICRC), Bombay and has been maintained in the inbred strain of mice SWR/IISc.

Table 1. *Benzimidazole mustards tested*



Compound No.	R^1	R^2	R^3	R^4	R^5	Chemical name
A	H	Cl	H	H	OH	4-chloro-1-[di(2-hydroxyethyl)aminomethyl]benzimidazole
B	H	Br	H	H	OH	4-bromo-1-[di(2-hydroxyethyl)aminomethyl]benzimidazole
C	H	Br	H	H	Cl	4-bromo-1-[di(2-chloroethyl)aminomethyl]benzimidazole
D	H	Cl	H	H	Cl	4-chloro-1-[di(2-chloroethyl)aminomethyl]benzimidazole
E	H	H	H	H	Cl	1-[di(2-chloroethyl)aminomethyl]benzimidazole
F	H	H	MeO	H	Cl	1-[di(2-chloroethyl)aminomethyl]-5-methoxybenzimidazole
G	H	H	Cl	Cl	Cl	5,6-dichloro-1-[di(2-chloroethyl)aminomethyl]benzimidazole
H	Ph	H	H	H	Cl	1-[di(2-chloroethyl)aminomethyl]-2-phenylbenzimidazole
I						1-[di(2-chloroethyl)aminomethyl]-1*H*-naphth[1,2-*d*]imidazole

Yoshida ascites sarcoma. This was obtained from ICRC, Bombay and has been maintained in a closely inbred substrain of Wistar rats A/IISc.

Benzimidazole mustards. Chemical structures and names are in Table 1. Solutions were prepared by dispersing the compounds in 30% propylene glycol in saline, to give the required concentration in 0·2 ml of solution, i.e. the volume injected intraperitoneally each time.

Screening studies were made in rats weighing 120–150 g and mice 20–25 g of the respective strains. The animals were provided with dry diet (cracked wheat 60%, cracked Bengal gram 20%, fish meal 8%, shark liver oil 2%, peanut oil 5%, commercial casein 4% and common salt 1%) and water *ad libitum*. The weights and general behaviour of all animals were recorded regularly.

The efficacy of the extract in controlling the growth of tumours was estimated by calculating the T/C values. These were calculated as follows: For solid tumours excised after two weeks the T/C value is the ratio of the mean tumour weight of treated animals divided by the mean tumour weight of control animals. T/C value for ascites tumour is the ratio (expressed as %) of the mean survival time of the treated group divided by the mean survival time of the control group.

T/C values of 0·5 or below in solid tumours and above 200 in ascites tumours were considered to be effective.

Design of experiments

Mouse fibrosarcoma. Tumour implantation was by aseptic subcutaneous injection of 0·1 ml of tumour homogenate (1 : 2 w/v in saline) at the axillary region. Animals were either injected with 0·2 ml of 30% propylene glycol in saline intraperitoneally or given five daily successive intraperitoneal doses of the compound 24 h after transplantation.

Yoshida ascites sarcoma. The rats received 10 million cells of actively growing tumour intraperitoneally and were either administered 30% propylene glycol in saline 0·2 ml intraperitoneally (20 animals) or in groups of 10 animals, treated in the same way as the mice.

RESULTS

Mouse fibrosarcoma. Doses and T/C values are shown in Table 2. Two of the animals receiving compound H had died by the end of the experiment. During treatment weights of the mice treated with compound G decreased but not significantly.

Yoshida ascites sarcoma. The survival period of rats treated with compounds C, D and F significantly enhanced (Table 3) with T/C values: in 534, 441 and 309 respectively. Compound H was inactive against this tumour.

Table 2. *Effect of intraperitoneal injections of benzimidazole mustards on mouse fibrosarcoma*

Compound	Dose (mg/kg)	Total dose (mg/kg)	Survivors	Weight* difference (g)	Tumour† weight (g)	T/C	Inhibition‡ (%)
Control	—	—	30/30	—	1·67 ± 0·15	—	—
A	8	40	10/10	+ 2·6	1·21 ± 0·23	0·70	30
B	8	40	10/10	— 1·2	1·85 ± 0·25	1·11	nil
C	8	40	10/10	— 0·7	0·97 ± 0·23	0·58	42
D	8	40	10/10	+ 0·4	0·99 ± 0·25	0·59	41
E	8	40	10/10	— 1·1	1·98 ± 0·63	1·19	nil
F	8	40	10/10	+ 0·2	1·26 ± 0·21	0·75	25
G	8	40	10/10	— 2·1	0·57 ± 0·22	0·34	66
H	8	32	8/10	— 3·8	0·65 ± 0·11	0·39	61
I	8	32	10/10	+ 0·4	1·25 ± 0·18	0·75	25

* The average animal weight change of treated hosts minus the average animal weight change of control hosts.

† Mean ± s.e.

‡ $\frac{\text{Control tumour weight} - \text{treated tumour weight}}{\text{Control tumour weight}} \times 100.$

Table 3. *Effect of intraperitoneal injections of benzimidazole mustards on Yoshida ascites sarcoma*

Compound	Dose (mg/kg)	Survival* period (days)	T/C
Control	—	8·2 ± 0·26	—
C	8	43·8 ± 12·39	534
D	8	36·2 ± 10·16	441
F	8	25·4 ± 10·86	309
H	8	7·8 ± 0·12	96

* Mean ± s.e.

DISCUSSION

In tests with mouse fibrosarcoma, compounds A, B and E were inactive. Introduction of a methoxy-group on the benzenoid ring in compound E gave a molecule (compound F) with slightly but not significantly increased activity compared to the parent molecule. Introduction of a chloro- or bromo-atom on the other hand resulted in molecules (compounds C and D) more active than compound F, while compound G, with two chloro-atoms on the benzenoid ring of compound E, was active. Similar activity was found in compound H, which is a 2-phenyl-substituted derivative of compound E.

The compounds C, D and F, though inactive with mouse fibrosarcoma, were active against Yoshida ascites sarcoma but the reverse applied to compound H.

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Interaction between phenylpropanolamine and monoamine oxidase inhibitors

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The role of monoamine oxidase in the potentiation of the pressor effect of phenylpropanolamine (norephedrine) by monoamine oxidase inhibitors has been investigated. *In vitro*, phenylpropanolamine was not a substrate of monoamine oxidase from guinea-pig liver. In spinal cats the pressor effects of both phenylpropanolamine and tyramine were potentiated by a monoamine oxidase inhibitor, nialamide, and by the microsomal enzyme inhibitor SKF 525-A, which is not a monoamine oxidase inhibitor. These results suggest that the enhanced pressor effect of phenylpropanolamine in the presence of monoamine oxidase inhibitors is caused by inhibition of other enzymes.

Pressor responses to indirectly acting sympathomimetic amines are greatly increased in the presence of monoamine oxidase inhibitors. An example in man is the potentiation of the pressor effect of phenylpropanolamine (norephedrine) by tranylcypromine (Cuthbert, Greenberg & Morley, 1969). This might occur as a result of an increase in the concentration of the amine at its site of action because its enzymatic metabolism by amine oxidase is inhibited. But phenylpropanolamine has a methyl group attached to the α-carbon atom of the aliphatic side chain, and it is known that some sympathomimetic amines of a similar structure (e.g. ephedrine, amphetamine) are not substrates of monoamine oxidase. Since it is uncertain if the enhanced pressor activity of phenylpropanolamine in the presence of a monoamine oxidase inhibitor is caused by inhibition of this or other enzymes, the interaction was investigated further.

EXPERIMENTAL

Manometry. Acetone dried guinea-pig liver powder as a source of amine oxidase was prepared according to Blaschko (1952). A suspension of the powder in M/15 phosphate buffer (pH 7·40) was incubated in oxygen at 37° in Warburg flasks in the presence of tyramine, phenylpropanolamine, or a mixture of the two amines. Their final concentrations were: liver powder in each flask 15 mg/ml, tyramine HCl 0·008M, phenylpropanolamine HCl 0·008M or 0·016M. The central well contained 0·3 ml 10% KOH to absorb CO_2. Endogenous substrate in the enzyme preparation was removed by washing with phosphate buffer before the final suspension was made. After equilibration, the uptake of oxygen by the enzyme during oxidative deamination was read for an hour at 10 min intervals.

Cat blood pressure. Cats, 1·5 to 2·0 kg, were anaesthetized with sodium pentobarbitone (50 mg/kg, i.p.) and then submitted to spinal section (Burn, 1952). The mean arterial b.p. was recorded from the carotid artery by a cannula and mercury manometer.

In one series of experiments duplicate control pressor responses to 100 and 250 μg of both tyramine and phenylpropanolamine were obtained by injecting the drugs into

the femoral vein at 15 min intervals. Such an interval avoided tachyphylaxis to the amines. After the control responses had been obtained, SKF 525-A (diethylaminoethyl diphenylpropyl acetate) (40 mg/kg) was infused intravenously for 1 h. Two h after the start of the infusion the injections of both amines were repeated in duplicate.

In another series of experiments cats were pretreated with nialamide, a monoamine oxidase inhibitor devoid of sympathomimetic activity (Ryall 1961), 50 mg/kg by intraperitoneal injection 20 h before the experiment. Pressor responses to 50 and 100 μg of both tyramine and phenylpropanolamine were obtained. These responses were compared with control responses from other untreated cats.

RESULTS

Manometry. Tyramine but not phenylpropanolamine was deaminated by the enzyme preparation and the second substance is therefore not a substrate of monoamine oxidase. However, when both amines were incubated with the enzyme simultaneously the rate of deamination of tyramine was decreased, perhaps because phenylpropanolamine competed with tyramine for attachment of the enzyme (Fig. 1).

Cat blood pressure. In each cat it was possible to obtain responses at two dose-levels for each amine before and after treatment with SKF 525-A (Table 1).

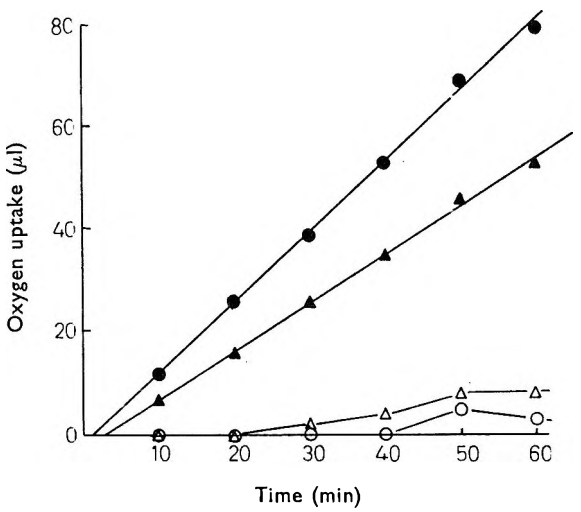

FIG. 1. Oxygen uptake (μl) by guinea-pig liver powder suspension with different substrates. Mean values of 4 experiments. ● Tyramine HCl 0·008M, ▲ Tyramine HCl 0·008M + phenylpropanolamine HCl 0·008M, △ Phenylpropanolamine HCl 0·008M, ○ Phenylpropanolamine HCl 0·0_6M.

Table 1. *Increases in mean arterial blood pressure (mm Hg) before and after SKF 525-A. Each value represents the mean of 2 determinations.*

	Tyramine				Phenylpropanolamine			
Cat	100 μg		250 μg		100 μg		250 μg	
No.	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A	Control	SKF 525-A
1	24	58	51	93	19	42	56	69
2	20	43	47	74	14	35	26	37
3	22	45	44	66	10	15	21	29
4	14	28	52	78	10	20	30	36
Mean	20·0	43·5	48·5	77·8	13·3	28·0	33·3	42·8

The difference in the pressor response of the two sympathomimetic amines was significant. There was a significant difference between the response to the low and high doses of each drug ($P < 0{\cdot}001$). The difference between the control pressor response and the response after treatment with SKF 525-A was significant ($P < 0{\cdot}001$). The estimates of the variance from the interactions "substrates x treatments" and "doses x treatments" were significantly different from the residual variance ($P < 0{\cdot}001$); therefore treatment with SKF 525-A significantly potentiated the effect of both tyramine and phenylpropanolamine at each dose-level.

Table 2. *Increases in mean arterial blood pressure* (mm Hg ± s.e.) *in untreated cats and in cats treated with nialamide*

		Untreated cats	After nialamide	Significance level
Tyramine	50 μg	3 ± 0·9	31 ± 2·1	$P < 0{\cdot}001$
	100 μg	22 ± 2·6	68 ± 3·2	$P < 0{\cdot}001$
Phenyl-propanolamine	50 μg	3 ± 0·6	34 ± 6·2	$P < 0{\cdot}01$
	100 μg	13 ± 1·9	81 ± 2·3	$P < 0{\cdot}001$

Tachyphylaxis to both sympathomimetic amines occurred to a varying degree after nialamide. In some cats it was not possible to obtain pressor responses to more than one dose of each sympathomimetic amine. Therefore it was impossible to use an experimental design similar to that devised for treatment with SKF 525-A where each cat received two different doses of each amine. In cats treated with nialamide there was considerable potentiation of the responses to both doses of each amine compared with the responses from untreated cats (Table 2).

DISCUSSION

Potentiation of the pressor effect of phenylpropanolamine by a monoamine oxidase inhibitor, tranylcypromine, has been observed in man by Cuthbert & others (1969). Phenylpropanolamine is a sympathomimetic amine with a methyl group attached to the α-carbon atom of the aliphatic side-chain. Such a structure usually precludes a substance from being a substrate of monoamine oxidases. *In vitro* experiments indicate that phenylpropanolamine is not a substrate of monoamine oxidase prepared from guinea-pig liver. Enhanced pressor activity to both phenylpropanolamine and tyramine was demonstrated in anaesthetized cats pretreated with nialamide, a monoamine oxidase inhibitor devoid of sympathomimetic activity (Ryall, 1961). However, SKF 525-A, a substance which inhibits liver microsomal enzymes (Brodie, 1956), but not monoamine oxidase (Dubnick, Morgan & Phillips, 1963), potentiated the pressor effect of both phenylpropanolamine and tyramine. These results show that phenylpropanolamine is not a substrate for monoamine oxidase and suggest that other enzymes are involved in its metabolism. The observation of Rand & Trinker (1968) that tyramine is metabolized by enzymes other than monoamine oxidase and that so called monoamine oxidase inhibitors are active against more than one type of enzyme system is confirmed. The interaction between phenylpropanolamine and monoamine oxidase inhibitors is thus probably due to inhibition of the binding or metabolism of the amine by these other enzymes.

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The evaluation of inflammation induced by material implanted subcutaneously in the rat

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Inflammation induced by cotton pellets, implanted subcutaneously in the rat, has been compared with that resulting from implantation of pellets impregnated with irritant substances. Time sequences of fluid exudation and granulation tissue formation have been evaluated by differential weighing. Increased irritancy of the pellets resulted in increased exudate volume, and heavier granulation deposition. Contact between granulation tissue and irritant pellets was delayed until the volume of exudate had subsided. Penetration of the pellet was also delayed. Systemic anti-inflammatory effects from granulomata surrounding implanted pellets were related to either the size of the implantation lesion or the degree of irritancy of the implanted material. Evaluation of granulation tissue formation is suggested as a test for irritancy of surgical fabrics.

The nature of the inflammatory response to subcutaneously implanted foreign material has been investigated mainly in experiments applied to the evaluation of anti-inflammatory drugs (Meier, Schuler & Desaulles, 1950; Eichhorn & Sniffen, 1964), the properties of materials used in prosthetic surgery (Newman, 1956; Arons, Sabesin & Smith, 1961), or the toxicity of plastics materials (U.S. Pharmacopeia, 1965; Lawrence, Mitchell & others, 1963). Such investigations may also be of value in assessing the relative degree of irritancy of other materials which might come into contact with tissue, e.g. surgical fabrics. For this purpose both qualitative and quantitative aspects of the tissue reactions to implanted irritant material have been studied. In addition, the systemic anti-inflammatory influence of one irritant lesion upon others has been investigated as a possible parameter for the assessment of irritant potency.

EXPERIMENTAL AND RESULTS

Material and Methods

Male Wistar rats weighing 200–250 g, were used. They were housed in well ventilated conditions with the temperature regulated at 65° F, and were maintained on unrestricted supplies of water and diet 41B (Oxoid).

Material for implantation. Either weighed portions of absorbent gauze B.P.C. (40 mg prepared as pellets by rolling vigorously between gloved fingers) or selected cotton wool dental pellets (Johnson & Johnson) (8 mg) were used. Pellets, with incorporated irritant substances, were similarly prepared from euflavine gauze B.P.C. 1954, boric acid gauze B.P.C. 1954, or iodoform gauze B.P.C. 1954, and also from dental pellets or absorbent gauze B.P.C. repeatedly soaked and dried in stronger tincture of capsicum B.P.C. 1934. Pellets were initially sterile and aseptic precautions were taken in their handling.

Implantation. One or more pellets were introduced in each animal under light

ether anaesthesia. The pellets were placed subcutaneously in a lateral abdominal position, either through a dorsal midline incision, or by means of a pellet implantation instrument inserted in the groin. Midline incisions were sealed with suture clips.

After periods varying from 3 to 56 days animals carrying implants were killed by a blow on the head and the pellets, together with surrounding granulation tissue, were dissected and removed. Each pellet and associated tissue was weighed wet, and then dried to a constant weight at 60° and reweighed. Where considerable accumulation of exudate fluid had occurred, the fluid was collected with a pasteur pipette and subsequently replaced with the pellet for weighing.

When dissecting, granulation tissue was identified as that firm vascular tissue surrounding a cavity filled with exudate, or adherent to the inserted pellet. It was generally well differentiated from normal areolar connective tissue. In sample cases the accuracy of dissection was confirmed histologically.

Time course of development of exudate and granulation tissue

Groups of five rats were selected at random and two identical cotton dental pellets were implanted one on each side in every animal. After given periods of time all animals in each group were killed and the pellets were removed. Pellets from four of the animals were weighed and those from the remaining rat in each group were examined histologically. Other groups of five rats were similarly implanted with cotton dental pellets impregnated with capsicum oleoresin, and the pellets were examined after corresponding periods of time. Wet and dry weights were as shown in Table 1.

The sequences of qualitative changes which occurred after the implantation of these pellets are illustrated diagrammatically in Fig. 1. These changes can conveniently be considered as divided into three phases: namely exudation, granulation and consolidation.

Exudative phase. At three days after implantation there was a marked rise in the wet weight of the normal pellets, attributable to the accumulation of a visible fluid exudate. The exudate filled a sac around each pellet, but at this time it was impossible, on the basis of naked-eye observation, to decide the boundary of the sac. The fluid was protein rich and contained numerous polymorphonuclear leucocytes—largely neutrophils. Subsequently the wet weights fell as the exudate was resorbed. After

Table 1. *Weights of granulation tissue at various times after subcutaneous implantation of cotton pellets, compared with tissue from pellets impregnated with capsicum oleoresin.* Original pellet weights 8 mg. Each value represents the mean from eight pellets

Days after implantation	Mean granulation tissue weight (mg) ± standard errors			
	Normal pellets		Impregnated pellets	
	Wet	Dry	Wet	Dry
3	79·8 ± 28·5	4·6 ± 1·9	130·2 ± 36·0	10·3 ± 3·1
7	108·0 ± 16·3	15·3 ± 1·6	166·4 ± 40·5	20·3 ± 2·6
13	97·9 ± 12·4	20·2 ± 1·9	173·8 ± 29·6	25·8 ± 4·8
20	96·0 ± 8·9	19·2 ± 2·3	150·0 ± 18·2	29·4 ± 1·8
28	59·5 ± 6·7	24·9 ± 2·0	105·2 ± 20·4	32·6 ± 2·6
42	46·1 ± 6·8	17·4 ± 1·5	78·0 ± 10·4	44·6 ± 4·8
56	42·5 ± 5·1	17·2 ± 1·2	64·9 ± 14·2	48·2 ± 3·3

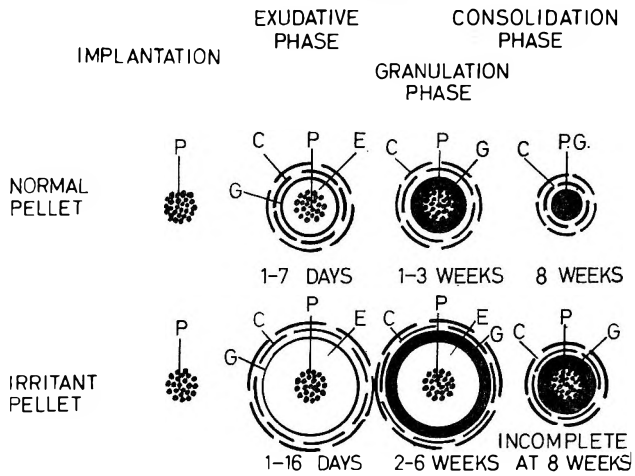

FIG. 1. Diagrammatic comparison of development of inflammation and granulation tissue, induced by implantation of non-irritant or irritant pellets. Key: P, pellet; C, normal connective tissue; G, granulation tissue; E, exudate.

seven days the granulation layer was discernible although this having scattered capillaries and a sparse layer of fibroblasts and collagen fibres.

The oleoresin-impregnated pellets provoked a much more severe and prolonged exudative reaction. The volume of exudate was higher than that from the normal pellets after three days, and increased further for up to two weeks. At seven days the granulation layer was thin and not well differentiated from surrounding tissue. No tissue adhered to the pellet for up to 28 days.

Granulation phase. At seven days the granulation tissue was loosely adherent to the normal cotton pellets. The granulation layer thickened during the following week, and its capillary network became more complex with noticeable budding of new vessels towards the pellet. Fibroblast and macrophage numbers had markedly increased with a thickening of the web of collagen fibres. Some penetration of outermost layers of the pellets had occurred and the tissue was firmly adherent. By 21 days after implantation, macrophages and fibroblasts had reached deeper layers, collagen fibres were deeply enmeshed with the cotton fibres, and capillaries were established in the fibre network at the pellet surface. During this period the wet weights had markedly declined and the dry weights approached maximal values. No significant increase in dry weights occurred after 21 days.

In contrast the granulation layer around the impregnated pellets was less well defined between 7 and 14 days and the pellets still lay unattached in sacs of exudate. Subsequently the granulation layer became much more apparent and developed in considerable thickness around the pellet, replacing the exudate, and then adhering to the pellet. A steady decline in wet weights, commencing after two weeks and accompanied by a sustained rise in dry weights, marked the development of granulation tissue much thicker than around normal cotton pellets. More fibroblasts and leucocytes were present and the capillary and collagen development was much denser.

Consolidation phase. For normal pellets observed after week three, there was a small decrease in dry weight of material deposited. This was accompanied by further decrease in wet weights. Histological changes occurring during this time

were further penetration of fibroblasts and collagen fibres to the centres of the pellets, and some giant cell formation around the cotton fibres. Thickness and vascularity of the outer layers decreased leaving a thinner but denser fibre barrier. After eight weeks numbers of fibroblasts and leucocytes had decreased in all fields.

Up to the end of the period investigated, penetration of impregnated pellets was poor, so that most of the granulation tissue remained in a weakly adherent but thick layer at the surface. Large numbers of leucocytes and fibroblasts clustered on and just within the outer interstices of the pellets.

Comparison of the effects of various impregnating substances on fabric implant induced granulation tissue

Using light ether anaesthesia, 40 mg pellets of absorbent gauze B.P.C., or of other gauze fabrics impregnated with euflavine, boric acid, iodoform, or capsicum oleoresin, were inserted. Groups of five rats were used, each animal receiving two pellets of the same type of fabric. Eleven days later each animal was killed and pellets with surrounding granulation tissue were dissected and removed. Wet and dry weights of granulation tissue were found, the amount in excess of 40 mg representing the weight of exudate or granulation tissue. Results were as shown in Table 2. In a separate experiment similar clean or impregnated fabric pellets were implanted for 11 days, but the pellets were dissected out with only tissue directly adherent to the pellets. The dry weights of such material were measured, and were as shown in Table 2.

Table 2. *Dry (I) and wet (II) weights of granulation tissue surrounding pellets and (III) adherent to pellets of gauze fabrics implanted subcutaneously for* 11 *days.* Dry weight of pellet (40 mg) subtracted from each weighing

	I			II			III		
Fabric	Mean wt (g)	Standard error	No. of observations	Mean wt (g)	Standard error	No. of observations	Mean wt (g)	Standard error	No. of observations
Absorbent gauze (B.P.C.)	0·1238	±0·015	10	0·636	±0·057	10	0·099	±0·010	10
Euflavine gauze (B.P.C. 1954)	0·1523	±0·010	8*	0·804	±0·060	8*	0·042	±0·012	5*
Boric acid gauze (B.P.C. 1954)	0·1487	±0·006	10*	0·771	±0·043	10*	0·064	±0·05	10*
Iodoform gauze (B.P.C. 1954)	0·1644	±0·012	8*	0·890	±0·201	—	0·071	±0·008	10*
Gauze impregnated with capsicum oleo resin	0·252	±0·028	8†	1·368	±0·089	8*†	No adherent tissue		5*

* Denotes significant difference from absorbent gauze ($P < 0·05$).
† Denotes significant difference from all other fabrics ($P < 0·05$).

It can be seen that after implantation for 11 days both the dry and wet weights of material deposited around the impregnated pellets, were significantly higher ($P < 0·05$) than those of tissue on pellets of absorbent gauze B.P.C. Weights of granulation tissue actually adhering to the pellets were significantly lower ($P < 0·05$) in the case of impregnated pellets.

Wet and dry weights of granulation tissue surrounding pellets impregnated with capsicum oleoresin were significantly higher ($P < 0·05$) than those associated with any of the other fabrics. There was no tissue directly adherent to these pellets.

Table 3. *Granulation tissue deposited on single "target" cotton pellets in animals with simultaneous implantation of four other pellets.* Dry weight of target pellet (8 mg) subtracted from each weighing. Each value represents the mean weight (mg ± s.e.) from five pellets

	Time of implantation			
	2 days	5 days	14 days	49 days
Wet weights (mg)				
Control—target pellet alone ..	239 ± 46	188 ± 19	128 ± 10	95 ± 13
Test—plus 4 other pellets ..	138 ± 21*	144 ± 15*	102 ± 6*	91 ± 10
Dry weights (mg)				
Control—target pellet alone ..	5·7 ± 1·2	10·7 ± 1·4	22·6 ± 2·0	19·9 ± 3·3
Test—plus 4 other pellets ..	3·4 ± 1·5	6·2 ± 2·0	15·3 ± 1·2	18·9 ± 2·4

* Denotes significant difference between means.

Anti-inflammatory influence of simultaneously implanted irritant material

Single cotton pellets (8 mg) were implanted in the lateral abdominal subcutaneous connective tissue on the left side of each animal in groups of five rats (*target pellets*). At the same time four pellets were implanted together on the opposite side of each of the animals (*test pellets*). Groups of animals thus treated were killed after 2, 5, 14 or 49 days, and the single target pellets, together with surrounding granulation tissue, were dissected and removed. Other groups of animals received only the single target pellets in the left flank; the implanting instrument being inserted on the opposite side but no pellets deposited. Wet and dry weights of the target granulation tissue were determined and the growth in presence or absence of other granuloma inducing material was as shown in Table 3. It can be seen that the development of both exudate and granulation tissue was retarded in those animals implanted with additional granuloma inducing material. This effect was observable over the first 14 days of the inflammatory reaction. To determine whether the degree of inhibition exerted by one granulation reaction upon another was dependent upon the relative size of the two sites of inflammation, the previous experiment was repeated with varying numbers of "test" pellets. Single pellets were implanted in the left flank of rats in groups of 5, while 1, 2, 4 or 8 pellets were simultaneously implanted on the opposite sides. One group received only single pellet implantations and these acted as control animals. Five days later the rats were killed and the target pellets removed and weighed. Results were as shown in Table 4. It was found that the degree of inhibition increased

Table 4. *Granulation tissue deposited on single "target" cotton pellets in rats with simultaneous implantation of additional pellets.* Dry weight of target pellet (8 mg) subtracted from each weighing. Implantation for five days.

	Mean "target" granulation tissue weight (mg) ± s.e.	
Number of "test" pellets	Wet	Dry
0	201 ± 37	11·2 ± 2·3
1	194 ± 18	11·8 ± 2·0
2	174 ± 26	7·8 ± 2·0
4	146 ± 20	4·0 ± 1·0
8	140 ± 16	6·4 ± 1·0

Table 5. *Granulation tissue deposited on single cotton "target" pellets, influenced by presence of "test" pellets (40 mg) impregnated with irritant substances* Implantation for 5 days. Dry weight of "target" pellet (8 mg) subtracted from each weighing.

Type of "test" pellets	Mean "target" granulation tissue weight (mg) ± s.e.	
	Wet	Dry
None (control)	145 ± 22	23·9 ± 2·1
Absorbent gauze	90 ± 10*	16·2 ± 2·4*
Capsicum gauze	63 ± 12*†	11·7 ± 2·2*†
Euflavine gauze	76 ± 10*	10·6 ± 1·9*†

* Denotes value significantly different ($P < 0·05$) from corresponding control value.
† Denotes value significantly different ($P < 0·05$) from corresponding result for absorbent gauze B.P.C.

with the number of "test" pellets. It was noted that although larger areas were involved the inflammatory reactions surrounding multiple pellets were qualitatively similar.

Evaluation of the indirect antigranulomatous influence of implants impregnated with irritant material. Single cotton dental pellets (8 mg) were implanted in the left side abdominal subcutaneous connective tissue of rats. Groups of five of these animals were implanted, at the same time, with single 40 mg pellets of either absorbent gauze B.P.C., euflavine gauze, or gauze impregnated with capsicum oleoresin, subcutaneously on the right side. Eight days after implantation the animals were killed and the target pellets and granulation tissue removed and weighed. Wet and dry weights were as shown in Table 5. The simultaneous presence of the 40 mg absorbent gauze pellet significantly depressed the target granulation tissues, while further significant depression was caused by the pellets impregnated with irritant materials. It was confirmed that the inflammatory reactions surrounding the impregnated pellets were more severe and there was greater exudate than those surrounding the absorbent gauze pellets. In the control animals no "test" implantation was made, but the incision was made and the connective tissue parted in simulation of the trauma of pellet insertion. After five days the connective tissue had not fully repaired but signs of inflammation were barely discernible.

DISCUSSION

In qualitative terms the inflammatory processes which result from the subcutaneous implantation of foreign material are well documented. Even if the material is relatively biologically inert it will become isolated from surrounding tissues, initially by fluid exudate and then by the development of a barrier of granulation tissue. It would be assumed that a more irritant material would, in general, induce a more severe inflammatory reaction. However, the relation between irritant potency and the severity of different components of inflammation do not appear to have been quantitatively defined. This applies also to the interrelation between the inflammatory components themselves, e.g. exudation and the subsequent granulation.

The present investigations have revealed that the presence of irritant material, such as capsicum oleoresin, results in both quantitative and chronological differences

in the development of inflammation, in comparison to the relatively non-irritant cotton fabrics alone. The most marked difference is the potentiation of the degree and persistence of the exudate formation in the presence of the capsicum impregnation. The formation of granulation tissue appeared to be markedly affected by the exudate process, since it first made its appearance at the extremities of the lesions. Thus, in the presence of the copious exudate induced by irritant pellets, the granulation tissue developed first as a rather poorly defined boundary, remote from the irritant focus. As the exudate volume declined, the granulation tissue front advanced in towards the pellet, contact with the pellet being considerably delayed. Even after such contact penetration of the pellet by granulation tissue elements was retarded, in contrast to an almost total colonization of the non-irritant pellets after a similar time. A possible explanation is that residual capsicum oleoresin remained within the pellet, and its influence was revealed both by the much thicker granulation barrier at the surface and the inability of fibroblasts and other cells to survive within the interstices of the cotton fibres.

With these effects of irritant material clearly defined, it is possible to utilize granulation tissue weight data to evaluate irritant potency. This has been applied to investigations of euflavine, boric acid and iodoform, which have been used clinically in medicated gauze fabrics. Such usage is largely obsolete; one reason being the mild irritant properties which retarded wound healing—a process analogous to granulation. Thus, significant increases in both exudate and granulation tissue formation were recorded in the presence of these mild irritants. Even greater and more significant increases were induced by the presence of the capsicum oleoresin, a material with powerful local irritant properties. Such an application, therefore, provides a discriminating test for relative potency of irritants. The value of making both wet and dry weight determinations in assessing granulation tissue growth, has previously been recognized by Penn & Ashford (1963). In conjunction with knowledge of the time course of the inflammatory reactions, such data may give information about both exudation and granulation. In the present case, there was consistent agreement between assessments of relative irritancy obtained by both wet and dry weight data. When pellet granuloma investigations are applied to evaluation of anti-inflammatory properties, inconsistencies between wet and dry weight data might occur, which could be of significance in interpreting modes of action.

The time course of the reactions to normal cotton pellets in the present investigation does not compare closely with either of those described in investigations by Penn & Ashford (1963) or Di Pasquale & Meli (1965). In the former, maximal development of granulation tissue had occurred after two days, and in the latter development continued up to 90 or more days. This disparity serves to emphasize the dangers in basing implantation tests for toxic hazards on absolute evaluations or uncontrolled observations. The differences between the three investigations could be due to variation between animals or laboratory conditions, or to the physical or chemical nature of the implanted materials.

It has been demonstrated that the induction of an inflammatory response in one area of an animal results in an indirect anti-inflammatory influence on other sites of inflammation (Laden, Blackwell & Fosdick, 1958; Di Pasquale, Girerd & others, 1963; Cygielman & Robson, 1963; Goldstein, Shemano & others, 1967). With particular reference to the present investigations, Robinson & Robson (1964) showed that implanted cotton wool or polyester sponge, inhibited the development of cotton

wool granulomata. Their observation that the degree of inhibition varied with the quantity of material implanted, is confirmed by the present results. These findings are consistent with the present observation that the degree of inhibition varies also with the irritant potency of the implanted material. It has been demonstrated that this phenomenon can be used in a test to discriminate between the inflammatory properties of irritant substances and those of relatively inert cotton material. This, however, appears to be less sensitive than tests based upon direct evaluation of granulation tissue formation.

The tests suggested by this investigation might have an increasing variety of applications as new fabrics and materials find their way into surgical and medical use. The main difficulties in such applications would probably be the choice of suitable materials of proven safety or known irritancy with which to make valid comparisons.

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The determination of the binding of salicylate to serum proteins

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The ratios of protein-bound to unbound salicylate in bovine, calf, horse and human sera were measured by equilibrium dialysis, ultrafiltration and frontal elution techniques. The equilibrium dialysis method gave the best measure of binding capacity, but several factors were found to affect the results. These factors include adsorption of the drug onto the dialysing membrane, the composition of the dialysing fluid, the necessity to allow sufficient time for equilibrium to be attained in the presence of serum, and the occurrence of dialysable substances in sera which may either compete with or displace salicylate from its binding sites.

A knowledge of the ratio of protein-bound to free, i.e. unbound, salicylate in the circulation is of obvious importance. The fraction of the drug in the unbound form is that available, at any one time interval, to enter the cells and initiate pharmacological and other actions. Some individuals show a reduced capacity to bind salicylate to their plasma proteins and may therefore be at higher risk after the ingestion of a toxic dose of the drug (Smith, 1968). Conventional laboratory methods (for example, Trinder, 1954) only measure the total salicylate concentration in the plasma and give no indication of the ratio between protein-bound and unbound drug. A convenient and reliable method for the separation and determination of protein-bound and unbound salicylate would therefore be of much practical value. It is relatively easy to assess such a method with respect to speed, simplicity and economy of sample. The evaluation of its precision and general accuracy is less straightforward. The usual practice is to compare it with either equilibrium dialysis or with ultrafiltration. The usefulness of the comparison depends on the reliability of these apparently established procedures as reference standards. They have been criticized (Cooper & Wood, 1968; Moran & Walker, 1968) because of possible changes in protein concentration due either to the prolonged manipulations or to absorption of the drug on to the dialysing membranes. We have therefore compared the results obtained from several sera and a purified bovine albumin fraction exposed to a range of salicylate concentrations, using ultrafiltration, equilibrium dialysis and frontal elution methods for the measurement of the ratio of protein-bound to unbound salicylate.

EXPERIMENTAL

Materials

Bovine albumin (fraction V) was obtained from the Sigma Chemical Co., St. Louis, and used as a 4% w/v solution. Horse serum, bovine serum and calf serum (natural clot, unheated) were obtained from the Wellcome Research Laboratories, Beckenham, and pooled human serum from the National Transfusion Service, Sutton. In some

experiments the sera were dialysed against the phosphate buffer before use; 50 ml samples of serum were dialysed against 1 litre of buffer, this being replaced three times over a 24 h period. Visking dialysis tubing (8/32 inch inflated diameter) was obtained from the Scientific Centre, London and G-25 Sephadex (medium grade) from Pharmacia Fine Chemicals, Uppsala. All chemicals were of analytical grade except for the sodium salicylate, which was of British Pharmacopoeial grade, and deionized water was used throughout. Unless otherwise stated, 0·1M phosphate buffer, pH 7·4, was used to prepare the solutions, to elute the gel columns and during the dialysis procedures.

Methods

Ultrafiltration was as described by Goldstein (1949) except that 1 ml of the reaction mixture was centrifuged at 3000 *g* and the first 50 μl of the ultrafiltrate used for analysis.

In the equilibrium dialysis experiments, the Visking tubing was soaked in two changes of water for 20 min before use. Sample solution (1 ml) inside the dialysis sac was dialysed against 10 ml of fluid in a vessel shaken 100 cycles per min on a Luckman rotary shaker for 20 h at room temperature (22°). Frontal elution was according to Cooper & Wood (1968); 7 ml of reaction mixture being applied to a 120 mm × 5 mm gel column and eluted with phosphate buffer. Salicylate was determined with an Aminco Bowman Spectrofluorometer, using an activating wavelength of 294 nm and a detecting wavelength of 413 nm.

RESULTS

Table 1 shows the results of experiments in which sufficient salicylate was added to various sera to give final concentrations of 50 μg/ml and the percentage of unbound salicylate measured by ultrafiltration, equilibrium dialysis and frontal elution. In some of the equilibrium dialysis experiments, the serum placed inside the sac was dialysed against a solution of salicylate in phosphate buffer sufficient to give a final concentration of approximately 50 μg/ml of salicylate inside the dialysis sac. The results with the sera showed wide variation. The results in Table 2 show that a prolonged soaking of the tubing (5 days) compared with a relatively short period (20 min), reduced the percentage of unbound salicylate measured by the equilibrium

Table 1. *Unbound salicylate in sera measured by different methods.* Each value represents the mean ± standard deviation of six determinations and is expressed as a % of the initial salicylate concentration (50 μg/ml) for the ultrafiltration and frontal elution methods. The results given for the equilibrium dialysis method represent the % of the final salicylate concentration (approximately 50 μg/ml), see text for details.

Serum	Equilibrium dialysis: protein-salicylate mixture inside sac	Ultrafiltration	Frontal elution
Bovine	4·3 ± 0·06	10·0 ± 0·16	10·8 ± 0·10
Calf	11·5 ± 1·60	41·7 ± 1·40	47·1 ± 0·30
Horse	7·3 ± 1·80	23·9 ± 0·30	22·0 ± 0·40
Human	1·0 ± 0·05	5·1 ± 0·10	5·0 ± 0·80

Table 2. *Adsorption of salicylate by dialysis tubing used in equilibrium dialysis method.* The Visking tubing was soaked in distilled water for either 20 min, with one change of water, or for five days, with 5 changes of the water. The lengths of open tubing were then exposed to 5 ml of either 10, 50 or 200 μg/ml salicylate solutions for 20 h and the salicylate concentration measured in appropriate solution at the end of this period. Each value represents the mean $\pm$ standard deviation of six determinations.

Length of tubing (cm)	Time in water	Salicylate concentration (μg/ml)		
5	20 min	10·1 $\pm$ 0·5	50·0 $\pm$ 1·3	202·9 $\pm$ 4·7
10	20 min	9·8 $\pm$ 0·4	50·7 $\pm$ 2·1	202·9 $\pm$ 6·7
20	20 min	9·7 $\pm$ 0·3	47·8 $\pm$ 3·7	197·0 $\pm$ 3·4
5	5 days	5·5 $\pm$ 0·2	47·0 $\pm$ 1·7	191·1 $\pm$ 4·9
10	5 days	1·4 $\pm$ 0·5	38·2 $\pm$ 3·0	182·2 $\pm$ 5·6
20	5 days	0·1 $\pm$ 0·1	18·9 $\pm$ 4·3	164·5 $\pm$ 5·6

Table 3. *Unbound salicylate in horse and human sera estimated by an equilibrium dialysis procedure with salicylate initially inside the dialysis sac, using different dialysing media.* Each value represents the mean $\pm$ standard deviation of six determinations and is expressed as a percentage of the final salicylate concentration (approximately 50 μg/ml).

Serum	Dialysing medium			
	0·1M Phosphate	Distilled water	0·9% NaCl	3·6% NaCl
Horse	12·9 $\pm$ 0·90	20·8 $\pm$ 0·70	32·0 $\pm$ 1·10	51·0 $\pm$ 7·10
Human	1·0 $\pm$ 0·05	0·6 $\pm$ 0·15	2·8 $\pm$ 0·14	5·9 $\pm$ 0·40

Table 4. *Unbound salicylate in sera measured by equilibrium dialysis method with salicylate initially either inside or outside the dialysis sac.* The results represent the percentages of the final salicylate concentration (approximately 50 μg/ml), see text for details. Each value is the mean $\pm$ standard deviation of six determinations.

Serum	Protein-salicylate mixture inside sac	Salicylate solution outside sac
Bovine	4·3 $\pm$ 0·06	4·3 $\pm$ 0·06
Calf	11·5 $\pm$ 1·60	22·7 $\pm$ 2·50
Horse	7·3 $\pm$ 1·80	10·4 $\pm$ 0·35
Man	1·0 $\pm$ 0·05	6·1 $\pm$ 0·40

dialysis method. The length of dialysis tubing subsequently used to make the dialysis sac also influences the results, but this effect only becomes apparent in the dialysis tubing which has been presoaked in water for the longer period. The results in Table 3 show the effect of varying the composition of the dialysing fluid in the equilibrium dialysis method. With the horse serum, the percentage of unbound salicylate increased when distilled water was substituted for the phosphate buffer and the values were even further enhanced when isotonic saline and hypertonic saline were used. The effects were similar, though less prominent, with the human serum,

except that virtually identical results were obtained with either distilled water or the phosphate buffer. Preliminary experiments showed that the dialysis in aqueous solution of salicylate in either direction in the equilibrium dialysis system was complete within 12 h. The results in Table 4 show that equilibrium occurred in the presence of bovine serum, but not in the presence of calf, horse or human sera, during a dialysis period of 20 h.

DISCUSSION

There are two aspects of the binding of drugs to circulating proteins. One is the capacity of a protein molecule to bind the drug; this is determined by the number of available sites. The second is the affinity of the drug for the binding site; this is determined by the dissociation of the drug-protein combination. There may be more than one type of binding site and the affinities for these may differ (see Davison & Smith, 1961). Both factors influence drug action. The binding capacity controls the pharmacological and other effects exerted at any one moment of time since these depend on the fraction of unbound drug available for entry into the cells. The affinity affects the rate of release of the bound drug from the circulating proteins and hence the duration of action. The separate determination of binding capacity and affinity for any drug-protein combination is desirable because these reflect different aspects of drug action. The methods used to investigate the binding of drugs to proteins are generally assumed to measure only the binding capacity but the results represent arbitrary combinations of binding capacity and affinity. The contribution of the affinity to the final result varies because the extent of dissociation of the drug-protein combination differs, depending on the experimental procedures employed. From this it can be inferred that the method which gives the lowest value for the amount of unbound drug has exerted the least effect on the dissociation of the drug-protein complex and gives the best measure of the binding capacity.

The results of the present work (Table 1) show that the equilibrium dialysis method meets this requirement for salicylate and the serum proteins of several species. However, using this method, several factors can influence the results. The first is adsorption of the drug to the dialysing membrane. Moran & Walker (1968) reported an appreciable adsorptive loss of salicylate from plasma samples into cellophane, and Goldstein (1949), working with methylene blue, commented that such loss may not only be large but variable from bag to bag. The present results (Table 2) also show that Visking tubing can adsorb salicylate. The extent of adsorption is principally affected by the nature of the pretreatment of the dialysing membrane. Thus, prolonged soaking (5 days) of the tubing in distilled water, compared with a short period (20 min) of soaking, caused a considerable increase in the adsorptive loss of the drug. Furthermore, with the tubing soaked for 5 days, the amount of salicylate lost from the drug solutions increased with the length of tubing eventually used to make the dialysis bag. The possibility of adsorptive loss on to dialysing membranes, and the influence of various forms of pretreatment of the membranes, must therefore be separately studied for each drug-protein or drug-serum combination.

A second factor is the composition of the dialysing fluid. The use of different media altered the values for unbound salicylate in horse and human sera and these alterations were not consistent (Table 3). Thus, substitution of distilled water for 0·1M phosphate buffer almost doubled the values with the horse serum but has the

reverse effect with the human serum. These results offer no guide to the choice of the most suitable dialysing fluid but only emphasize that the fluid used must be carefully described.

A third factor is whether sufficient time has been allowed, during an equilibrium dialysis method, to ensure that dialysis is complete and that equilibrium has been reached. A conventional method of testing this is to measure the time it takes for the drug, in aqueous solution, to reach equilibrium on both sides of the dialysing membrane. In the present work, preliminary experiments, in which the salicylate solutions were initially placed either inside or outside the dialysis sacs, showed that equilibrium was attained within 12 h. However, when experiments were repeated with various sera, the results (Table 4) showed that although equilibrium has been reached in the presence of the bovine serum, this has not occurred with the calf, horse and human sera, despite the dialyses being allowed to continue for 20 h. It must be concluded that data obtained from aqueous solutions of drugs cannot be used to predict the time necessary to reach equilibrium in experiments using different sera. Each combination of drug and serum must be separately investigated in order to establish this time when an equilibrium dialysis method is used to determine binding capacity.

A further factor may also be concerned because preliminary dialysis of the bovine serum before admixture with the salicylate reduced the values for unbound salicylate from $4{\cdot}3 \pm 0{\cdot}06$ (see Table 4) to $2{\cdot}6 \pm 0{\cdot}02$. When a purified bovine albumin solution was used instead of the bovine serum, the value was further reduced to $1{\cdot}3 \pm 0{\cdot}06$. One possible explanation is that other dialysable molecules, which could interfere with the binding of salicylate to the proteins, are present in varying amounts in the different sera. These molecules would be expected to be removed during dialysis of the sera or in the preparation of the purified bovine albumin fraction. It also follows that they may be removed to a variable extent during any method of equilibrium dialysis, the extent depending on the duration of dialysis and the ratio between the volumes of fluid inside and outside the dialysis sac. Thus, it is not possible because of these small molecules to obtain an absolute value for the binding capacity of serum proteins for a particular drug with any equilibrium dialysis technique. However, if the experimental conditions were such that the ratio of serum containing the drug to dialysate were high, then this would minimize the removal from the serum of any small molecules originally present which might interfere with the binding of the drug to the serum proteins. For example, if the ratio of 100 parts of serum plus salicylate to one part of dialysing solution were employed, then the value obtained for the binding capacity under these conditions would be similar to that existing in the circulation. This value, which for convenience could be termed the "actual binding capacity", may be of some clinical significance, since it is a good approximation of the *in vivo* binding capacity for salicylate at the time when the sample was collected. On the other hand, if the ratio of serum plus drug to dialysate was very low or if the serum sample was exhaustively dialysed before being analysed, then the small dialysable molecules would be largely removed and the value obtained would reflect the ability of the proteins to bind the drug only. Thus if, in the equilibrium dialysis technique, the ratio of serum to dialysing fluid was 1 to 100, then a measure of what may be termed the "potential binding capacity" of the serum sample for salicylate would be obtained. Many of the published techniques for estimating binding capacity by equilibrium dialysis methods measure neither actual nor potential

binding capacity but an intermediate value between the two. It is possible to estimate them separately as described above and this may be of some relevance in serum samples from patients receiving multiple drug therapy.

Although the results of the present work suggest that equilibrium dialysis is the method of choice for determining binding capacity, it must be stressed that there are several factors which influence the results obtained by this method and they must be separately investigated for each drug-protein or drug-serum combination being studied.

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The effect of ultrasonic energy on the extraction of anthraquinones from senna pericarps

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Ultrasonic irradiation increased the extraction rate of rhein glycosides, free anthraquinones and total water-soluble solids from whole senna pericarps, irrespective of the temperature conditions employed. An increase in ultrasonic power from 35 to 100 W increased the yield of extractable constituents over a 15 min period, the ratio of rhein glycosides to total water-soluble solids being higher in extracts prepared using ultrasound than in preparations obtained by simple maceration, but that ratio decreased slightly with an increase in ultrasonic power. Ultrasonic irradiation produced occasional visible damage to epidermal cell walls and the significance of this in the extraction of senna pericarps is discussed.

Among those factors considered to be involved in the ultrasonic extraction of plant material are dispersion of adhering material, particle size reduction, partial cell wall disruption, gross stirring effects, selective agitation at phase boundaries, thermal effects and liberation of active constituents from bound sites within the cell (Boswart & Blazek, 1954; Thompson & Sutherland, 1955; Golian & Tamas, 1956; Drabent & Podeszewski, 1958; Wray & Small, 1958; DeMaggio, Lott & Gerraughty, 1963).

Morrison & Woodford (1967) showed that ultrasonic irradiation of an aqueous suspension of senna pericarps produced an increase in the extraction rate of both sennosides and free anthraquinones over a 90 min period. The temperature rise produced by insonation accounted for about 70% of the increase in those constituents and the hydrogen peroxide formed in the extraction liquid was not, apparently, a factor in the specific effect of ultrasonic energy.

In the present work the effect of ultrasonic irradiation on the extraction of senna pericarps has been examined more fully to seek possible explanations for the results obtained, and to determine the effect of ultrasonic power on that process.

EXPERIMENTAL

Effect of temperature

Ultrasonic irradiation of aqueous suspensions of whole senna pericarps was carried out as described by Morrison & Woodford (1967), 12 ml samples of the extract being withdrawn after 15, 30, 45, 60, 75 and 90 min into empty test tubes immersed in ice-water and the content of free and combined anthraquinone derivatives determined in 10 ml at 20°. The remaining 2 ml was returned to the extraction vessel and the 10 ml volume removed at each time interval was replaced by 10 ml of purified water, the solvent volume being maintained constant throughout. The temperature increase was noted and the experiment repeated in the absence of ultrasonic energy using a stirrer-hot plate apparatus to duplicate the temperature rise. Stirring was

necessary to produce an even temperature rise but preliminary experiments showed that the rate of stirring had no effect on the yield of extractable constituents.

To determine the effect of insonation at room temperature, the normal ultrasonic technique was employed except that the extraction vessel was partly immersed in an ice-salt mixture maintained at −5°. By controlling the degree of immersion of the extraction vessel in this mixture the temperature of the liquid in the beaker was maintained at 20 ± 0·5°. The experiment was repeated at room temperature (20°) by replacing the ultrasonic probe with a glass stirrer and omitting the cooling system.

The rhein glycoside content of the aqueous senna extracts was determined as described in Appendix II of the Recommended Methods for the Evaluation of Drugs: The Chemical Assay of Senna Fruit and Senna Leaf (1965) after removal of the free compounds using anaesthetic ether. The amount of free anthraquinones in the ether was determined after extraction with N sodium hydroxide solution by measuring the absorbance of the coloured solution at the wavelength of maximum absorption 500 nm. The result was expressed as rhein by reference to a linear calibration graph prepared using a sample of pure rhein (E 1%, 1 cm 500 nm = 330 in N sodium hydroxide. Melting point = 321–322°; Oestele & Tisza (1908) give 321–321·5°).

All experiments were made in triplicate and the results are shown in Figs 1 and 2. At the completion of each experiment the amount of total water-soluble solids

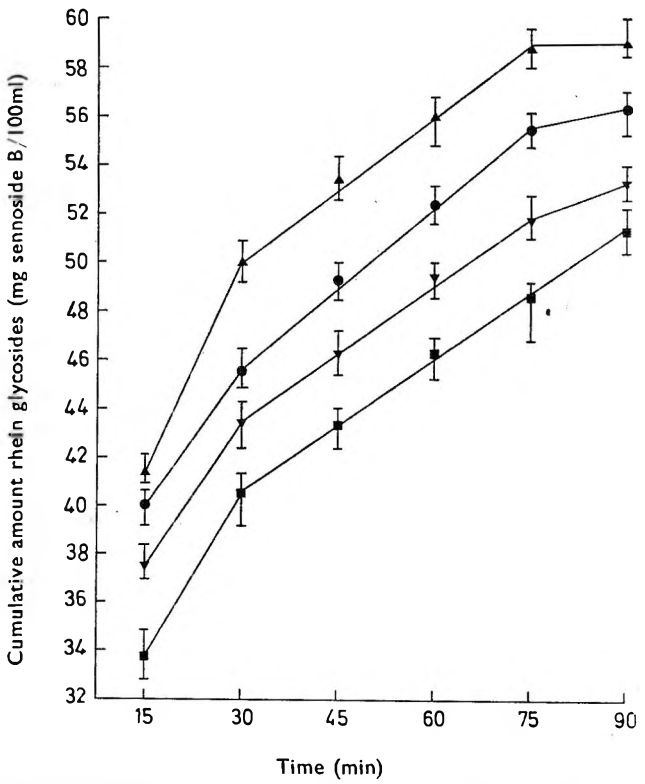

FIG. 1. Effect of ultrasonic energy, controlled heating and ultrasonic energy at room temperature on the extraction of rhein glycosides in aqueous extracts of senna pericarps. ■, Room temperature; ▼, ultrasonic energy at room temperature; ●, controlled heating; ▲, ultrasonic energy. Symbols indicate mean of 3 experiments and vertical lines indicate variation in 3 experiments.

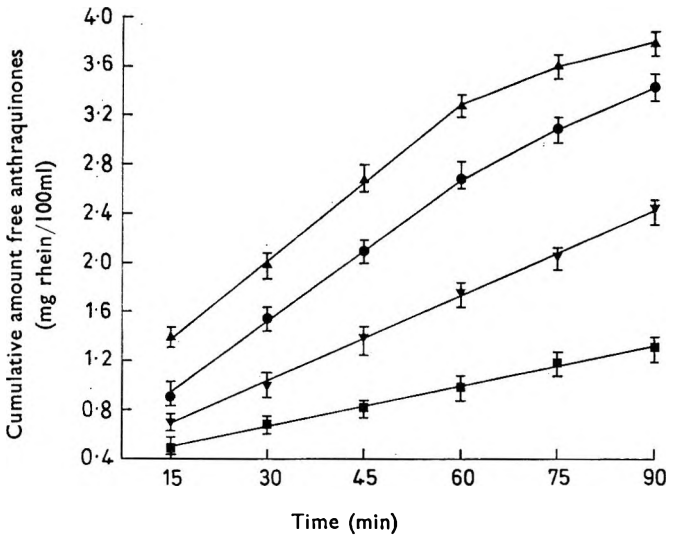

FIG. 2. Effect of ultrasonic energy, controlled heating and ultrasonic energy at room temperature on the extraction of free anthraquinones in aqueous extracts of senna pericarps. ■, Room temperature; ▼, ultrasonic energy at room temperature; ●, controlled heating; ▲, ultrasonic energy. Symbols indicate mean of 3 experiments and vertical lines indicate variation in 3 experiments.

extracted was determined by filtering the solution through a No. 4 Whatman filter paper and drying two 30 ml portions to constant weight at 105°.

Effect of ultrasonic power

The extraction technique was repeated in triplicate by subjecting the pericarp suspension to ultrasonic energy for 15 min using a Soniprobe type 1130* (frequency 20 kHz, probe end diameter $\frac{1}{2}$ inch) adjusted to generator power outputs of 35, 60 and 100 W. The procedure was also carried out at $20 \pm 0{\cdot}5°$ by surrounding the extraction vessel with an ice-salt mixture and the results compared with those obtained using an MSE 60W ultrasonic disintegrator† (frequency 20 kHz, probe end diameter $\frac{3}{8}$ inch).

Effect of ultrasonic energy on pericarp epidermal cells

Portions of epidermal cell tissue were removed from pericarps which had been ultrasonically irradiated for 45 min and the result compared microscopically with samples from non-insonated material. Cell debris in the extraction vessel after 90 min ultrasonic irradiation was similarly examined and the results are shown in Fig. 3.

Effect of size reduction

Whole pericarps (1 g) were suspended in purified water (100 ml) in a stoppered conical flask and the latter left at room temperature for 24 h with constant agitation. The content of rhein glycosides and total water-soluble solids in the filtered extract was determined as described above and the method repeated using pericarps in moderately fine powder.

* Dawe Instruments Limited, London, W.3.
† Measuring and Scientific Equipment Limited, S.W.1.

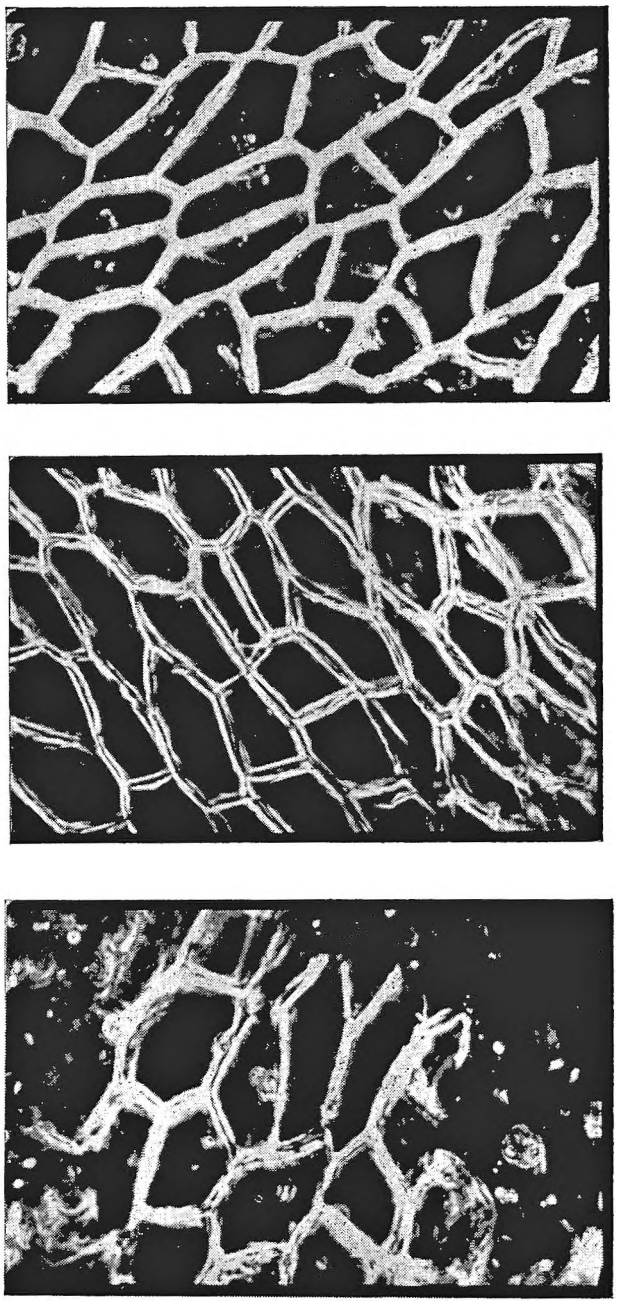

FIG. 3. Effect of 60 W ultrasonic energy on pericarp epidermal cells. (a) Non-insonated pericarp. (b) Pericarp insonated for 45 min. (c) Pericarp insonated for 90 min. Cell debris in extraction vessel.

DISCUSSION

Preliminary experiments showed that ultrasonic irradiation increased the extraction rate of rhein glycosides from whole pericarps but not the total amount extracted.

Over a 90 min period the mean increases over room temperature maceration due

Table 1. *Effect of ultrasonic energy, controlled heating and ultrasonic energy at room temperature* (20°C) *on the extraction of total water-soluble solids after* 90 *min in aqueous extracts of senna pericarps*

	Amount of total water-soluble solids in aqueous extracts (mg/100 ml)			
	Ultrasonic energy	Controlled heating	Ultrasonic energy at room temperature	Room temperature
Mean value of 6 determinations	227	219	210	200
Standard deviation	4·9	3·7	4·2	4·2
% increase over room temperature	13·5	9·5	5·0	—

Value of t for ultrasonic energy and controlled heating = 4·84, ultrasonic energy at room temperature and room temperature = 6·32, ultrasonic energy and room temperature = 17·68 ($P' = 0{\cdot}05$ and 10 degrees of freedom, $t = 2{\cdot}23$).

to ultrasonic energy at room temperature, controlled heating and normal ultrasonic extraction were: 6·6%, 13·2% and 20·4% for the rhein glycosides; 71%, 151% and 204% for free anthraquinones; and 5·0%, 9·5% and 13·5% for total water-soluble solids (Table 1). In all instances these results are in the approximate ratio of 1 : 2 : 3. Neither the temperature rise produced by insonation (31·5° over 90 min) nor ultrasonic energy itself produced any obvious chemical degradation of senna extracts as shown by spectrophotometric examination and paper and thin-layer chromatography using 10 solvent systems.

The rate of hydrogen peroxide formation in water insonated using the MSE disintegrator (Morrison & Woodford, 1967) was independent of temperature over the range 20 to 60°. The consistency of the results obtained from successive experiments using the same container and depth of probe immersion indicated that no significant change in cavitation intensity occurred during the extraction of senna pericarps.

The results in Table 2 show that the temperature of the insonated liquid and the yield of extractable constituents increased with ultrasonic power, the proportion of

Table 2. *Effect of ultrasonic power on the extraction of rhein glycosides, free anthraquinones and total water-soluble solids after* 15 *min in aqueous extracts of senna pericarps*

Ultrasonic power W	Temperature of extract after 15 min	Rhein glycosides (mg sennoside B/100 ml) (a)	Free anthraquinones (mg rhein/100 ml)	Total water-soluble solids (mg/100 ml) (b)	a/b × 10^3
—	20	33·7	0·57	270	125·0
35	20	38·1	0·63	281	135·5
	29	41·1	1·01	301	136·7
60	20	39·5	0·76	293	134·8
	38	43·2	1·46	318	135·8
60*	20	37·5	0·74	285	131·6
	37	41·3	1·40	312	132·4
100	20	45·7	1·03	341	134·1
	67	54·1	1·90	401	135·0

Each value is the mean of 3 experiments. * MSE 60W ultrasonic disintegrator; other values refer to Soniprobe equipment.

active principles in the extract being slightly lower when ultrasonic irradiation was at 20° than when the temperature was uncontrolled. The greatest yield of rhein glycosides was obtained using high power irradiation but although the ratio of sennosides to total extractive increased by 1·04% at 20° using low power ultrasound this was not statistically significant. Little information is available about the effect of ultrasonic power on the yield of active principles but Kubiak (1962) reported that an increase in intensity produced an increase in glycoside yield from frangula bark over a 20 min insonation time.

The ratio of rhein glycosides to total water-soluble solids obtained using the MSE disintegrator was 2·5% lower than that observed for the Soniprobe adjusted to the same power output but this was not statistically significant.

Microscopical examination of epidermal tissue showed that exposure to ultrasonic energy for 45 min produced visible damage to occasional cell walls (Figure 3b) as well as more rapid extraction of mucilage and other cell contents. In addition, where the pericarp had been directly in the path of cavitation streamers, complete removal of small areas of epidermal tissue was noted and after 90 min the extraction vessel contained obvious cell debris (Figure 3c).

The results in Table 3 indicate that cell wall rupture using a high speed mill increased the extraction rate of rhein glycosides from senna pericarps. Cell walls not ruptured during milling did not show the occasional abraded appearance of those exposed to ultrasonic energy but extracts prepared using comminuted or insonated tissue contained significant amounts of colloidal matter absent from normal whole-pericarp macerates. The $E(1\%, 1\text{ cm})$ value at 440 nm for the red solution produced during the rhein glycoside assay is raised when impurities are present (Recommended Method) but the mean value for the ratio E (515 nm)/E (440 nm) obtained was 1·42, irrespective of the presence of colloidal matter.

The results of this study indicate that the specific effect of ultrasound on the extraction rate of senna pericarps is not due to the chemical affects of cavitation. Sennoside determinations using different sieve fractions of comminuted material showed that the rhein glycosides are located in the non-fibrous tissue and damage to epidermal cells, together with facilitated removal of mucilage, may contribute to the increased extraction rate of active and inactive constituents produced by ultrasonic energy.

Table 3. *Effect of size reduction on the aqueous extraction of senna pericarps*

	Whole pericarps			Pericarps in moderately fine powder		
	Rhein glycosides (mg sennoside B/100 ml) (a)	Total water-soluble solids (mg/100 ml) (b)	$a/b \times 10^3$	Rhein glycosides (mg sennoside B/100 ml) (a)	Total water-soluble solids (mg/100 ml) (b)	$a/b \times 10^3$
Mean value of 10 determinations	53·7	327	164·3	59·3	371	159·9
Standard deviation	0·94	2·2	0·11	1·46	9·6	0·16

Value of t for rhein glycosides = 10·16, total water-soluble solids = 14·09, ratio of rhein glycosides to total water-soluble solids = 2·48 ($P' = 0{\cdot}05$ and 18 degrees of freedom, $t = 2{\cdot}10$). Rhein glycoside content of senna pericarps = 63·8 mg sennoside B/g (mean of 5 determinations, standard deviation = 0·58).

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The diffusion of sennoside A through a cellulose membrane

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The diffusion rate of sennoside A through a cellulose membrane into water increased irrespective of the temperature conditions under which diffusion took place, when the membrane was irradiated with ultrasound. The results are consistent with the hypothesis of boundary layer disruption at the phase interface and their significance in the effect of ultrasonic energy on the aqueous extraction of senna pericarps is discussed.

Although ultrasonic energy may increase the extraction rate of plant material, the means whereby that result is achieved remains obscure. Morrison & Woodford (1967) observed that ultrasonic irradiation of an aqueous suspension of senna pericarps resulted in an increase in the extraction rate of both sennosides and free anthraquinones, and further work (Woodford & Morrison, 1969) showed that although insonation produced visible damage to occasional epidermal cell walls, only slight gross physical damage to whole pericarps resulted. Because many pericarp cell walls were visibly undamaged by insonation, experiments were made to see if ultrasonic energy is capable of increasing the diffusion rate of sennoside molecules through an intact cellulose barrier.

EXPERIMENTAL

Construction of diffusion cell

The diffusion cell was constructed in Perspex to the design of Wood, Rising & Hall (1962). An RBT 16 thermoelectric unit* was embodied into the rear chamber of the cell so that the inner face of the unit was continuous with the wall of the diffusion chamber. The unit was connected in series with a 6 V accumulator through an ammeter and rheostat so that a current of 1 to 10 A could be passed through the thermoelectric junctions. By adjustment of the direction and amount of current the unit could be used either to increase or decrease the temperature of the liquid in the cell at a predetermined rate.

Modification of cellulose film permeability to sennoside A

The permeability of additive-free regenerated cellulose film (British Cellophane) to sennoside molecules was increased by a zinc chloride treatment modified from the methods of McBain & Stuewer (1936), Craig & Konigsberg (1961) and Pierce & Free (1961).

Suitably-sized squares of film were immersed in a 64% w/w aqueous solution of zinc chloride at 35° for 20 min. The squares were placed in 0·01N hydrochloric acid for 2 min, the procedure repeated using a similar volume of acid, and the membranes washed with purified water until free from chloride. Squares of both treated and

* Salford Electrical Instruments Limited, Salford, Lancs.

untreated cellulose film were stored in purified water at room temperature for 14 days before being used. The thickness of these samples was measured using a horizontal metroscope (Zeiss). The thickness of the dry film was 21·6 μm, of the untreated film soaked in water 42·8 μm (98% increase over dry film); the thickness of the treated film soaked in water was 136·5 μm (532% increase over dry film). Each value is the mean of 10 measurements. The increase in area of soaked treated film over dry untreated film was 88%.

Aqueous solutions of sennoside A [decomposition range 215–220°; Stoll, Becker & Kussmaul (1949) give 200–240°] were prepared using potassium bicarbonate and their pH adjusted to 5·40, the pH of aqueous extracts from senna pericarps. The diffusion of sennoside A through untreated and treated cellulose film into purified water at 20° was determined using the diffusion cell and the results are shown in Fig. 1. The amount of sennoside diffused was determined from a linear calibration graph of absorbance against concentration at the wavelength of maximum absorption 268 nm (E 1%, 1 cm = 226 in purified water).

Effect of ultrasonic energy on the diffusion of sennoside A through treated cellulose film

The potassium salt of a (28 ml of a 90 mg% w/v) solution of sennoside A, adjusted to pH 5·40, was placed in the donor chamber of the diffusion cell and the same volume of purified water in the receptor compartment. Small glass stirrers were inserted into each chamber and the amount of sennoside diffused after 10 min was determined spectrophotometrically at 268 nm. The cell was emptied and the experiment repeated using diffusion times of 20 and 30 min. Separate experiments showed that the size of the stirrer and the rate of stirring had no effect on the diffusion rate of sennoside A.

The effect of ultrasonic energy was determined by subjecting the liquid in the receptor chamber to insonation from a model 60W MSE ultrasonic disintegrator (frequency 20 kHz, probe end diameter $\frac{3}{8}$ inch) for 10 min. The experiment was

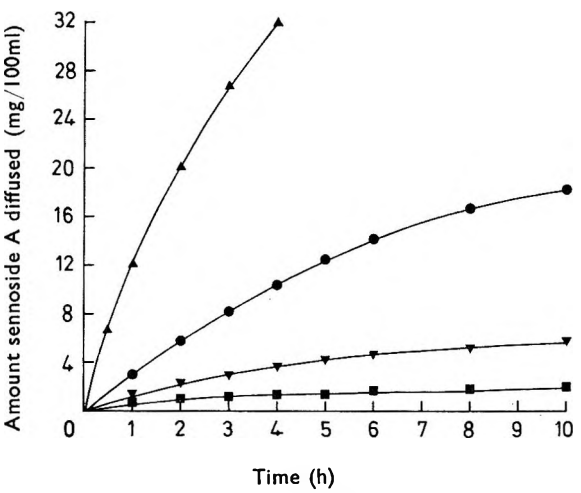

FIG. 1. Diffusion of sennoside A as potassium salt through untreated and zinc chloride-treated cellulose film into purified water at 20°. ■. Initial concentration of sennoside in donor chamber 50 mg/100 ml, untreated membrane; ●, ditto, treated membrane; ▼, initial concentration of sennoside in donor chamber 90 mg/100 ml, untreated membrane; ▲, ditto, treated membrane. Micro-glass stirrers (240 rev/min) were used in donor and recipient chambers.

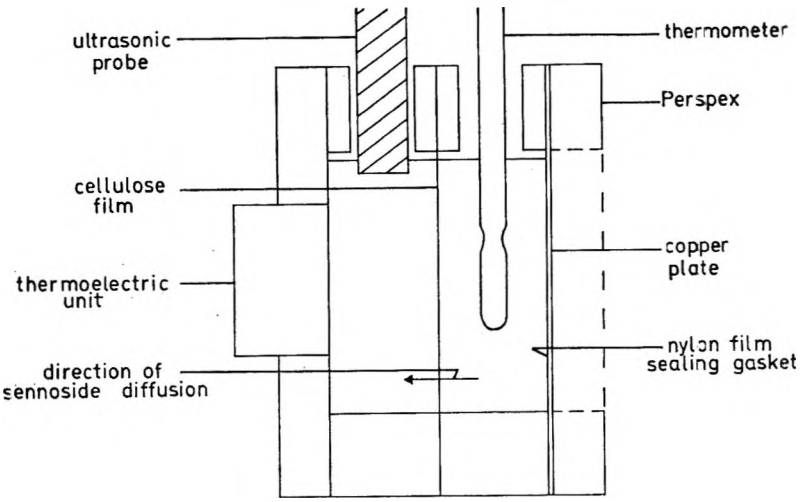

FIG. 2. Diffusion cell employed in the study of the effect of ultrasonic energy on the diffusion of sennoside A through cellulose film.

repeated with fresh sennoside solutions using diffusion times of 20 and 30 min, the absorbance of all solutions being measured at 20°. The rise in temperature of the liquids on either side of the membrane was (time, min/temp.°): 0/20, 5/33·5, 10/43·5, 15/49·5, 20/56, 25/60, 30/63. Preliminary experiments were made to determine the volume of purified water required to be added to each chamber to replace that lost by evaporation.

The contribution of this temperature rise in the diffusion process was determined by passing a current of 7·5 to 8·5 A through the thermoelectric unit in the absence of ultrasonic irradiation. By adjustment of the rheostat the temperatures obtained above could be repeated with an accuracy of $\pm 0{\cdot}3°$ during diffusion periods of 10, 20 and 30 min; in each case fresh sennoside solution was used.

The effect of ultrasonic energy at room temperature was determined in the cell in which the front face was replaced by 0·3 mm thick bright copper plate and the complete assembly was surrounded by solid carbon dioxide (Fig. 2). This removed heat away from the cell at the same rate as it was produced. The plate was held in place by a nylon film sealing gasket impermeable to sennoside A. The carbon dioxide served to augment the cooling action produced by passing a 5 A current through the thermoelectric unit in the reverse direction to that used in assessing the effect of temperature rise on the diffusion process in the absence of ultrasonic irradiation.

All experiments were in triplicate. The diffusion of sennoside A at room temperature through cellulose film previously subjected to ultrasonic waves for 30 min was measured at the completion of the investigations to see if insonation affected the permeability of the membrane. The results are in Fig. 3.

DISCUSSION

The increase in permeability of regenerated cellulose film produced by zinc chloride depended on the concentration of the zinc chloride solution, the temperature, and the time of immersion of the film in the zinc chloride solution. Modified membranes produced as described were of reproducible permeability to sennoside A; film samples

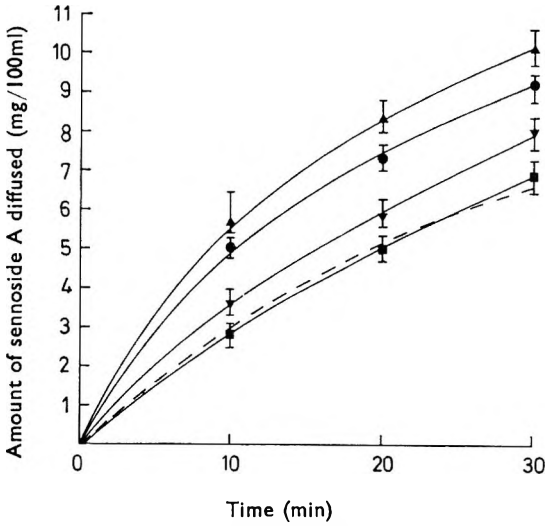

FIG. 3. Effect of ultrasonic energy, controlled heating, and ultrasonic energy at room temperature on the diffusion of sennoside A through cellulose film. ■, Room temperature; ▼, ultrasonic energy at room temperature; ●, controlled heating; ▲, ultrasonic energy. Symbols are the means of 3 experiments, the vertical lines show the range. — — — — Diffusion of sennoside A at room temperature through cellulose film previously subjected to ultrasonic energy. Initial concentration of sennoside in donor chamber 90 mg/100 ml, volume in each chamber 28 ml, surface area of membrane 15·9 cm^2. Micro-glass stirrers (240 rev/min) were in the chambers not containing the ultrasonic probe.

stored in purified water for 14 days did not yield substances absorbing in the ultraviolet region.

Spectrophotometric examination of sennoside A solutions before and after ultrasonic irradiation for 90 min showed that no chemical degradation resulted from insonation. This was confirmed using paper and thin-layer chromatographic techniques.

The use of the diffusion cell enabled the overall heating effect of insonation to be separated from the other effects of ultrasound. The results show that ultrasonic energy produced an increase in the amount of sennoside A passing through the cellulose membrane during a 30 min period, irrespective of the temperature conditions under which diffusion took place. The effect of ultrasonic energy and controlled heating on the diffusion rate of sennoside A through a cellulose membrane was similar to that observed in the extraction of active and inactive constituents from senna pericarps (Woodford & Morrison, 1969). In both cases diffusion of molecules occurred through a cellulose barrier into water which was irradiated with ultrasound.

Ginzburg & Katchalsky (1963) assumed the existence of an unstirred layer adjacent to the cellulose membrane while Nozdreva (1964) stated that the accelerating effect of ultrasound on diffusion rates is due to its disruptive effect on the boundary layer at the phase interface. The present work shows that ultrasonic energy was superior to the stirring procedure previously described in accelerating the passage of sennoside A through cellulose film and that insonation did not produce a permanent alteration in membrane permeability to that glycoside. During the ultrasonic diffusion process marked turbulence together with occasional bubble formation occurred at the membrane surface. These results are consistent with the hypothesis of boundary

layer disruption at the phase interface and indicate that this phenomenon may be an important factor in the non-thermal effect of ultrasonic energy on the extraction of plant material.

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Calibration and application of high torques to a reaction air turbine viscometer

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A reaction air turbine viscometer has been calibrated with the air bearing placed both horizontally and vertically. It has been shown that changing the position of the bearing varied the result by less than 2%. The air bearing pulley employed in the calibration is described; this may also be used to apply high torques during a rheological analysis.

Warburton & Barry (1968) have described a concentric cylinder creep viscometer in which an air bearing was used to centre, and a berilium-copper wire was used to support, the inner cylinder. The torque was applied either by weights hanging over pulleys, or from a modification of a chemical balance.

Davis, Deer & Warburton (1968) improved the viscometer by using a P.C.B.1 Westwind air turbine in which bearing air was used to centre and support the inner cylinder, and turbine air was used to apply the torque. They calibrated the applied turbine pressure in terms of torque at a constant bearing pressure of 2080 torr (approximately 39 p.s.i.) by attaching at 1 cm radius bobbin to the end of the spindle of the turbine rotor, and, with the bearing horizontal, they applied weights directly. They found a linear relation between turbine pressure and torque over a pressure range of 100–2000 torr, with a constant difference of 33 torr between the turbine pressures at which upward and downward movement of the weight just occurred. This difference was attributed to standing friction in the bearing.

To overcome the problem of this residual friction, and to extend the range of the viscometer to lower torques, the P.C.B.I. turbine was replaced by an improved air turbine, the Westwind P.C.B.III, which has been described by Davis (1969). He calibrated the P.C.B.III for low torques by the method described above, keeping the bearing pressure constant at 120 torr (approximately 2·25 p.s.i.). Again there was a linear relation between turbine pressure in the range 0–100 torr, and torque. The standing friction in the bearing was reduced to the equivalent of 3 torr turbine pressure. On increasing the bearing pressure gradually from 50 to 250 torr, Davis found the turbine pressure required to counteract a constant torque (5 g cm) increased, that is, the torque produced by the turbine pressure was reduced. Doubling the standard bearing pressure of 120 torr caused a 3% change in turbine calibration.

We have constructed a similar viscometer using the Westwind P.C.B.III. The applied turbine pressure was calibrated in terms of torque over a wide range of torques (50–16,000 dyne cm), with the bearing horizontal, by a similar method to that of Davis & others (1968). On computer analysis of the results of eight complete calibrations with a constant bearing pressure of 40 p.s.i. an excellent linear relation was found between turbine pressure and torque over the pressure range 5–370 torr (the resultant calibration graph had a regression coefficient of 0·9997 and a gradient of 43·3 dyne cm

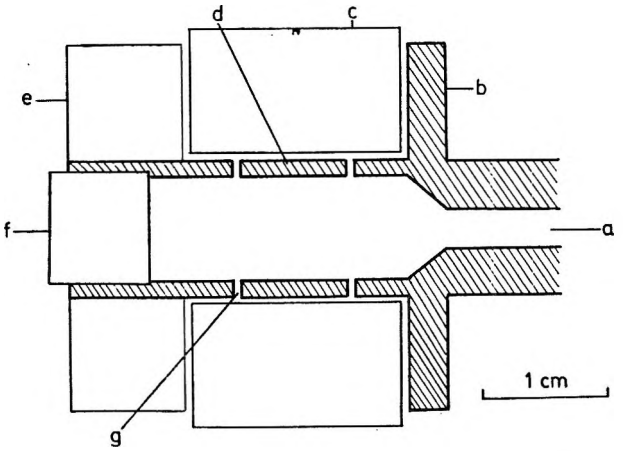

FIG. 1. Sectional diagram of brass air bearing pulley. Key: a. compressed air. b. Inner thrust collar. c. Rotor. d. Journal. e. Outer thrust collar. f. Taper screw. g. Journal air hole. For clarity, the thrust and bearing air gaps and the journal holes are not to scale.

torr^{-1}). The graph however did not pass through the origin, but computer extrapolation gave an intercept of 1·26 torr, which is similar to that found by Davis (1969). This value is also attributed to a small standing friction in the bearing. On repeating the calibration for bearing pressures 25, 30, 35, 40, 50 and 80 p.s.i., the calibration parameters did not change within the limits of experimental error.

The air bearing is placed vertically when used as part of the viscometer, and supports the weight of the inner cylinder assembly. As the distribution of air, and hence the standing friction in the bearing may possibly vary with position, we considered it important to check the calibration with the bearing vertical, and the inner cylinder and the transducer arm in place. In the vertical position, weights cannot be applied directly to the turbine rotor, and so a second air bearing was constructed to function as a frictionless pulley wheel (Fig. 1).

The air pulley is inexpensive, robust and simple to manufacture. The journal has two sets of six holes drilled radially, through which air (at about 30 p.s.i.) passes to the bearing faces. Manufacture is simplified as air at the thrust faces is leakage bearing air, which is sufficient to centre the rotor and thrust holes are not required. The air gap between the journal and the rotor is approximately 0·001 inch (this is not critical) and the thrust air gap can be adjusted and maintained by a taper screw, which expands against the shaft to hold the outer thrust collar firmly in place. The pulley is essentially frictionless. For example, it could be made to rotate for a long time in either direction by giving the rotor an initial spin. There was a slight tendency for it to rotate preferentially in one direction, i.e. to "windmill". This is a common effect in air bearings due, in this case, to the air holes not being drilled absolutely radially. However, during the calibration the weights were hung both in the direction of the windmill effect and against it, and no difference in response was found.

Computer analysis of the results of eight complete calibrations in the vertical position again showed an excellent linear relation between turbine pressure and torque over a turbine pressure range 27–300 torr (regression coefficient 0·9997, gradient 44·1 dyne cm torr^{-1}, intercept 1·38 torr). Typical results for the horizontal and vertical positions are shown in Fig. 2.

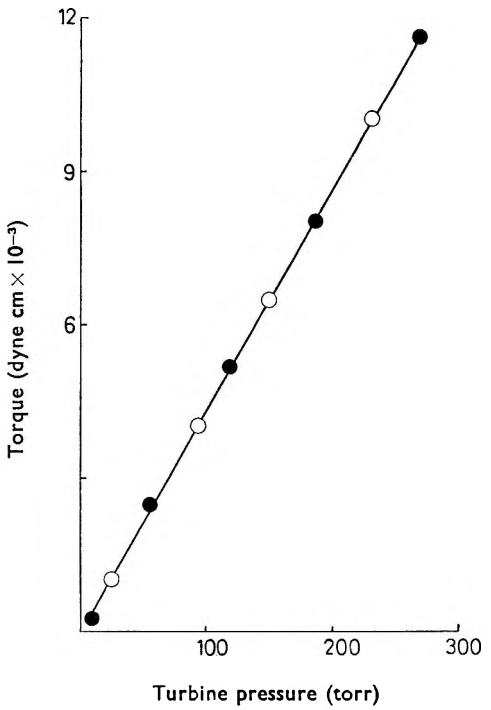

FIG. 2. Representative data from the calibration of the reaction air turbine. –●– bearing in the horizontal position, –○– bearing in the vertical position. Single calibration line drawn through both sets of data.

As the gradients for the graphs differed by less than 2% (which we attribute to alteration in the distribution of air), it was concluded that movement of the bearing from the horizontal to the vertical position did not *markedly* alter the calibration. However, although less than 2% variation may be satisfactory for many uses of the instrument, in particular when used as a creep viscometer, for more precise work, for example when used as a rotational viscometer, we consider that the bearing should be calibrated in the final working position.

The air pulley is also useful for applying a torque directly during a test if forces in excess of those available from the laboratory air supply are required.

Acknowledgement

The authors wish to thank Mr. A. G. Pillidge for constructing the air bearing pulley.

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Quantitative analysis of 4′-chloro-2-ethylaminopropiophenone, 4′-chloro-2-aminopropiophenone and the corresponding aminoalcohols in a mixture of the four compounds

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A method specific for the quantitative analysis of 4′-chloro-2-ethylaminopropiophenone, and 4′-chloro-2-aminopropiophenone in the presence of their corresponding aminoalcohols in urine is described. The instability of the aminoketones makes extraction from alkaline solutions impractical. Hence they are quantitatively reduced to their corresponding alcohols and analysed using gas-liquid chromatography.

Some α-aminoaromatic ketones, like diethylpropion (2-diethylaminopropiophenone), are used as drugs. Their assay is difficult because they are unstable in alkaline solution and therefore they cannot be extracted from urine before gas-liquid chromatography without some decomposition occurring. The related aminoalcohols like methylephedrine, ephedrine and norephedrine are, however, stable in alkaline solution and can be so analysed (Beckett & Wilkinson, 1965).

We have devised a method of quantitative analysis for the aminoketone 4′-chloro-2-ethylaminopropiophenone (compound I, Table 1), the aminoketone (compound III) and aminoalcohols (compounds II and IV) in the presence of each other in urine in which they are found after oral doses of compound I are given to man. The assay is applicable to other α-aminoketones.

EXPERIMENTAL

Apparatus

Perkin-Elmer Model F11 Gas Chromatograph (F.I.D.). Hitachi Perkin-Elmer Model 159 recorder. Lab-tek Aliquot Mixer. Centrifuge tubes with ground glass stoppers. Evaporating tubes with finely tapered base (Beckett, 1966). Pye Dynacap pH meter.

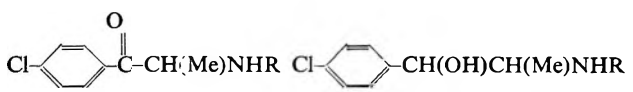
Materials and reagents

Compound I HCl, compound II HCl, compound III HCl and compound IV HCl were supplied by Smith, Kline and French Labs., Philadelphia. The buffer contained 52·4 g dipotassium hydrogen orthophosphate and 46·8 g of sodium dihydrogen phosphate in 200 ml of water. The internal marker solution contained pethedine HCl (9·69 μg/ml water). 20% NaOH. Sodium borohydride. Freshly distilled Analar diethyl ether.

Chromatography

A 1 metre stainless steel tube ($\frac{1}{8}$ in.o.d.) packed with 80–100 mesh Chromosorb G

Table 1. *Structures of compounds investigated*



Compound	Aminoketone Structure	Aminoalcohol Chemical names
I	Ketone R=Et	4′-Chloro-2-ethylaminopropiophenone
II	Alcohol R=Et	1-(4-Chlorophenyl)-1-hydroxy-2-ethylaminopropane
III	Ketone R=H	4′-Chloro-2-aminopropiophenone
IV	Alcohol R=H	1-(4-Chlorophenyl)-1-hydroxy-2-aminopropane

(acid-washed, dimethyldichlorosilane treated) which was coated with 10% KOH and 10% Apiezon L was used. The column was conditioned for 24 h at the following operating conditions, oven temp. 157°, injector block temp. *ca* 160°, hydrogen pressure 14 lb/in²; air pressure 24 lb/in²; nitrogen flow 70 ml/min; stream split ratio *ca* 1:8. The column was then silanized with 2 × 5 μl hexamethyldisilazane.

Procedure

(A) *Analysis in urine of the aminoketones (I and III) in the presence of the aminoalcohols (II and IV).* The stable aminoalcohols (II and IV) were measured by extracting them into ether, followed by gas-liquid chromatographic analysis (Analysis 1). Compounds I and III were determined by reducing them in the urine to produce the corresponding aminoalcohols II and IV (Analysis 2).

Analysis 1 (for aminoalcohols). A urine solution (4 ml) containing compounds I–IV was placed in a centrifuge tube containing the internal marker solution (1 ml) and NaOH (0·5 ml). The compounds were extracted with 4 × 2·5 ml of ether using a shaker (2 min), centrifuged (5 min) and the ether extracts were transferred to a 15 ml Quickfit test tube (tapered base), concentrated (*ca* 50 μl) in a water bath (40°) and the tube was placed in ice. 5 μl of the concentrate was analysed by gas-liquid chromatography. The drug to marker ratios for the two alcohols were determined by measuring the heights of the peaks and these ratios were multiplied by the appropriate calibration factor, determined as described below, to give the concentration (μg/ml) of drug in the urine.

Analysis 2 (for aminoketones). A second aliquot (4 ml) was placed in a centrifuge tube with the internal marker solution (1 ml). The tube was placed in an ice bath for 10 min, buffer solution (0·5 ml) was added and the tube returned to the ice bath for 5 min. Sodium borohydride (*ca* 10 mg) was added, the tube was slowly inverted once and returned to the ice bath for 20 min. The addition of sodium borohydride was repeated twice at 20 min intervals. The NaOH (0·5 ml) was added and the compounds (II and IV) were extracted, analysed and the concentrations calculated as above. The value for each compound obtained in Analysis 2 (ketones I and III, and alcohols, II and IV measured as alcohols II and IV) less the value obtained for the alcohols II and IV all in Analysis 1, give the amount of reduced ketones I and III calculated as the corresponding alcohols.

B) *Calibration factor of compounds II and IV*

Urine (4 ml) containing known quantities of compounds II or IV, or both, was placed in a centrifuge tube. The compounds were extracted, analysed and the drug to marker ratio for each compound calculated using procedure A1. The calibration

factor (μg base/ml urine ÷ the drug to marker ratio) was calculated. The coefficient of variance was calculated for the calibration factor obtained using both amino-alcohols.

(C) *Stability of compounds I-IV in alkaline urine*

Urine (4 ml) containing known quantities of compounds I–IV plus NaOH (0·5 ml of 20%) was allowed to stand (2 h) at room temperature. The internal marker (1 ml) was then added and the contents of the tubes were analysed using procedure A.

(D) *Stability of compounds I–IV stored in urine*

A volumetric flask containing known quantities of compounds I–IV had urine and solutions of either HCl or NaOH added to adjust the urine pH to 4·5, 6·5 or 8·5. The solution was placed in clear glass bottles and stored at either room temperature in the laboratory or in darkness at 4°. "Blank" urine samples were prepared and stored as above.

(E) *Error of injection*

An ethereal concentrate containing internal marker and either compound II or IV was repeatedly injected into the chromatograph. All drug to marker ratios for each alcohol were averaged and the coefficients of variance were calculated.

RESULTS AND DISCUSSION

Injection of an ethereal solution, containing compound II or IV and the internal marker, into the chromatograph (procedure E) led to a ±2% coefficient of variance (8 samples). Extraction of compound II from urine followed by chromatography resulted in a coefficient of variance of ±2·5% (8 samples), whereas with compound IV there was a ±6·2% coefficient of variance (8 samples). This latter error for compound IV could have been decreased with the use of an appropriate gas-liquid chromatographic column and solvent for extracting, but, for the purpose of analysis one column and solvent which could be used satisfactorily for compound II in the presence of ketones and urinary constituents was adopted.

The aminoketones (I and III), but not the aminoalcohols (II and IV), were unstable when left in 4 ml of alkaline urine (0·5 ml of 20% NaOH) for 2 h. Typical results before and after this storage were compound I, 1·56/0·21; compound II, 1·53/1·46; compound III, 1·58/0·24 and compound IV, 1·62/1·57 μg/ml. Thus there would have been negligible decomposition of the aminoalcohols during their extraction from alkaline urine into ether.

At acid pH values (4·5 and 6·5) the aminoketones and aminoalcohols were stable in the dark at 4° for at least 2 days (ketones) and 5 days (alcohols) without interfering

Table 2. *Determination of the compounds I–IV in urine*

Compound	Amount of compound added to urine (μg/ml)				Amount of compound determined in urine (μg/ml)			
	I	II	III	IV	I	II	III	IV
	1·56	1·53	1·58	1·62	1·76	1·46	1·71	1·53
	3·13	1·53	1·58	—	3·11	1·45	1·44	—
	1·56	3·05	—	1·62	1·38	3·13	—	1·60
	1·56	—	3·15	1·62	1·49	—	3·23	1·47
	—	1·53	1·58	3·24	0·01	1·49	1·65	3·31
	1·56	3·05	1·58	—	1·49	2·93	1·37	—

peaks developing in the urine. Thus, immediate analysis of urine samples is not necessary provided the sample is acidic.

Straight line calibration curves were obtained for the aminoalcohols within the experimental error previously mentioned. As the aminoketones were quantitatively reduced to the alcohols the quantitative determination of compounds I–IV in urine (Table 2) was within the experimental error discussed above and thus, this method of analysis is suitable for following the distribution and metabolism of compound I in man.

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LETTERS TO THE EDITOR

Peripheral factors in the mediation of the effects of L-dopa on locomotor activity

Injection of 3,4-dihydroxyphenylalanine (dopa), the metabolic precursor to dopamine, noradrenaline, and adrenaline, produces pronounced autonomic symptoms such as salivation, piloerection, exopthalamus, and elevated blood pressure and respiratory rate (Blaschko & Chrusciel, 1960; Smith & Dews, 1962; Marley, 1966) and also has been reported to both increase and decrease locomotor activity. Smith & Dews (1962) described some of the autonomic effects mentioned above and found that DL-dopa decreased spontaneous motor activity in doses ranging from 100–1000 mg/kg. In contrast, Blaschko & Chrusciel (1960) reported increases in motility after L-dopa in doses greater than 500 mg/kg. Boissier & Simon (1966) found that DL-dopa in lower doses (<500 mg/kg) suppressed locomotor activity but caused hyperkinesia at 1000 mg/kg. Further, several authors have reported suppression of various types of conditioned behaviour after dopa (Eiduson, 1959; Boff & Heise, 1963; Scheckel, Boff & others, 1965). The excitatory effects of dopa are thought to be mediated centrally, probably by the catabolites dopamine and noradrenaline (Carlsson, 1965). To our knowledge, however, no explanations have been given for the suppression of spontaneous motor activity and conditioned behaviour observed particularly after lower doses.

In a previous paper (Butcher & Engel, 1969) we reported that if the enzyme dopa decarboxylase was selectively inhibited in the periphery by 50 mg/kg Ro 4–4602 (seryltrihydroxybenzylhydrazine), then a subsequent injection of L-dopa produced only excitatory effects (i.e. increase in lever-pressing rate on a free-operant avoidance schedule). At the dose of Ro 4–4602 used, only peripheral decarboxylase activity is inhibited whereas the enzyme in brain is relatively unaffected (Bartholini & Pletscher, 1968). Since in our experiments the autonomic effects of dopa were absent after peripheral decarboxylase inhibition, we suggested that the suppressant effects on behaviour reported by some investigators may have been attributable to the action of physiologically active dopa catabolites in the periphery. We now present further evidence for this view.

Sixty male Sprague-Dawley rats, ~200 g weight, had their individual motor activities recorded every 5 min for 1 h, in activity boxes described by Svensson & Thieme (1969). The drugs studied were (doses are expressed in terms of the drug forms shown): L-dopa, 150 mg/kg; dopamine HCl, 150 mg/kg; and Ro 4–4602, 50 mg/kg. In addition to investigating the effects of these drugs alone, we also examined the effects on locomotor activity of Ro 4–4602 in combination with dopa and of dopamine in rats pretreated with Ro 4–4602 and dopa. The drugs were intraperitoneally administered at the following times before the start of the testing session: L-dopa, 45 min; dopamine, 5 min, and Ro 4–4602, 75 min. In the regimen in which the effects of dopamine were studied after prior treatment with Ro 4–4602 and dopa, the dopamine was injected 10 min after the start of the session. Each drug-treatment group consisted of 10 rats. The results were statistically evaluated using analysis of variance with a P × Q factorial design (Winer, 1962). *A posteriori* analyses comparing each treatment condition with every other were performed using the Newman-Keuls procedure (Winer, 1962). A $P < 0{\cdot}05$ was arbitrarily required for significance.

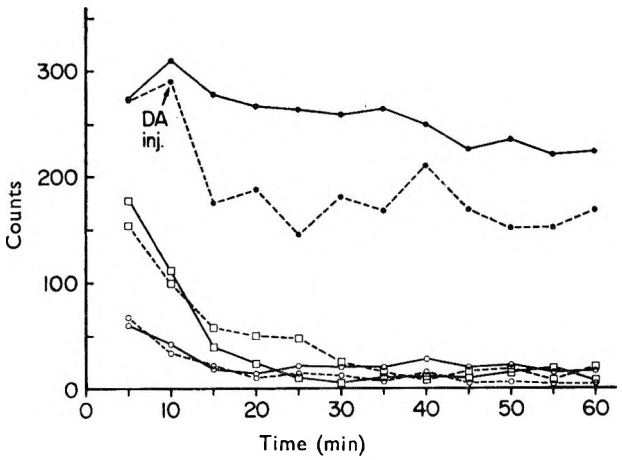

FIG. 1. Effects on locomotor activity of each drug regimen. The doses and time of injection before the start of session are as follows: Ro 4–4602, 50 mg/kg, 75 min; L-dopa, 150 mg/kg, 45 min; dopamine (DA), 150 mg/kg, 5 min. In the Ro 4–4602-dopa-dopamine regimen, the time of dopamine administration is indicated at the arrow. Each point represents the mean of 10 values. Ro 44602 + L-dopa ●—●. Ro 44602 + L-dopa + dopamine ●— —●. Control □—□. Ro 44602 □— —□. L-Dopa ○—○. Dopamine ○— —○.

No significant differences were found between control levels of performance and motor activity after Ro 4–4602 at any of the time intervals (Fig. 1). But dopa and also dopamine significantly reduced ($P < 0{\cdot}01$, compared to control) locomotor activity at the 5 and 10 min intervals (Fig. 1). This is consistent with the results of Smith & Dews (1962). No significant differences were found at the remaining time intervals due to the fact that control activity itself decreased at later portions of the session (Fig. 1). Dopa and dopamine also produced marked automatic symptoms characterized primarily, as assessed by gross observation, by piloerection, salivation, and exopthalmus.

In agreement with the recent findings of Bartholini, Blum & others (1969), dopa in combination with Ro 4–4602 caused an increase in spontaneous motor activity (Fig. 1) and also stereotyped movements of the head and forepaws. In contrast to dopa and dopamine alone, no autonomic manifestations were observed. Locomotor activity was significantly increased over control ($P < 0{\cdot}01$) at all time intervals measured. Introduction of peripheral symptoms by an injection of dopamine in rats pretreated with Ro 4–4602 and dopa was correlated with a significant decrease ($P < 0{\cdot}05$) in motor activity, compared to the Ro 4–4602 dopa treatment, at all time intervals following dopamine administration except the 40 min interval (Fig. 1). Before dopamine injection, no significant difference was found between the Ro 4–4602-dopa animals and the Ro 4–4602-dopa-dopamine group. At all time intervals, however, the activity under the Ro 4–4602-dopa-dopamine regimen was significantly increased over control ($P < 0{\cdot}01$). In addition, 5 of the 10 rats died within 3 h after receiving Ro 4–4602, dopa, and dopamine, whereas no animals died after the other drug treatments. This latter finding suggests that central stimulation may contribute to the lethal effects Ro 4–4602 and of dopa in combination with dopamine.

The dopa-induced suppression of locomotor activity observed in our experiments was probably attributable to physiologically active dopa catabolites in the periphery, although the precise mechanism for this effect is unknown. The following data are compatible with this contention: since the behavioural and autonomic effects of dopa can be blocked after combined central and peripheral decarboxylase inhibition and potentiated after monoamine oxidase inhibition, dopa itself is thought to be

pharmacologically inert and its effects mediated by the catecholamines formed from it at different central and peripheral sites (Carlsson, 1965). Further, injection of dopamine in our experiments produced the same autonomic signs as after dopa, and locomotor activity was suppressed to approximately the same extent. Noradrenaline and adrenaline administration has also been found to suppress conditioned behaviour in pigeons and cats (Sharpless, 1955; Wurtman, Frank & others, 1959). Since catecholamines do not appreciably cross the blood-brain barrier (Weil-Malherbe, 1960), these effects are probably due to actions at peripheral sites. When extracerebral dopa decarboxylase was inhibited in our experiments, L-dopa produced marked stimulation of locomotor activity without autonomic symptoms. A subsequent injection of dopamine in these latter animals resulted in a decrease in motor activity which was accompanied by marked autonomic manifestations. It is possible, therefore, that when dopa is injected alone, catecholamines are preferentially formed in the periphery; their action at peripheral sites may therefore mask the effect of the centrally formed amines. The fact that the enzyme dopa decarboxylase has a higher activity extracerebrally than in the brain (Blaschko & Chrusciel, 1960) supports this view.

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The effect of purity on the surface tension behaviour of a homogeneous non-ionic detergent

The surface tensions of aqueous solutions of both commercial and homogeneous monoalkyl ethers of polyoxyethylene glycols have been described by several authors (Becher, 1967; Lange, 1967). The results have not always been in agreement, and the purity of the compounds studied may be suspect. Homogeneous compounds of this type are not particularly stable in solution (Corkill, Goodman & Ottewill, 1961; Hudson, R. A., personal communication) and although analytical data may suggest that the compound is pure, discrepancies in the surface tension versus log concentration plots can often indicate the presence of impurities which are not detected by other methods.

Most published work indicates a constant value for the surface tension above the critical micellar concentration (CMC), but measurements using highly purified sodium dodecyl sulphate (Elworthy & Mysels, 1966) and a homogeneous monodecyl ether of octaoxyethylene glycol (Hudson & Pethica, 1964) have shown a decrease of surface tension with increasing detergent concentration above the CMC. A few other instances of this behaviour appear in the literature (Brady, 1949; Clayfield & Matthews, 1957; Williams, Woodberry & Dixon, 1957).

As a means of investigating the effect of chemical purity on surface tension, 3,6,9,12,15,18-hexaoxahexacosan-1-ol (C_8E_6) was prepared by two routes, both based on the Williamson ether reaction

(A) by two consecutive additions of triethylene glycol to the octyl chain, i.e. $C_8 \rightarrow C_8E_3 \rightarrow C_8E_6$

(B) direct addition of hexaoxyethylene glycol to the octyl chain, i.e. $C_8 \rightarrow C_8E_6$

In method A the C_8E_6 was obtained from the reaction mixture by extraction and purified by distillation and chromatography; in method B, distillation was avoided. Final purification of both batches was achieved by foam fractionating a 0·35% aqueous solution of the compound (just below the CMC) until the solution was reduced to 60% of its original volume. The foamed solution was then freeze-dried.

Surface tension measurements were determined by the Wilhelmy-plate method at a temperature of 25° ± 0·02°.

The plot of surface tension versus concentration of C_8E_6 is shown in Fig. 1 (e). Comparison of results from a series of experiments showed that although method A produced what appeared initially to be the best results these were not consistent between batches. This we attributed to breakdown of the compound during the final distillation giving rise to impurities which were not removed by the subsequent purification. Despite the occurrence of minima and decreased slopes above the CMC in samples of B which had not been foamed (see insert to Fig. 1, curve f) this method did produce a final product which gave reproducible values. Increasing the relative amount of solution foamed off beyond 40% did not alter the surface tension curves. It is interesting to note that despite the difference in the shapes of the surface tension curves and the surface tension at the CMC between our results and those of other workers (Corkill, Goodman & Ottewill, 1961; Corkill, Goodman & Harrold, 1963) the CMC values are in reasonable agreement.

Addition of hexaoxythylene glycol, E_6; the mono-octyl ether of tetraoxyethylene glycol, C_8E_4; and the disubstituted compound, $C_8E_6C_8$, to foamed samples of C_8E_6 prepared by the method B gave curves d, c and a respectively. As expected $C_8E_6C_8$ gave the most dramatic effect, both lowering the surface tension and producing a large minimum. Comparison of this curve with the unfoamed solution would suggest that the minimum in the latter could be due to a trace of the di-compound.

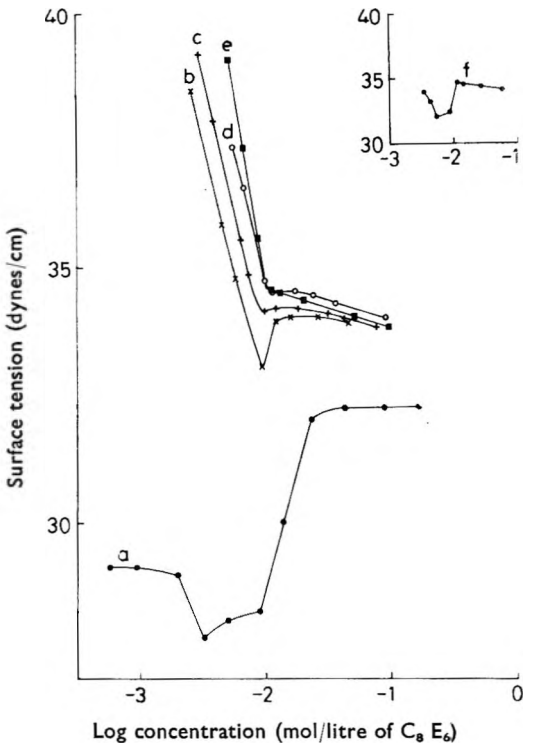

FIG. 1. Variation of surface tension with concentration of C_8E_6 with various additives. (a) a mixture of C_8E_6 and $C_8E_6C_8$ (98·7 : 1·3 w/w); (b) the product of the collapsed foam from the C_8E_4 contaminated solution; (c) a mixture of C_8E_6 and C_8E_4 (98·5 : 1·5 w/w); (d) a mixture of C_8E_6 and E_6 (98·7 : 1·3 w/w); (e) foamed C_8E_6 prepared by method B; (f) unfoamed C_3E_6 prepared by method B.

C_8E_4 and E_6 each produced a small minimum but whereas C_8E_4 reduced the surface tension above the CMC, E_6 slightly increased it. Subsequent foam fractionation and freeze-drying of the contaminated solutions appeared to successfully remove both the $C_8E_6C_8$ and the E_6 as the residue gave identical surface tension curves to curve e.

With C_8E_4, although the solution obtained from the collapsed foam had a lower surface tension and an increased minimum (curve b) to that of the originally contaminated solution (curve c), and the minimum disappeared from the surface tension curve of the refoamed solution, the gradient of the slope of this curve below the CMC was slightly less than that of the originally purified compounds.

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Micellar polydispersity of the non-ionic detergent cetomacrogol

The determination of the micellar molecular weights of detergents in aqueous systems has been restricted mainly to the evaluation of the weight average figure ($\overline{M}_w$) using light scattering and ultracentrifuge techniques (Elworthy & Macfarlane, 1965). To obtain an idea of the degree of polydispersity of macromolecules in solution, $\overline{M}_w$ is compared with the number average molecular weight $\overline{M}_n$; if $\overline{M}_w/\overline{M}_n$ is significantly larger than unity, the macromolecules, or micelles, are said to be polydisperse. A direct comparison of $\overline{M}_w$ and $\overline{M}_n$ has been reported for only a few detergents. Sirianni & Gingras (1961) using a vapour pressure technique found values for $\overline{M}_n$ of pure polyoxyethylene glycol ethers to be significantly lower than the published value for $\overline{M}_w$ of similar detergents, a fact corroborated by later work on non-ionic detergents of industrial origin (Sirianni & Coleman, 1964). Both Ikeda & Kakiuchi (1967) and Schott (1966) however, report a fairly close agreement between $\overline{M}_w$ and $\overline{M}_n$ of polyoxyethylene ethers in aqueous solution.

Membrane osmometry is used here for the first time to ascertain $\overline{M}_n$ of a detergent forming large micelles, with the intention of comparing the values with $\overline{M}_w$ obtained by light scattering.

A commercial sample of cetomacrogol was ion-exchanged twice in methanol on a mixed bed resin column and dried for 6 h at 50° C/30 mm Hg. The ethylene oxide chain length was estimated using the method of Siggia, Starke & others (1958) and corresponded to 23 units. $\overline{M}_n$ of the detergent in aqueous solution was determined using a Hewlett Packard 503 Membrane Osmometer with B19 Cellulose Acetate Membranes (Hewlett Packard Ltd.).

Two major problems are associated with this technique when it is applied to micellar systems. The first involves the reduction to the minimum of any monomer diffusion across the membrane. This was achieved by placing a solution of cetomacrogol just in excess of the critical micellar concentration (CMC) below the membrane. Since the number of monomers in solution does not significantly increase with concentration once the CMC is exceeded, any flow of monomers across the membrane, when a more concentrated solution is placed above it, is obviated. Hence, any contribution the monomers might make to the osmotic pressure may be neglected.

The second difficulty is associated with diffusion of micelles. Since the osmotic pressure readings were shown to remain constant for 3 h, it was concluded that the membrane was impermeable to micelles. Verification of this assumption was obtained by measuring the partition coefficient of the solute with respect to the solutions above and below the membrane (Gardon & Mason, 1957) using a diffusion cell similar to that of Hartley & Runnicles (1938) and Stokes (1950). An average value of 0·003 was obtained for this coefficient over the concentration range of the test solutions, as against a theoretical value of zero for a completely impermeable membrane and unity for complete permeability.

$\overline{M}_w$ values were obtained using the light scattering apparatus described by Attwood (1968).

Table 1 $\bar{M}_w$ *and* $\bar{M}_n$ *of cetomacrogol micelles in aqueous solution*

Temperature °C	Weight average micellar weight (light scattering)	Number average micellar weight (osmometry)
18	101,000*	
25	108,000	103,000
36	110,000	106,000

* Elworthy (1960).

The differences between the two sets of values do not exceed 5%, indicating no significant discrepancy within the error of experimental technique. Thus the micelles are monodisperse or have a very narrow range of sizes.

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Effect of tyramine and octopamine on lipolysis in isolated fat cells of the rat

Injection of tyramine increases plasma free fatty acid (FFA) levels in man (Mueller & Horwitz, 1962), in rats (Stock & Westermann, 1965) and in guinea-pigs (Maier, Maitre & Staehelin, 1967). The present study shows that tyramine and its metabolite, octopamine (Musacchio & Goldstein, 1963), has little direct lipolytic action, and in high concentration reduces noradrenaline-induced lipolysis in isolated rat fat cells.

Male Holtzman rats, 180–220 g, were fasted overnight and killed. Immediately after the epididymal fat pads were removed, and the fat cells prepared by a slight modification (Nakano, Ishii & others, 1968) of the method described by Rodbell (1964). The isolated fat cells, suspended in Krebs-Ringer-bicarbonate buffer (pH 7·4) containing 3% bovine albumin (gassed with 5% carbon dioxide in oxygen), were incubated in a temperature-controlled bath shaker (37°) with noradrenaline, tyramine or octopamine for 1 h. Then the FFA concentration of an aliquot of the mixture was determined (Duncombe, 1963). Triglyceride content of fat cells was measured by van Handel & Zilversmit's method (1957).

Table 1. *Effect of noradrenaline, tyramine and octopamine on free fatty acid release from isolated rat fat cells*

	Free fatty acid release (μmol/h g^{-1} triglyceride) at concentration (M):				
	0	$5{\cdot}9 \times 10^{-9}$	$5{\cdot}9 \times 10^{-8}$	$5{\cdot}9 \times 10^{-7}$	$5{\cdot}9 \times 10^{-6}$
Noradrenaline	$3{\cdot}6 \pm 0{\cdot}2$	$4{\cdot}8 \pm 0{\cdot}3$*	$14{\cdot}5 \pm 0{\cdot}5$*	$48{\cdot}5 \pm 0{\cdot}7$*	—
Tyramine	$4{\cdot}9 \pm 0{\cdot}3$	$4{\cdot}8 \pm 0{\cdot}5$	$5{\cdot}0 \pm 0{\cdot}6$	$6{\cdot}1 \pm 0{\cdot}4$*	$13{\cdot}2 \pm 0{\cdot}6$*
Octopamine	$4{\cdot}5 \pm 0{\cdot}5$	$4{\cdot}3 \pm 0{\cdot}3$	$4{\cdot}9 \pm 0{\cdot}7$	$6{\cdot}8 \pm 0{\cdot}6$*	$15{\cdot}1 \pm 0{\cdot}7$*

The numerical values in each column represent mean $\pm$ s.e.
* $P < 0{\cdot}05$.

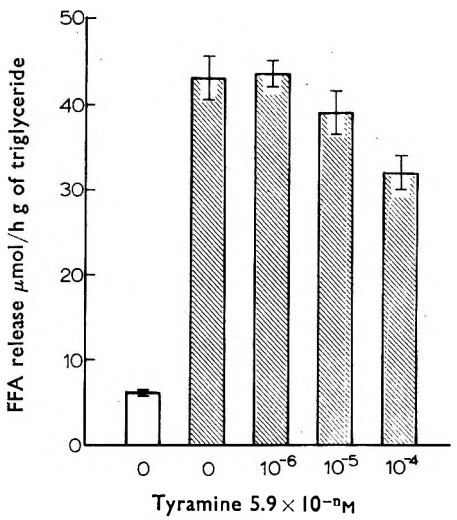

FIG. 1. Effect of tyramine on pyrolysis induced by noradrenaline, $5{\cdot}9 \times 10^{-7}$M in rat isolated fat cells. Bars represent the mean $\pm$ s.e.

Data in Table 1 show that $5{\cdot}9 \times 10^{-9}$ to 10^{-7} M noradrenaline increased markedly the FFA release from the fat cells. On the other hand, $5{\cdot}9 \times 10^{-9}$ to $5{\cdot}9 \times 10^{-8}$ M tyramine and octopamine did not produce lipolysis although higher concentrations ($5{\cdot}9 \times 10^{-7}$ to 10^{-6} M) increased FFA release slightly but significantly ($P < 0{\cdot}05$). Fig. 1 shows that the effect of $5{\cdot}9 \times 10^{-6}$ to 10^{-4} M tyramine on noradrenaline (10^{-7} M)-induced FFA release from the fat cells. Concentrations less than $5{\cdot}9 \times 10^{-5}$ M of tyramine did not influence significantly the noradrenaline-induced FFA release from the isolated rat fat cells. However, tyramine, 1×10^{-4} M, decreased significantly the noradrenaline-induced lipolysis.

Stock & Westermann (1965) showed that the subcutaneous injection of 5 mg/kg of tyramine increased plasma FFA concentrations in rats. They ascribed tyramine-induced lipolysis *in vivo* to the release of noradrenaline from the adrenergic nerve endings (Burn & Rand, 1958), since the blockade of noradrenaline release from the storage sites with cocaine or its depletion by pretreatment with syrosingopine reduced markedly or blocked completely the lipolytic action of tyramine. The present study shows that only concentrations greater than 1×10^{-7} M of tyramine or octopamine directly increased FFA release from the isolated rat fat cells. In addition, only concentrations of 10^{-4} M of tyramine significantly reduced noradrenaline-induced lipolysis. The magnitude of the direct lipolytic action of tyramine and octopamine is approximately 1/100 of that of noradrenaline. The present observations do not appear to be in disagreement with those made previously. *In vivo*, the doses of

tyramine given by other workers would not have interfered with the lipolytic action of the endogenous noradrenaline it released, because the lipolytic action of tyramine caused by it releasing noradrenaline is apparent at concentrations lower than those at which tyramine modifies its response to added noradrenaline.

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Effect of capsaicin on the guinea-pig isolated atrium

Capsaicin (decanoic acid vanillylamide), the pungent principle present in various species of capsicum, is recognized as one of the most active substances in causing excitation of sensory nerve endings. In the cat or dog, circulatory and respiratory effects like hypotension, bradycardia and apnoea are especially pronounced. Since these symptoms occur when capsaicin is injected intravenously but are abolished by vagotomy, the mechanism of these effects is generally believed to be the result of stimulation of the chemo- or stretch receptors in the lung or coronary regions. Additional experiments are needed to fully elucidate this mechanism (Pórszász, György & Pórszász-Gibiszer, 1955; Coleridge, Coleridge & Luck, 1965; Mitchell, Dwarka & Stephen, 1967; Molnár & György, 1967; Molnár, Makara & György, 1967).

In studying the effects of thiamine derivatives on the guinea-pig atrium, Fujiwara & Fukuda (1969) discovered that the extract of *Capsicum annuum* caused a marked increase in the heart rate and enhanced the contraction of the atrium in a manner similar to adrenaline and that this action is due to capsaicin, an ingredient of *Capsicum annuum*.

Guinea-pigs, either male or female, 250 to 300 g were bled out by cutting the common carotid arteries without severing the vagi. The heart was quickly removed and immersed in oxygen saturated Locke-Ringer solution of the following composition (mM): NaCl 154, KCl 56, $CaCl_2$ 2·2, $NaHCO_3$ 2·4, and glucose 5·6 in 1 litre of distilled water. After the extraneous tissues were removed the atrium was suspended in a 50 ml bath containing Locke-Ringer solution aerated with oxygen at 30° and spontaneous contractions of the atrium recorded with an isotonic lever. After the beat of the atrium reached equilibrium, each drug was administrated into the bath. Pure crystalline capsaicin (Kusuge, Inagaki & Uehara, 1958) from the fresh fruits of *C. annuum* var *parvo-acuiminatum* Makino, was used in the experiment. As capsaicin was not readily soluble in water, the reagent was prepared as follows: capsaicin

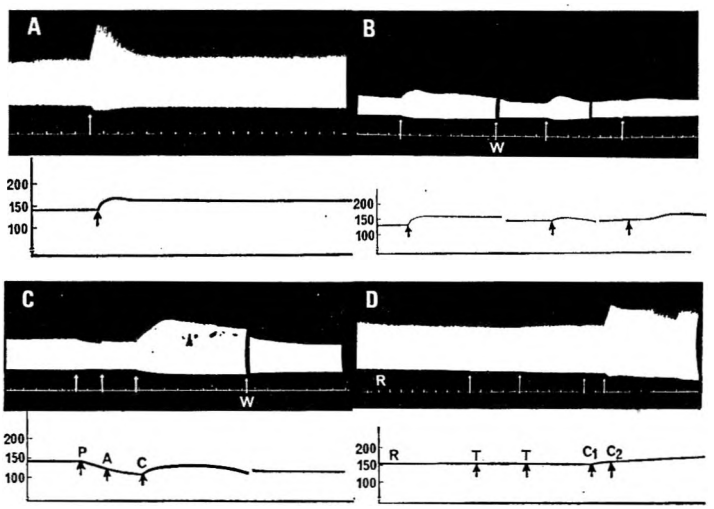

FIG. 1. Responses of atrium (upper traces) and heart rate/min (lower traces) to capsaicin. A. Addition of capsaicin (0·5 μg) to bath at arrow. B. Repeat of inotropic effect of capsaicin (0·5 μg at arrows) after the atrium was washed with Ringer-Locke solution. C. Response of atrium to capsaicin (C↑ 0·5 μg) made unresponsive to adrenaline (A↑ 10 μg) by pretreatment with propranolol (P↑ $7{\cdot}7 \times 10^{-8}$ M). D. Response of atrium to capsaicin (C_1 0·1, C_2 0·4 μg ↑) after it had been made unresponsive to tyramine (T↑ 100 μg) by pretreatment with reserpine (5 mg/kg) 24 h previously.

(1 mg) was dissolved in ethanol (5 ml) and then diluted to 100 ml with distilled water. Tests were made on both the synthetic capsaicin and capsaicin II (the pungent principle present in capsicum; its chemical structure differs from capsaicin at the point of saturation of the double bond of decylenic acid only (Kusuge, Inagaki & Niwa, 1958).

The addition of capsaicin to the bath caused a sudden and marked increase in the amplitude of the atrium contraction, an effect demonstrable at concentrations as low as $1{\cdot}6 \times 10^{-9}$ M (Fig. 1A). This inotropic effect was repeated when capsaicin was added again after the atrium was washed with Locke-Ringer solution as shown in Fig. 1B.

Fig. 1C shows the results when capsaicin was added to the atrium which had been made unresponsive to adrenaline by pretreatment with $7{\cdot}7 \times 10^{-8}$ M propranolol. A marked effect of capsaicin on atrial contraction can still be seen under these conditions. Capsaicin also exhibited the same inotropic effect when the atrium had been made unresponsive to tyramine (Fig. 1D) by pretreatment with reserpine (5 mg/kg weight) which was injected intraperitoneally 24 h beforehand.

From these observations there seems little doubt that the capsaicin effect is different from and independent of adrenaline.

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Relative blocking effectiveness of propranolol and of practalol [4-(2-hydroxy-3-isopropylaminopropoxy) acetanilide] on isoprenaline in β-1 receptor mediated calorigenesis*

Barrett, Crowther & others (1968) have shown practalol [ICI 50172; 4-(2-hydroxy-3-isopropylaminopropoxy) acetanilide] to be about one third as effective as propranolol in blocking isoprenaline in previously defined (Arnold, McAuliff & others, 1966; Lands, Arnold & others, 1967) β-1 receptor mediated lipolytic or cardiac effects. However, practalol was only about 1/100 as effective as propranolol in antagonizing reference catecholamines in previously defined (Arnold & others, 1966; Lands & others, 1967) β-2 receptor mediated bronchodilatation or vasodepression. Since we have shown (Arnold & McAuliff, 1968) that calorigenesis (non-shivering thermogenesis) in the rat, based on oxygen uptake, is β-1 receptor mediated, we were prompted to compare the blocking effectiveness of propranolol and of practalol on isoprenaline under these *in vivo* conditions.

The method we used was modified slightly from that of MacLagan & Sheahan (1950) for mice. Briefly, groups of three, 60 to 90 g, *ad libitum* fed, conscious rats were placed in a small but adequate sized wire basket which was placed, in turn, in a 10-inch dessicator at 28°. The dessicator previously had been flushed with oxygen for a few minutes. The oxygen uptake of the rats was monitored by an appropriately inter-connected Med Science Electronics (St. Louis) Model 160 Spirometer. The comparisons are based on the oxygen taken up over the 10- to 25-min period after administering a test compound, a 10 min equilibration having been judged to be adequate.

Table 1. *Comparison of blockade of (−)-isoprenaline in calorigenesis in the rat by propranolol or by practalol. Three rats per trial*

Isoprenaline* (μg/kg)	Blocking agent† (mg/kg)	No. of trials	O_2 Uptake Mean ± s.e. (cc/100 g/min)	O_2 Uptake % Control
None	None	6	3·57 ± 0·13	
Isoprenaline, 4	None	5	4·45 ± 0·25	125
Isoprenaline, 12	None	6	6·21 ± 0·53	170
None	None	4	3·19 ± 0·12	
Isoprenaline, 12	Propranolol, 3·16	4	4·39 ± 0·53	140
Isoprenaline, 12	Propranolol, 10·0	4	3·46 ± 0·17	110
None	None	8	2·91 ± 0·18	
Isoprenaline, 12	Practalol, 31·6	4	4·20 ± 0·12	140
Isoprenaline, 12	Practalol, 100	3	3·19 ± 0·10	110

* Test agents as base. Compounds used as the hydrochlorides. Test agents given s.c.
† Blocking agent given ½ h before isoprenaline.

* Presented, in part, at the Fall Pharmacology Meetings, Pittsburgh, August 24–28, 1969.

The blocking agents were given subcutaneously in the back of the neck a half hour before administration of isoprenaline by the same route.

Isoprenaline, 12 μg/kg, increased oxygen uptake well above 50% of the control intake (Table 1). At 4 μg/kg there was a 25% increase in oxygen uptake over the control value. Propranolol at 10·0 mg/kg or practalol at 100 mg/kg antagonized the effect of isoprenaline, 12 μg/kg, almost completely. Lesser amounts, propranolol 3·16 mg/kg, or practalol 31·6 mg/kg, had an intermediate antagonizing effect. Neither propranolol nor practalol alone manifested any effect on the oxygen uptake of the rats. On the basis of this comparison, we conclude practalol to be about 1/10 as effective as propranolol in antagonizing the effect of a modestly calorigenic dose of isoprenaline (12 μg/kg).

Barrett & others (1968) indicated that practalol was less effective in blocking isoprenaline-effected tachycardia in conscious dogs than in anaesthetized animals. The use of conscious rats in the present comparisons, accordingly, may explain the practalol/propranolol ratio, on a weight basis, of about 1/10 noted here compared with the ratio of about 1/3 indicated by Barrett & others (1968) on the basis of lipolytic and cardiac effect comparisons. Burns, Salvador & Lemberger (1967) noted that butoxamine {α-[1-(t-butylamino)ethyl]-2,5-dimethoxybenzaldehyde alcohol} antagonized the effect of isoprenaline on heart rate in conscious dogs but not in anaesthetized animals.

By way of contrast with the relatively similar effectiveness of propranolol and of practalol in antagonizing catecholamines in β-1 receptor mediated effects (lipolysis, heart rate and force, calorigenesis) compared with their significantly unequal effect in blocking catecholamines in β-2 receptor mediated effects (bronchodilatation, vasodepression), may be mentioned the converse findings of Moran (1966). He noted that DCI, dichloroisoprenaline [3,4-dichloro-α-(isopropylaminomethyl) benzyl alcohol] and α-methylDCI were essentially equally effective in antagonizing the vasodilator effect of isoprenaline in the dog. However, α-methylDCI was only about 1/15 as effective as DCI in blocking the effect of isoprenaline on heart rate.

Thus, evidence is at hand to support the view previously proposed (Arnold & others, 1966; Lands & others, 1967) that adrenergic receptor mediated-effects are readily explainable by a three receptor concept. This view is based both on studies with agonists (Arnold & others, 1966; Lands & others, 1967) as well as with antagonists (Barrett & others, 1968; Moran, 1966) along with the above antagonist comparison.

We are glad to acknowledge the careful technical assistance of Miss Anne R. Pytell.

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The potency of heparin fractions obtained by gel filtration

It is generally agreed that heparin is polydisperse in molecular weight, and there is evidence that blood anticoagulant activity increases with increasing molecular weight. Thus, Laurent (1961) precipitated the cetyl pyridinium salt of heparin in a series of fractions, and found the least soluble fraction to have the highest molecular weight, as measured in the ultracentrifuge, and also the highest anticoagulant activity. Successive fractions in decreasing order of molecular weight were of decreasing activity.

Gel filtration separates molecules according to their mean molecular size (Stokes radius). Ricketts, Walton & Bangham (1966) found that samples of heparin of higher potency contained more heparin molecules of larger size, as shown by the position of peaks, from a column of Sephadex G-100. It therefore seemed very likely that if heparin fractions from a Sephadex column could be assayed individually for anticoagulant activity, early fractions from the column containing larger molecules would show higher potency than later fractions.

Heparin of porcine mucosal origin* (50 mg/ml of solution), having a potency of 212 units/mg, was submitted to gel filtration on a column of Sephadex G-200 45 cm long by 2·5 cm diameter, in 0·15 M sodium chloride solution (9 vol), 0·12 M sodium phosphate buffer pH 7·4 (1 vol.) containing 0·05% sodium azide as preservative. The column was eluted at 11·55 ml/h into 5 ml fractions. The effluent solution was monitored by a differential refractometer (Waters Model R4). This provided a record of the difference in refractive index between solution entering and leaving the column, as shown in Fig. 1, curve I. Void volume and total volume were measured with blue dextran and sodium iodide respectively in a preliminary experiment; K_{av} was calculated from these and the elution rate. Previous experiments had shown that the recorder reading was proportional to the concentration of polysaccharide in

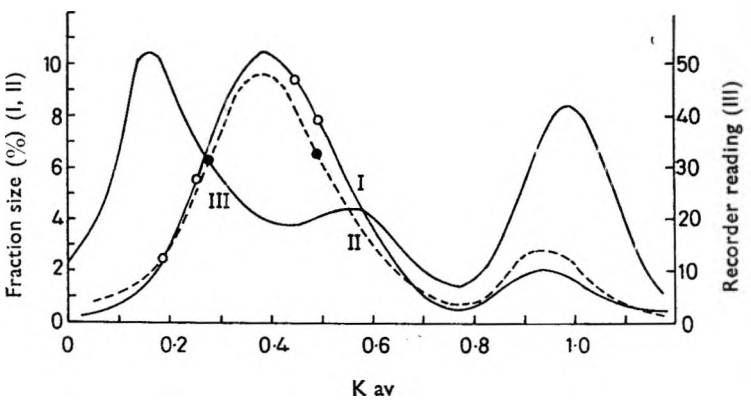

FIG. 1. Curve I. Gel filtration of pig mucosal heparin (212 units/mg) on a column of Sephadex G-200. Fraction size as percentage of whole sample calculated from refractive index change and plotted against K_{av} ($= V_e - V_o/V_t - V_o$ where V_t = total volume, V_e = elution volume and V_o = void volume of column). The heparin peak is at K_{av} 0·4; the peak at K_{av} 0·95 was due to inactive impurities. ○ Fractions selected for assay of anticoagulant activity.

Curve II. Gel filtration of a further sample of pig mucosal heparin (159 units/mg), details as above. Fractions with K_{av} value 0·27 and 0·49, symmetrically placed on each side of the peak, were selected for measurement of anticoagulant activity. ● Fractions selected for assay.

Curve III. Three fractions from each side of the heparin peak of Curve A having K_{av} values 0·14–0·21 and 0·60–0·68, were mixed and the mixture was separated on the same column of Sephadex G-200 into two heparin peaks with K_{av} values 0·16 and 0·56 showing that the gel filtration was separating molecules according to size and that the peak of Curve A was not simply due to diffusion. The third peak at K_{av} 1·0 is due to salts.

* Samples of high potency heparin were supplied by Weddel Pharmaceuticals Ltd., West Smithfield, E.C.1. where all assays were made.

Table 1. *Heparin fractions obtained by gel filtration*

Sample	Fraction No.	K_{av}	Weight (mg)	Activity (units)	Potency (units/mg)
	23	0·17	1·04	240	230
A	24	0·21	1·74	312	179
(212 units/mg)	30	0·45	4·65	1120	240
	31	0·49	3·87	700	180
B	26	0·27	2·86	540	188
(159 units/mg)	32	0·49	3·18	550	173

the solution. The percentage of the sample appearing in each fraction was calculated from the area under the curve. All fractions were freeze-dried and sealed in glass ampoules. Fractions selected from each side of the main peak were dissolved in distilled water and the number of units of anticoagulant activity present in each fraction was assayed.

The assays (B.P. 1963, p. 1136) were in terms of the "First British Standard Mucous Heparin" using the coagulometer described by Walton & Wright (1964). The methods for the determination of potency and calculation of variance were those of the B.P. 1963 (p. 1088).

Table 1 lists the fractions assayed, their K_{av} value, the number of units of anticoagulant activity found and the weight of heparin present, calculated from the refractive index recording. To minimize dependence on calculation of the weight of heparin in each fraction it was thought desirable to repeat the experiment selecting fractions having the same recorder reading. This was done using a further sample of mucosal heparin of potency 159 units/mg. The elution curve, Fig. 1, curve II, obtained closely resembled curve I, as indeed did the elution curves of several other samples of heparin of mucosal origin. Results of this second experiment are also given in Table 1. When the potency of the fractions investigated is plotted against their K_{av} value, taking all the results together there is no systematic variation with K_{av} values. Thus, the expectation that fractions of heparin containing molecules of greater molecular diameter would prove more potent has not been realized.

It remained to be shown that gel filtration under these conditions was in fact separating molecules reproducibly and that the almost symmetrical peak obtained was not simply due to diffusion. A group of fractions from the high molecular weight side of the peak of curve I in Fig. 1 was mixed with a group from the low molecular weight side of the peak. The solution was concentrated by ultrafiltration and dialysed against the buffer solution used for elution; 1 ml of the concentrate was applied to the Sephadex column and eluted as before. The elution curve, Fig. 1 III, showed two peaks at the appropriate K_{av} values due to heparin and a third peak due to salts. The two heparin peaks are not of equal height; it is possible that some of the smaller molecules were lost through the Cellophane tubing during ultrafiltration. It is believed therefore that under these experimental conditions heparin molecules were being separated according to their size.

The samples of mucous heparin used in this work were of relatively high potency, 159 and 212 units/mg. The experiments have shown that while this heparin is certainly polydisperse in molecular size it is relatively homogeneous in anticoagulant activity. It seems quite likely that a highly potent preparation of a natural product would be more homogeneous in respect of biological activity than a less potent sample, part of which may have undergone some small alteration of chemical structure during its isolation. This may very well be true of heparin samples where the exact relation between detail of chemical structure and biological activity remains incompletely understood.

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A use of the isomeric ratio as a criterion to differentiate adrenergic receptors

The general concept of α- and β-adrenergic receptors is well recognized. Rossum (1965) indicated, however, that different tissues may contain different types of α-adrenergic receptors. Although this is a theoretical possibility, Furchgott (1967) found similar K_B values for phentolamine in three different tissues of the rabbit and, on this basis, suggested that the α-adrenergic receptors in thoracic aorta, muscle from the corpus of the stomach and duodenum of rabbit are of a single type.

As with other pharmacologic receptors, a very characteristic property of adrenergic receptors is that of stereoselectivity. If α-adrenergic receptors in various tissues are of a single type, and if their ability to interact with optical isomers is stereoselective, it follows that the activity difference or the isomeric ratio between (−) and (+)-noradrenaline should be the same in each tissue. Under normal circumstances, this isomeric ratio is obscured by several factors operative at the adrenergic nerve terminals; the stereoselective uptake, the unequal distribution of antagonistically-acting α- and β-adrenergic receptors in the same tissue, and the presence of enzymes which can cause selective degradation of isomers of noradrenaline. If all these factors were properly controlled, it is possible that the isomeric ratios of noradrenaline in different tissues containing α-adrenergic receptors would be identical. Data in Table 1 were readily available from previous reports from ours and other laboratories. It can be seen that in normal tissues, the isomeric ratios vary from 2 to 64 (a 32 fold variation). Since acute treatment with reserpine does not significantly change the neuronal uptake, it did not change the isomeric ratio. However, if neuronal uptake was inhibited by cocaine or a cocaine-like agent, imipramine, the isomeric ratios were markedly altered. For cat blood pressure, nictitating membrane, spleen and rat vas deferens, the isomeric ratios for noradrenaline in the presence of cocaine or cocaine-like agents only ranges between 50 and 80. These ratios, within the limits of experimental error, may be considered as essentially equal. Rabbit jejunum has a low density of adrenergic innervation (0·5 μg/g). This is reflected in a high isomeric ratio in the normal tissue. In other words, after inhibiting the uptake, this isomeric ratio of 64 may not change significantly. Similarly, in the rabbit aorta, due to low adrenergic innervation relatively high isomeric ratio was obtained. Thus, the isomeric ratios in all these six tissues which mainly contain α-adrenergic receptors, there is a tendency for isomeric ratios to be equal. It varies from 42 to 80 (*i.e.*,

Table 1. *Relative activities of optical isomers of noradrenaline (NA) on various tissues which mainly contain α-adrenergic receptors*

Test parameter	Procedure or treatment	Approximate ratio[a] (+)-NA (—)-NA	Reference
Cat blood pressure ..	Normal	40	Tye & others (1967);
	Reserpine*	47	Seidehamel & others (1966)
	Cocaine (R)*	60	
Cat nictitating membrane	Normal	8	Tye & others (1967);
	Reserpine*	8	Seidehamel & others (1966)
	Cocaine (R)*	80	
	Denervation (R)*	128	
Cat spleen	Normal	2	Green & Fleming (1968)
	Cocaine (R)*	65	
	Denervation	7	
Rabbit aorta	Normal	42	Swamy, Tye & Patil (unpublished)
Rabbit jejunum ..	Normal	64	Rossum (1965)
Rat vas deferens ..	Normal	5	Patil & others (1967a)
	Reserpine*	5	Patil & others (1967b)
	Desipramine	50	Benvenuti & others (1967)

[a] A dose that will cause equivalent effect was selected as a criterion for calculation of dose ratio.
* Reserpine 3 to 5 mg/kg, i.p. was used 24 to 48 h to deplete catecholamine.

only two fold variation). Furthermore, it should be emphasized that these experiments were not particularly designed to test the present hypothesis. A study of isomeric ratios in different tissues of different animals would be an interesting approach to support or reject the concept of a single type of α-adrenergic receptor. Conversely, if β-adrenergic receptors in the different tissues are of different types, under proper conditions one should obtain markedly different isomeric ratios in different tissues. Unfortunately, at present such information is not available for many tissues containing β-adrenergic receptors. These hypotheses are currently being examined in our laboratories and details will be published elsewhere in the near future.

Furthermore, the same approach can be used to answer the question of a qualitative change in the α-adrenergic receptors after post ganglionic sympathetic denervation (Varma, 1966; Trendelenburg, 1965), and it can be seen that after such a denervation in the cat nictitating membrane and spleen, the isomeric ratios are markedly different than that after cocaine. It is possible that so called qualitative change of α-receptors can alter the steric configuration and hence, its ability to interact with optical isomers of noradrenaline. This is then reflected in the isomeric ratios.

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The analgesic and respiratory depressant activities of *N*-allyl noretorphine and morphine in the mouse

It is generally accepted that equally analgesic doses of all narcotic analgesics are accompanied by the same degree of respiratory depression. No narcotic analgesic would seem preferable to morphine in this respect. Even the narcotic antagonist analgesics like pentazocine depress respiration to the same extent as will equi-analgesic doses of narcotic analgesics (Dyrberg, Hennirgsen & Johansen, 1967).

The recent report by Blane, Boura & others (1968) that the *N*-allyl derivative of noretorphine (R & S 218-M) showed a dissociation between analgesia and respiratory depression was thus of interest. The published evidence upon which this claim was made did not include experiments in which analgesia and respiration in the same animal were measured concurrently. We have now examined the analgesic and respiratory depressant activities of 218-M and compared it with morphine.

Groups of not less than six mice were used. Drugs were administered intra-peritoneally in a volume of about 0·3 ml/25 g mouse. Dilutions, where necessary, were made in saline. In each experiment a control group treated with an equal volume of saline was investigated concurrently. Analgesia was estimated by the hot plate technique—the temperature of the plate being 55° and the end point taken as a shaking movement of a hind limb (Beecher, 1957). Respiratory rate was measured by placing the mouse's snout into the barrel of a syringe connected to a pressure transducer the output of which was recorded on a pen recorder.

Respiratory movements were recorded for at least 10 s then hot plate reaction time was determined. A full investigation of time course of drug action was made in each experiment—measurements being made at either 15 or 30 min intervals. The two parameters were investigated until they did not differ from those seen in the concurrently investigated controls.

Hot plate reaction time is expressed as the difference between the mean hot plate reaction time in a drug-treated group and the mean of the concurrently investigated control group. Depression of respiratory rate is calculated as the difference between the mean respiratory rate in a drug-treated group and that in its control, expressed as a percentage.

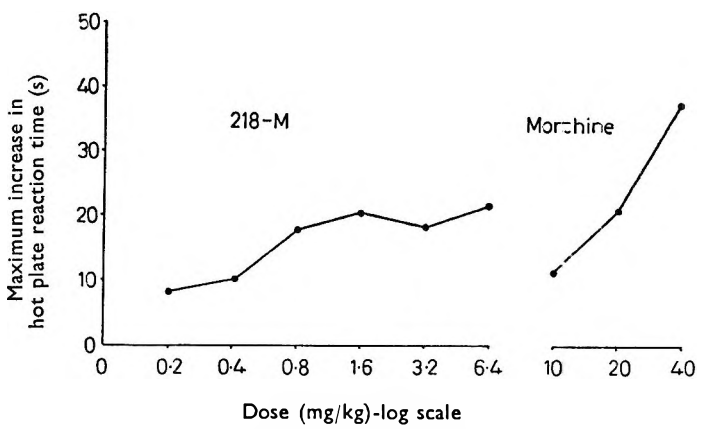

FIG. 1. The relation between the maximum increase in hot plate reaction time(s) and log dose for 218-M and morphine. Each point represents the mean based upon observations in not less than 12 and not more than 30 mice.

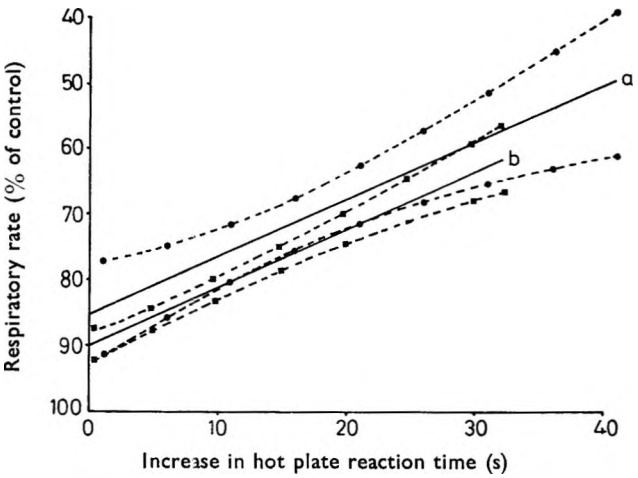

FIG. 2. The regression lines relating increase in hot plate reaction time to depression of respiratory rate for morphine (a) and 218-M (b). The broken lines about each regression line represent the 95% confidence limits for morphine (- - - ● - - -) and 218-M (- - - ■ - - -).

Fig. 1 shows the log dose response curves for morphine and 218-M for maximum increase in hot plate reaction time. Increasing the dose of 218-M from 0·8 to 6·4 mg/kg produced a negligible increase in reaction time. On the other hand doubling the equi-analgesic dose of morphine from 20 to 40 mg/kg produced a marked increase in reaction time. The maximum effect of morphine was not determined because of causing tissue damage during the long exposure of the mice to the hotplate. 218-M therefore has a lower peak effect than has morphine in our experiments.

Fig. 2 relates the degree of analgesia to the concurrent depression of respiratory rate produced by morphine and 218-M at all the various dose levels and intervals after injection. The relation between analgesia and respiratory depression is expressed as a regression line based upon 102 mean observations (218-M) and 42 mean observations (morphine). The intercepts for the regression lines for 218-M and morphine were 90·49 and 85·64% of control respiratory rate. The slopes for 218-M and morphine were 0·92 and 0·89 respectively. The 95% confidence limits for each regression line are included. There is no significant difference ($P > 0.5$) between the two slopes.

These results indicate that there is little dissociation between the analgesic and respiratory depressant effects of 218-M in the mouse as measured by our technique, and that this drug has a lower peak effect than has morphine.

Blane, Boura & others (1968) state that 218-M has a lower peak depressant effect on respiratory rate than has morphine, but for analgesia the log dose response curves are parallel. Amongst the extensive evidence presented by these authors, it is stated that the potency ratio for analgesia in mice compared with morphine is 131 to 1, but for respiratory rate depression is about 0·5 to 1. The standard for analgesia which they used—their ED50—was the dose to increase hot plate reaction time by 100% in 50% of the mice (personal communication). This is a relatively low dose, for in our experiments, in which control reaction time was about 5 s, an increase of 5 s is at the bottom end of our log dose response curve (Fig. 1). On the other hand their standard for respiratory depression from which their potency ratio was calculated was the dose to depress respiratory rate by 50%. This is a relatively enormous dose—indeed we infrequently saw rate depression of this magnitude. Thus the potency ratio for analgesia was established at one extreme end of the log dose response curves and that for rate depression at the other. To interpret the results obtained

in this way as indicating dissociation between analgesia and respiratory depression relies on the two drugs having log dose response curves which are identical with respect to slope and maximum. We were unable to confirm that morphine and 218-M share these characteristics in the mouse.

We wish to express our thanks to Reckitt and Sons Ltd of Hull for the sample of 218-M.

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An improved method of filtration in the determination of morphine by precipitation with fluorodinitrobenzene

In the high ambient temperatures (70–90° F) frequently encountered during the Australian summer, the method for the determination of morphine described by Garratt, Johnson & Lloyd (1957) and Garratt (1964), in which the dinitrophenyl ether is precipitated with 1-fluoro-2,4-dinitrobenzene, is difficult to carry out because of the increased solubility of the precipitate as the solution warms up during filtration. The transference of the precipitate to the filter crucible is made more difficult by the loss of liquid through evaporation.

To overcome these difficulties, the precipitation of the dinitrophenylether is effected in a pear-shaped flask (Quickfit & Quartz FP 50/1) maintained at 60° F in a constant-temperature water bath. When precipitation is complete, the supernatant liquid is drawn off by vacuum through an Emich filter stick with a porosity 3 sintered glass disc and the flask and precipitate are then washed with 4 × 2 ml portions of acetone cooled to 60° F.

In this way precipitation and filtration are carried out at 60° F and there is no troublesome transference of precipitate to the filter. The use of a pear-shaped flask facilitates washing of the precipitate since there is little dead volume, but it is necessary to dry the flask with the filter and precipitate in a vacuum oven to remove all traces of acetone.

The Emich filter stick is made by fusing a porosity 3 sintered glass disc (8 mm diameter × 2 mm thick) to the flared end of a Pyrex tube (1·5 mm i.d. and 4 mm o.d.), the overall length being approximately 14 cm.

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Original Papers

Letters to the Editor