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Effect of cold stress on the subcellular distribution of noradrenaline in the rat heart

C. C. CHANG AND C. Y. SU

Rats were exposed to cold (4°) for 2 hr to study the effect of increased sympathetic activities on the subcellular distribution of noradrenaline in the heart. Cold-exposure caused about 30% decrease of total noradrenaline contents in both auricles and ventricles of normal or adrenalectomized rats. This depletion of noradrenaline caused by cold was completely prevented by pretreatment of the rat with hexamethonium chloride. Measurement of the subcellular distribution of noradrenaline revealed that the percentage depletion in particle-bound amine in both auricles and ventricles was greater than in the supernatant noradrenaline. It is suggested that the noradrenaline in the particulate fraction is the functional pool of the amine available for release by nerve impulses.

SINCE the existence of two or more pools of noradrenaline stores in the sympathetically innervated organs was postulated by Trendelenburg (1961) and by others (see Kopin, 1964) there has been speculation about the functional pool of noradrenaline available for release by nerve impulses. Spontaneous junction potentials recorded in the vas deferens of the guinea-pig (Burnstock & Holman, 1962) indicate that in the adrenergic nerve, as in the cholinergic system, the neurotransmitter may be released in quanta. On the other hand, Euler & Lishajko (1962) suggested that noradrenaline in the free form might be the mobile pool, while Stjärne (1966) pointed out the impossibility of noradrenaline in granular vesicles being a functional pool on the basis of the number of the granular vesicles found in the sympathetic nerve.

Recently, Chang & Chang (1965) showed that when the isolated vas deferens of the rat was stimulated coaxially and then homogenized the decrease of noradrenaline was only found in the particulate fraction of the homogenized preparation. In contrast to this finding, Stitzel, Campos & Shideman (1965) reported that stimulation of the right accelerans nerve of the isolated perfused rabbit heart caused a decrease of the soluble noradrenaline fraction only.

It seemed, therefore, desirable to investigate the functional noradrenaline pool in intact animals and to attempt its localization. We have subjected rats to cold stress to increase the activity of the sympathetic innervation, and the effect of this treatment on subcellular distribution of noradrenaline in the heart has been examined. The results so far indicate that the content of noradrenaline in the particulate fraction decreased more than that in the supernatant fraction.

Methods

COLD STRESS

Long Evans rats of either sex, 250 to 300 g, were placed in a cold room at 4° for 2 hr after which the rats were killed by a blow on the head. The heart was then rapidly excised.

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SUBCELLULAR INVESTIGATION

The auricles, after being washed in cold saline, were homogenized in a loosely fitting glass homogenizer with 30 volumes of 0·25M sucrose in the cold for 2 min. Ventricles were homogenized with 10 volumes of 0·25M sucrose. A coarse fraction of the homogenates was removed by centrifugation at 1500 *g* for 5 min. This fraction usually contained about 40% of the total noradrenaline. The supernatant left after this initial centrifugation of the auricles (1 ml) was then layered on the surface of 0·4M sucrose (4 ml) and centrifuged at 39,000 rev/min (125,000 *g*) for 45 min in a Spinco Model L Ultracentrifuge with swinging bucket rotor SW 39. With this procedure, dilution of the auricle homogenate can be largely avoided. For the ventricles, aliquots (5 ml) of supernatant left after initial low speed centrifugation were centrifuged directly.

ASSAY OF NORADRENALINE

A modification of the trihydroxyindole method of Chang (1964) was used. The total content of noradrenaline in the auricle or ventricle was determined on 0·25 ml of the homogenate. For the assay of the noradrenaline in the supernatant from auricles, 1·5 ml of the topmost layer was used. High speed sediments were resuspended in 1 ml of 0·01N hydrochloric acid and assayed for particulate noradrenaline. Extraction of noradrenaline from each fraction was achieved by shaking with 10 volumes of acid-butanol to which excess of sodium chloride was added.

Results

EFFECT OF EXPOSURE TO COLD ON THE TOTAL NORADRENALINE CONTENT OF AURICLES AND VENTRICLES

Table 1 shows the effect of cold-exposure on the noradrenaline contents of auricles and ventricles. In agreement with the results of Leduc (1961),

TABLE 1. CHANGES OF THE TOTAL NORADRENALINE IN THE HEART OF RATS EXPOSED TO 4° FOR 2 HR (MEANS OF 6 EXPERIMENTS)

	Noradrenaline (μg/g ± s.e.)			
	Normal		Adrenalectomized	
	Auricle	Ventricle	Auricle	Ventricle
Control	3·54 ± 0·27	1·61 ± 0·21	4·36 ± 0·21	1·55 ± 0·12
Cold stressed	2·32 ± 0·25	1·11 ± 0·14	3·02 ± 0·25	0·97 ± 0·11
% Deviation from control	−34	−31	−33	−39
Statistical significance	P <0·02	P <0·1	P <0·01	P <0·01

cold exposure caused the noradrenaline of both auricle and ventricle to decrease by about 30%. Rats, which were adrenalectomized three days before the experiment and kept on normal saline, also showed a depletion of noradrenaline to a similar extent on exposure to cold (Table 1).

EFFECT OF GANGLIONIC BLOCKING AGENT

The decrease of the amine content of the rat heart in response to cold might be due to a decreased rate of synthesis, or to impairment of the storage mechanism, induced by a lowering of the body temperature. Hexamethonium chloride (10 mg/kg) was given to rats subcutaneously 20 min

before cold-exposure. Table 2 shows that treatment with this ganglionic blocking agent completely protected the amine both in auricles and ventricles from being decreased in response to cold. This would indicate

TABLE 2. EFFECT OF HEXAMETHONIUM ON THE CHANGE OF NORADRENALINE INDUCED BY COLD STRESS

Hexamethonium chloride (10 mg/kg) was given to rats subcutaneously 20 min before exposure to cold (means of six experiments)

	Noradrenaline (μg/g ± s.e.)	
	Auricle	Ventricle
Control	3·76 ± 0·25	1·71 ± 0·18
Cold stressed	3·58 ± 0·55	1·70 ± 0·30
Statistical significance	P >0·4	P >0·5

that the loss of noradrenaline in the animals not treated with hexamethonium (Table 1) may be mediated through the increased sympathetic nerve activities.

CHANGE IN THE SUBCELLULAR DISTRIBUTION OF NORADRENALINE

Measurement of noradrenaline in the subcellular fractions of the auricles from cold-stressed animals revealed that the particulate fraction lost more than 50% of its amine, while supernatant lost only 25% (Table 3).

TABLE 3. CHANGES IN THE SUBCELLULAR DISTRIBUTION OF NORADRENALINE IN RESPONSE TO COLD (MEANS OF 6 EXPERIMENTS)

	Noradrenaline (μg/g ± s.e.)					
	Auricle			Ventricle		
	Supernatant (S)	Particulate (P)	Ratio (P/S)	Supernatant (S)	Particulate (P)	Ratio (P/S)
Control	0·96 ± 0·04	0·58 ± 0·05	0·61 ± 0·06	0·37 ± 0·04	0·36 ± 0·04	0·99 ± 0·05
Cold stressed	0·72 ± 0·11	0·24 ± 0·04	0·37 ± 0·07	0·26 ± 0·03	0·20 ± 0·03	0·82 ± 0·09
% Deviation from control	−25	−59		−30	−44	
Statistical significance	0·05 < P <0·1	P <0·01	P <0·05	P <0·05	P <0·01	P = 0·1

The ratio of the amount of noradrenaline in the particulate fraction to that in the supernatant was thus reduced from 0·61 to 0·36 (P <0·05). The loss of particulate noradrenaline of ventricles also exceeded that of supernatant amine (44% as against 30%) although the difference is not as marked as in the auricles and is statistically not significant.

Discussion

Hsieh, Carlson & Gray (1957) pointed out the importance of noradrenaline released from the sympathetic nerve endings for the generation of heat other than by shivering. The circulatory level of noradrenaline in the cat exposed to cold was increased (Hemingway & Price, 1964) and urinary excretion of the amine in the rat exposed to 3° was also elevated (Leduc, 1961). The view that noradrenaline released in response to cold comes largely from sympathetic nerves is supported by evidence that, in adrenalectomized animals, the urinary excretion of noradrenaline still

increases to the same extent, so the response is not a secondary effect of adrenaline release from the adrenal medulla which might displace noradrenaline in the heart (Angelakos, Bloomquist & King, 1965), and also that a ganglionic blocking agent inhibits the increase of urinary noradrenaline excretion (Leduc, 1961). It was further shown by Leduc that the noradrenaline content in the rat organs innervated by sympathetic nerves decreased on exposure to cold, whereas Johnson (1964) reported a negative result.

In the present experiments we have confirmed the finding of Leduc that noradrenaline in the heart decreases in response to cold. Since this decrease of noradrenaline content was prevented by pretreatment of the animal with hexamethonium, it is unlikely that the decrease of tissue noradrenaline after exposure to cold is due to an impaired rate of noradrenaline synthesis caused by the fall in body temperature, to an impaired storage mechanism, or to increased metabolic turnover. If it were a temperature effect, hexamethonium treatment, which prevents the calorigenic effect in response to cold (Hsieh & others, 1957; Gilgen, Maickel, Nikodijevic & Brodie, 1962; Brück & Wünnenberg, 1965), should on the contrary enhance the decrement of noradrenaline. It can be concluded therefore that the decrease of heart noradrenaline in the rat exposed to cold is the result of increased release of noradrenaline from sympathetic nerve endings due to increased nervous activity.

The data in the present experiments show that, in the auricle, noradrenaline in the particulate fraction decreased more than that in the supernatant fraction on exposure of the rat to cold, suggesting that the particulate noradrenaline rather than the supernatant amine may be immediately involved in sympathetic nerve transmission. Although cold stress did not significantly change the pattern of subcellular distribution of noradrenaline in the ventricles, the result shows a similar tendency to depletion. It might be that the size of the functional pool of the transmitter amine in the ventricles is smaller than that in the auricle. The result obtained with the auricles agrees with that obtained by coaxial stimulation of the isolated vasa deferentia of rats (Chang & Chang, 1965), but is different from that obtained in the isolated rabbit heart (Stitzel & others, 1965). By stimulation of the right accelerans nerve of the isolated perfused rabbit heart for 5 to 10 min, Stitzel & others (1965) found a decrease of soluble noradrenaline in both auricle and left ventricle while no significant change was found in the particulate fraction. However, in their experiment, stimulation for more than 10 min did not increase the effect further. The results of Iversen, Glowinski & Axelrod (1965) showed that the ratio of particulate to supernatant [^{3}H]noradrenaline of the isolated perfused heart was markedly different from the same ratio of the heart, *in situ* (0·6 and 1·5 respectively). This might suggest that in the isolated perfused heart the ability of 'particles' to hold noradrenaline is impaired and such an existing impairement of storage or release mechanism in this preparation must be taken into consideration. It is interesting that Hift & Campos (1962) also found a greater decrease of noradrenaline from the particulate fraction in dogs under irreversible

haemorrhagic shock, a condition in which sympathetic discharge is greatly increased (Millar, Keener & Benfey, 1959; Neil, 1962). It appears that sympathetic activities induced either by cold-exposure or by haemorrhagic hypotension tend to deplete particulate noradrenaline more than the supernatant noradrenaline from the heart. The soluble fraction of noradrenaline is therefore unlikely to be the functional pool on which nerve impulses would directly act, as suggested by Euler & Lishajko (1962) and Stitzel & others (1965).

The electrophysiological finding on vas deferens by Burnstock & Holman (1962), of the quantal nature of the spontaneous junction potentials, strongly indicates that the transmitter released from the sympathetic nerve is contained in packets as in other systems. In studies on the particulate binding of noradrenaline and related compounds Musacchio, Fischer & Kopin (1966) found that only those sympathomimetic amines, which are retained in noradrenaline storage vesicles, can be released by sympathetic nerve stimulation. They suggested that the vesicles or a binding mechanism with similar properties is the site from which noradrenaline is released when impulses reach the nerve endings. Our results, which show that increased sympathetic activities in the rat heart induced by exposure to cold primarily release noradrenaline from the particulate fraction, also point to the possibility of particle-bound noradrenaline as the functional pool available for release by nerve impulses. The same conclusion has been reached from experiments using the isolated vas deferens preparation of the rat (Chang & Chang, 1965).

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A new method for the determination of globule size distribution of emulsions by dielectric constant measurement

R. C. KAYE AND H. SEAGER

A new method for the determination of globule size distribution of emulsions prepared with non-ionic surfactants by ascent rate analysis is described. The proportion of dispersed phase in a narrow layer of dilute emulsion situated at a known height in a capacitance cell is determined at suitable time intervals and by the application of Stokes' Law, the droplet size distribution is calculated.

ALTHOUGH a great deal of attention has been paid to the determination of particle size of finely divided solids, comparatively little interest has been shown in the determination of the droplet sizes of emulsions. The principles of incremental sedimentation analysis used for the size-grading of solid powders (see for example Rose, 1958; Orr & Dallavalle, 1960; British Standards No. 3406: Part 2, 1963) could be applied to the determination of droplet sizes in emulsions, but this seems to have been neglected because of the difficulty of determining oil concentrations in withdrawn samples or in narrow layers of emulsions.

Recently, Kaye & Seager (1965) described a method for the detection of composition changes which occur in the upper layers of creaming emulsions. In this method, a glass cell provided with a pair of capacitor plates at its upper end is filled with emulsion. As the emulsion gradually separates and the concentration of oil in the upper layers increases, the dielectric constant of the emulsion in this region decreases. The change in dielectric value results in a decrease of cell capacitance which is measured by the heterodyne beat method.

Since the relation between dielectric constant and oil concentration in dilute emulsions is known, it seemed likely that an incremental method of sedimentational analysis could be based on dielectric constant measurement.

We now describe the accurate determination of the proportion of dispersed phase in a narrow zone of emulsion situated at a known height in a capacitance cell. The proportion of dispersed phase in this zone is obtained at suitable time intervals, and by the application of Stokes' law the droplet size distribution of the emulsion is calculated.

Experimental

APPARATUS FOR MEASUREMENT OF DIELECTRIC CONSTANT

Capacitance cell. The capacitance cell (Fig. 1) is a long glass vessel having two concentric walls (1 and 2) defining an annular space (3) between them. The vessel is filled with emulsion through an opening (4) provided near one end. The capacitor plates near the top of the cell are two conducting layers (5 and 6) formed by the deposition of silver

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onto the glass surface. The inner conducting plate (7) is electrically connected to the outer earthed plate (6) and acts as a guard ring which reduces the effect of fringe capacitance between plate (6) and the upper edge of plate (5). Electrical connection to the plates is made through a coaxial plug (8) which is used to connect the cell to the heterodyne beat oscillator.

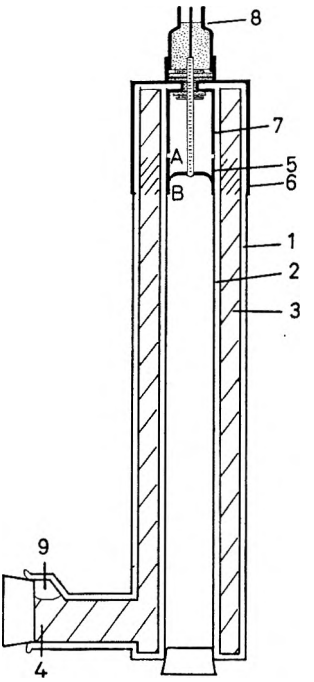

FIG. 1. Capacitance cell. For explanation see text.

Heterodyne beat oscillator. The capacitance of the emulsion-filled cell was measured by the heterodyne beat method. A block diagram of the apparatus is shown in Fig. 2. Radio-frequency signals, f_v and f_c, are generated by a variable frequency and a fixed frequency (crystal controlled at 7 Mc/sec) oscillator respectively. Both signals are fed into a mixer circuit, the output of which, $(f_v - f_c)$ or $(f_c - f_v)$, is monitored on a

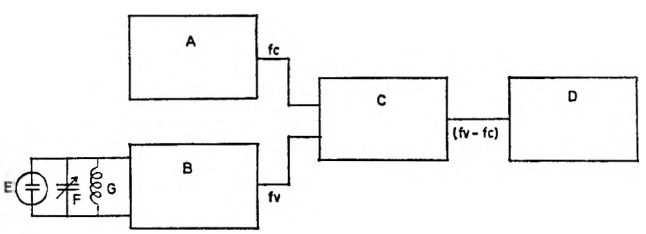

FIG. 2. Block diagram of the heterodyne beat apparatus. A, crystal oscillator. B, variable frequency oscillator. C, mixer. D, beat detector. E, capacitance cell. F, precision condenser. G, inductance.

loudspeaker and on a "magic eye" indicator tube. When the frequencies of the R.F. outputs from each oscillator are the same, i.e. $f_v = f_c$, there is no note from the loudspeaker and no flickering on the "magic eye" tube.

The frequency of the variable oscillator R.F. output is given approximately by the equation

$$f = \frac{1}{2\pi\sqrt{LC}} \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where L and C are respectively the inductance and capacitance of its tuned circuit. The capacitance in the frequency determining circuit is made up of the capacitance cell (the dielectric of which is formed by the emulsion under investigation) and a precision variable condenser, both connected in parallel with the inductance (of constant value). With the emulsion-filled cell in position, the variable frequency oscillator is tuned by the precision variable condenser to the same frequency as that of the crystal controlled oscillator. Any composition change in the emulsion shifts the frequency of the R.F. output of the variable frequency oscillator and gives rise to a note in the loudspeaker and flickering of the indicator tube. The amount by which the precision condenser must be adjusted to return the frequency to that of the crystal oscillator is a direct measure of the capacitance change brought about by the change of emulsion composition.

METHOD OF MEASURING CREAMING RATES

Emulsions containing up to 2% w/w of liquid paraffin B.P. in a 1% w/w aqueous solution of cetomacragol 1000 B.P.C. were prepared in an Atomix M.S.E. Emulsifier. A uniform sample of emulsion was taken and any air removed by gentle rotation for 10 min in a flask under vacuum. Gentle shaking was continued to ensure uniform globule distribution as the emulsion was warmed to 25°. The cell, maintained at 25° in a Perspex air chamber, was disconnected from the oscillator and filled with the emulsion. The rubber stopper was inserted and the cell inverted so that the remaining air bubble was trapped in the small space provided (Fig. 1, 9). The gap between the capacitor plates was thus filled with emulsion free from air bubbles.

A clock was immediately started, the cell quickly reconnected to the oscillator and the variable capacitor adjusted so that the frequency of the variable oscillator was equal to that of the crystal reference oscillator. As the emulsion gradually separated and the concentration of oil between the capacitor plates decreased, the dielectric changes caused a corresponding increase in cell capacitance. The frequency of the R.F. output of the variable frequency oscillator was returned at suitable time intervals to that of the reference oscillator by adjusting the variable capacitor.

CONVERSION OF CAPACITANCE READINGS INTO DIELECTRIC VALUES

The cell capacitance C_c is related to the dielectric constant of its contents by the following equation (Sherrick, Dawe, Karr & Ewen, 1954; Blaedel & Petitjean, 1956; Kaye & Seager, 1966).

$$C_c = \frac{[(2\pi f)^2 C_0\epsilon(C_0\epsilon + C_g) + K^2]C_g}{(2\pi f)^2(C_0\epsilon + C_g)^2 + K^2} \quad .. \quad .. \quad .. \quad (2)$$

where C_0 = capacitance due to air in empty cell
C_g = capacitance due to glass walls of cell
ϵ = dielectric constant of emulsion
K = conductance of emulsion
f = frequency of electrical field

For the system studied, the conductance term K^2 may be neglected in comparison with the capacitance terms $(2\pi f)^2 C_0\epsilon(C_0\epsilon + C_g)$ and $(2\pi f)^2(C_0\epsilon + C_g)^2$ and the equation reduces to

$$C_c = \frac{C_0\epsilon C_g}{C_0\epsilon + C_g} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

Since the capacitance of the cell when empty is given by

$$C_c = \frac{C_0 C_g}{C_0 + C_g} \quad .. \quad .. \quad .. \quad .. \quad (4)$$

the capacitance C due to the emulsion may be obtained by subtracting equation (4) from (3)

$$C = \frac{C_0\epsilon C_g}{C_0\epsilon + C_g} - \frac{C_0 C_g}{C_0 + C_g} \quad .. \quad .. \quad .. \quad (5)$$

and the dielectric constant of the emulsion may then be determined from capacitance measurements by employing the rearranged form of equation (5)

$$\epsilon = \frac{C_g + C(C_g/C_0 + 1)}{C_g - C(C_0/C_g + 1)} \quad .. \quad .. \quad .. \quad (6)$$

CONVERSION OF DIELECTRIC DATA INTO EMULSION COMPOSITION

Many equations relating the dielectric constant of an emulsion to its composition have been proposed (Lorenz & Lorentz, 1880; Rayleigh, 1892; Wiener, 1912; Wagner, 1914; Lichtenecker, 1926; Piekara, 1932; Bruggeman, 1935; Fradkina, 1950; Kubo & Nakamura, 1953; Reynolds & Hough, 1957). Because of the unknown effect of the oil-water interface on the electrical field strength between the capacitor plates, no single equation is suitable for use over a wide range of concentrations (Piekara, 1929, 1930; Heymann, 1934; Kruyt, 1952; Smyth, 1955; Fradkina & Khmunin, 1956; Naiki, Fujita & Matsumura, 1959; Khmunin, 1959; Hanai, Koizumi & Gotoh, 1962).

Most authors agree that the experimental results, especially in the case of dilute emulsions, are best fitted by the Bruggeman equation

$$\frac{\epsilon - \epsilon_0}{\epsilon_w - \epsilon_0}\left(\frac{\epsilon_w}{\epsilon}\right)^{1/3} = 1 - \phi \quad .. \quad .. \quad .. \quad (7)$$

where ϵ = dielectric constant of emulsion; ϵ_0 = dielectric constant of dispersed phase; ϵ_w = dielectric constant of continuous phase; ϕ = volume fraction of dispersed phase. A theoretical plot of this equation is shown in Fig. 3, along with the experimentally determined dielectric constants

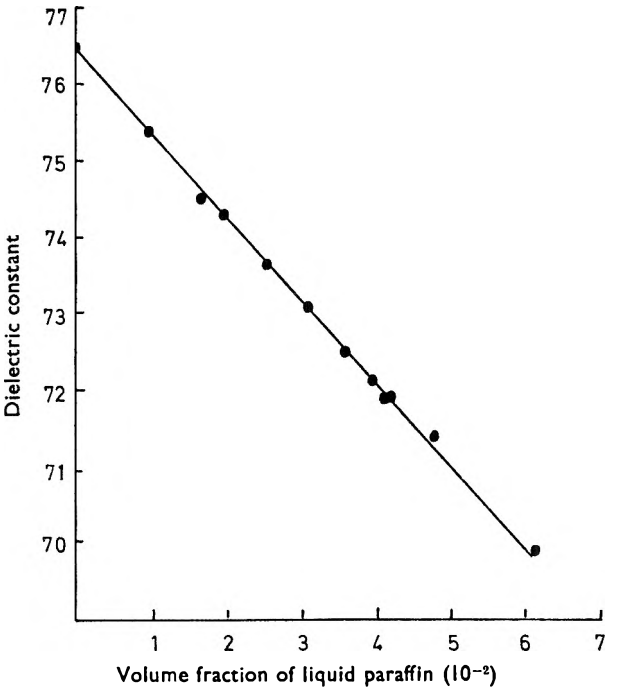

FIG. 3. Relation between volume fraction of dispersed phase and dielectric constant for liquid paraffin-in-water emulsions. — Theoretical curve calculated from the Bruggeman equation. ● Experimental points.

of a series of dilute liquid paraffin-in-water emulsions. The agreement between the theoretical and practical values is excellent. The Bruggeman equation was therefore used to calculate the composition of the emulsion lying between the capacitor plates from the dielectric values.

CALCULATION OF GLOBULE SIZE DISTRIBUTION

Consider the behaviour of a dilute monodispersed emulsion in a cell having very narrow capacitor plates, i.e. the distance AB (Fig. 1) is similar to the size of the globules themselves. The globules cream at the same velocity and the concentration of oil in the zone between the capacitor plates remains constant for a time because all disappearing globules are replaced by others rising from the layers below. When all the globules which were in the lowest regions of the emulsion have risen beyond the zone, the oil concentration suddenly falls to zero.

If the emulsion is not monodispersed, the globules of different sizes cream at different rates and the concentration of oil within the zone does not change suddenly but decreases gradually with time due to the continual disappearance of globules of certain sizes. At zero time, the emulsion between the capacitor plates is made up of globules of all sizes but after time t, only globules with an ascent velocity less than x/t (where x is the distance from the base of the cell to the capacitance zone, and t is

the time of creaming) are still to be found in the zone. All globules with ascent velocities greater than x/t will have risen completely from the lowest region of the emulsion to a point above the zone leaving the zone with a reduced concentration of oil. Similarly, after time t_1, the concentration of oil within the zone is further reduced due to the complete disappearance of all globules with a velocity greater than x/t_1.

If the change in concentration within the zone is measured at suitable time intervals and the equation

$$d_{st} = \sqrt{\frac{18\eta x}{g(\rho_1 - \rho_2)t}} \quad .. \quad .. \quad .. \quad .. \quad (8)$$

where d_{st} = diameter of globules; η = viscosity of continuous phase (0·009621 poise); x = height of globule ascent 25·952 cm); ρ_1 = density of dispersed phase (0·8797 g/cc); ρ_2 = density of continuous phase (0·99602 g/cc); t = time of globule ascent; g = acceleration due to gravity, derived from Stokes' law is used to calculate the sizes of droplets which have risen above the capacitance zone at these times, a cumulative weight undersize curve may be constructed from the results; from this a weight distribution of globule sizes may be obtained.

In practice, a cell with such a narrow capacitance zone would be insensitive to small changes in emulsion composition. To increase the sensitivity therefore, a cell with a wider capacitance zone was employed. This does not affect the above argument except that since each droplet now spends a significant proportion of its total ascent time within the capacitance zone, its contribution to capacitances does not fall instantaneously but decreases rapidly to zero as it rises through the zone In the following experiments, the distance x was taken to be the distance from the bottom of the cell to a point midway between the capacitance zone AB, i.e. to the point in the zone where the globules spend their average time. The error introduced by this step was expected to be small (see Seager, 1966).

Examples of results obtained for an emulsion of liquid paraffin in water are given in Table 1. The cumulative weight undersize and weight distribution curves for the same emulsion are shown in Fig. 4.

TABLE 1. RESULTS FOR AN EMULSION OF LIQUID PARAFFIN IN WATER

Time		Zone capacitance (in μμf)	Dielectric constant of sample in zone	Composition of sample in zone			Diameter of globules leaving zone (μ)
min	sec			Volume fraction	Weight fraction	Weight undersize %	
0		29·84	74·44	0·0265	0·0233	100	—
4	35	29·84	74·44	0·0265	0·0233	100	120
8	8	29·85	74·55	0·0250	0·0220	94·3	90
13	27	29·89	75·01	0·0217	0·0191	81·9	70
18	18	29·92	75·41	0·0174	0·0153	65·7	60
26	22	29·96	76·02	0·0121	0·0106	45·1	50
41	11	30·01	76·62	0·00710	0·00625	26·8	40
73	14	30·03	77·00	0·0030	0·00264	11·3	30
166	26	30·05	77·27	0·00130	0·00114	4·91	20
292	55	30·06	77·31	0·00075	0·00066	2·83	15

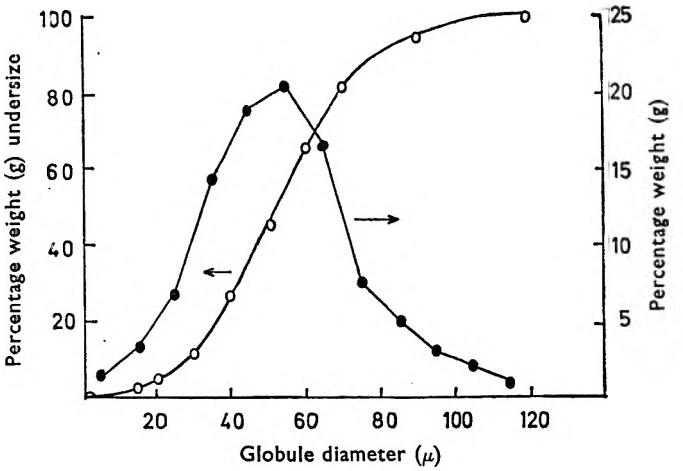

FIG. 4. Cumulative weight undersize curve (–○–) and weight distribution curve (–●–) for an emulsion of liquid paraffin in water stabilized by cetomacrogol 1000.

MICROSCOPIC MEASUREMENT OF GLOBULE SIZE

Size analysis by microscopy has been used for many years and while it is laborious, the results are often among the more satisfactory. It was therefore decided to compare the results obtained by the dielectric method with weight distribution curves obtained by microscopic measurement.

A sample of each emulsion was placed in a haemocytometer and photomicrographs of randomly chosen fields were taken concurrently with the determination of creaming rates. The magnification of all finished photographs was the same and the diameters of all globules were measured to 0·5 μ. In order to minimize the error which often occurs in the calculation of weight distribution curves from microscopic measurements (i.e. due to the accentuated effect of larger globules), at least 2000 globules were recorded for each emulsion.

Results and discussion

Six emulsions of different globule size distribution were prepared and the droplet size distributions obtained as described. The size distributions, expressed by weight, are shown in Fig. 5 along with the distributions obtained by microscopy. The agreement between the results from these two methods is excellent, showing that the dielectric method offers a simple and accurate alternative to microscopy for the droplet size grading of emulsions.

The systems used were coarser than many commercially produced emulsions. Coarse emulsions were used to avoid the complicating effects of Brownian motion and diffusion. Coarse emulsions also shortened the experimental time required to establish the correlation between the results obtained by dielectric constant measurement and those obtained by microscopy. With finer emulsions, much longer times would have been

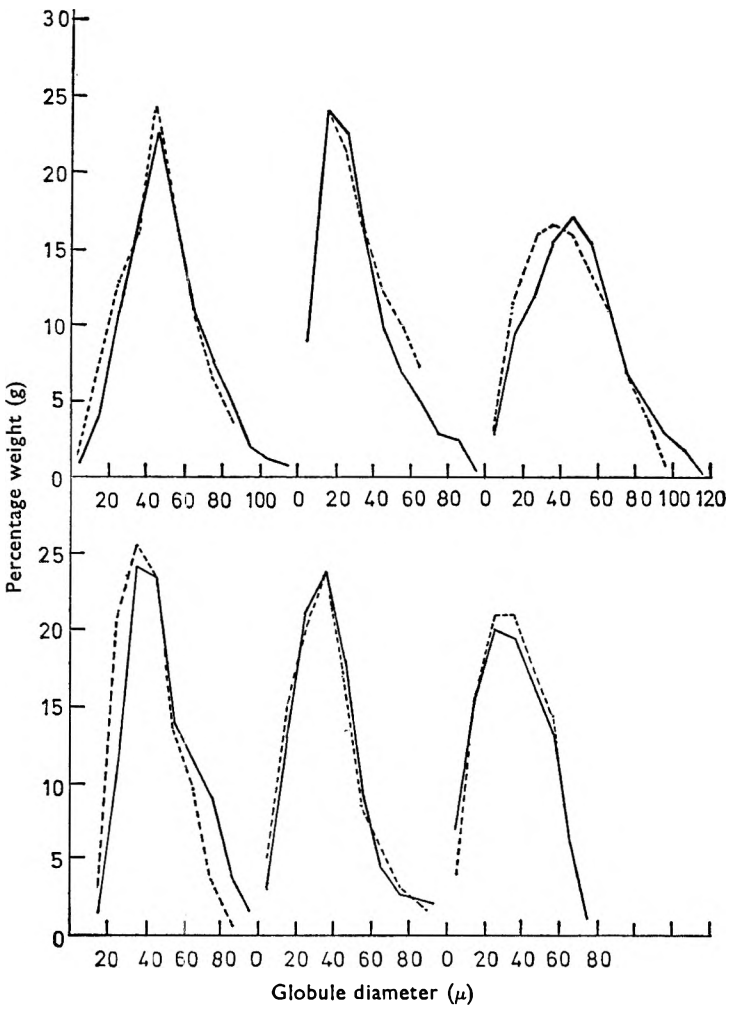

FIG. 5. Weight distribution curves for six different liquid paraffin-in-water emulsions. — Results by dielectric method. – – – Results by microscopy.

required to complete the experiments unless the time had been shortened by centrifugation.

One limitation of the method is that it is applicable only to emulsions of low electrical conductivity (Kaye & Seager, 1965).

The apparatus described is the subject of United Kingdom Patents Application No. 4885/64.

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Chemical and pharmacological observations on some *Hebe* species

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Examination of oven-dried leaves and twigs of four species of *Hebe* indigenous to New Zealand—*H. stricta*, *H. odora*, *H. bollonsii* and *H. corriganii*—has revealed D-mannitol and condensed tannins to be major constituents. Identification of some of the minor constituents, namely alkanes, fatty acids and aliphatic alcohols was achieved by means of gas-liquid chromatography. Pharmacological studies with the condensed tannin fractions of *H. stricta* and *H. odora* have indicated that the reputation of *H. stricta* as a constipatory agent is attributable to tannins; this agrees with earlier assumptions.

SOME of the larger-leaved shrubs of the genus *Hebe* (Family Scrophulariaceae)* have long enjoyed a medicinal reputation in New Zealand, the leaves and tender shoots being employed by the Maoris in the treatment of certain skin diseases (Goldie, 1905) and by both Maoris and Europeans for the alleviation of diarrhoea (e.g. Newman, 1879; Kesteven, 1880; Baber, 1886; Bell, 1890; Martindale & Westcott, 1898; Best, 1905; Beattie, 1920; Gardner, 1923; Wall & Cranwell, 1943; Brooker & Cooper, 1961). In the absence of any conclusive pharmacological studies, it has been generally assumed that the ability of these plants to arrest loose bowel movements could be attributed to the astringent action of tannins, although this view has not gone unchallenged (see Gardner, 1924). The total aqueous extracts from several *Hebe* species are without action against certain micro-organisms producing amoebic and bacillary dysentery (Professor L. H. Briggs, personal communication).

We have made a chemical and pharmacological examination of two *Hebe* species—viz. *H. stricta* (Benth.) L. B. Moore (which is one of several species included by earlier writers under the broad name *H. salicifolia* or *Veronica salicifolia*) and *H. odora* (Hook f.) Ckn. (syn. *H. buxifolia*)—in an attempt to isolate and characterize any active principles present. In addition a chemical investigation was made of two other species, *H. bollonsii* and *H. corriganii* (specimens of which were kindly supplied by Miss Lucy B. Moore) and the leaf alkane distribution patterns determined by gas-liquid chromatography as an extension of earlier work (Eglinton, Hamilton & Martin-Smith, 1962a) directed towards a possible chemotaxonomic differentiation of individual members of the genus, which is characterized by extreme ease of hybridization (Cockayne & Allan, 1934).

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* Botanical names as in H. N. Allan, *Flora of New Zealand*, Vol. 1, 1961, Wellington, Government Printer.

Materials and methods

ISOLATION PROCEDURE

The same method was employed for the isolation of the chemical constituents of the four species, *Hebe bollonsii*, *H. corriganii*, *H. odora* and *H. stricta*. Finely ground oven-dried twigs and leaves (80 g) were exhaustively extracted with 95% ethanol (200 ml) in a Soxhlet apparatus and the resulting solution evaporated to dryness under reduced pressure. Standard tests (Paech & Tracey, 1955) showed the absence of alkaloids in the solid residue in each case. Thorough extraction of each residue with redistilled light petroleum (b.p. 40–60°) gave material exhibiting -OH, C=O and $[CH_2]_4$ absorption in the infrared region.

Each residue, which was insoluble in light petroleum, was fractionally crystallized from 95% ethanol giving, as the least soluble fraction, crystalline material of m.p. 163–165°, which was undepressed on admixture with authentic D-mannitol. Infrared spectra (KCl discs) of the natural and authentic specimens were identical. Yields of D-mannitol (based on dry weight of plant material) were *H. stricta*, 0·25%; *H. odora*, 1·0%; *H. corriganii*, 3·7%; *H. bollonsii*, 2·9%. The bitter-tasting glassy residue remaining after removal of the D-mannitol and ethanol showed reactions characteristic of catechin-type condensed tannins. Each residue developed a pink tinge on prolonged exposure to air and was very hygroscopic (cf. Haworth, 1961), and all gave a deep green colouration with ferric chloride and afforded precipitates with gelatin solution, phenazone lead acetate and bromine water. On boiling with dilute hydrochloric acid phlobaphenes were formed, confirming the materials to be condensed tannins. Paper chromatography on Whatman No. 1 sheet and thin-layer chromatography on silica employing butanol-acetone-water showed the presence of several components, but individual components could not be obtained in crystalline form after column chromatography over paper rolls, silica gel, charcoal-kieselguhr or alumina pretreated with acetic acid. Application of standard colour tests (Campbell, 1959) indicated the absence of compounds of the chromone type and application of the Gibbs' test (1927) indicated the absence from the tannin fraction of phenols possessing a free *para* position.

ISOLATION OF ALKANES

In all instances the total alkane fraction, uncontaminated with compounds of other chemical groups, was isolated by the procedure described previously by Eglinton & others (1962a), and then subjected to gas-liquid chromatographic analysis on a 0·5% Apiezon L column. Identification of alkanes in the natural mixture was achieved through appropriate intensification experiments with added n-nonacosane and n-untriacontane followed by a plot of log retention time against carbon atom number. The resulting straight line plot (cf. James, 1960) then permitted identification of the remaining n-alkanes present. Percentages of individual components were obtained through integration of the areas under each peak

on the gas-liquid chromatographic traces of the natural mixtures. Repetition experiments indicated an accuracy of $\pm 2\%$.

ANALYSIS OF FATTY ACIDS AND n-ALKANOLS

In the cases of *H. odora*, *H. corriganii* and *H. bollonsii*, analyses were made of the total fatty acids and n-alkanols present without distinction between those occurring free and those occurring combined as esters. Glycerol was not detected in the products of saponification.

Total light petroleum solubles (1 g) from the particular *Hebe* species under investigation were refluxed for 2 hr in aqueous ethanol (1 : 2, 20 ml) containing sodium hydroxide (3 g). The solution was then evaporated to dryness under reduced pressure and the residue thoroughly extracted with dry ether. The combined ethereal solutions were taken to dryness under reduced pressure and the residue refluxed in acetic anhydride (5 ml) for 4 hr to convert the mixed alcohols into the corresponding acetates which were obtained free from coloured impurities by filtration in ethanol through neutral alumina and then subjected to gas-liquid chromatographic analysis on a 10% polyethyleneglycol adipate column.

Intensification experiments with added n-octyl acetate and n-decyl acetate and plots of log retention time against carbon atom number permitted identification of the individual components.

The ether-insoluble residue resulting from the aqueous ethanolic saponification of the light petroleum extractives from each plant was taken up in water (30 ml), and the solution acidified with dilute hydrochloric acid to liberate the free carboxylic acids from their sodium salts before being exhaustively extracted with ether. After removal of the solvent from the ethereal solution, the residue of mixed carboxylic acids was dissolved in methanol and treated with an excess of an ethereal solution of diazomethane. Removal of solvents under reduced pressure then afforded the methyl esters which were taken up in ethanol, filtered through a column of neutral alumina and subjected to gas-liquid chromatographic analysis on a 10% polyethyleneglycol adipate column.

Intensification experiments with added methyl laurate, methyl palmitate and methyl stearate and plots of log retention time against carbon atom number permitted identification of individual components.

GAS-LIQUID CHROMATOGRAPHY

The instrument employed was a standard Pye Panchromatograph, giving preheating of the argon carrier gas and fitted with standard glass tubes, containing the column packing, of 5 feet in length and internal diameter about $\frac{3}{16}$ inch. The detector was the standard Lovelock argon ionization type, fitted with a ^{90}Sr source and the current from the detector was fed into a Honeywell Brown (Newhouse, Lanarkshire, Scotland) pen recorder with sensitivity 0–10 mV.

Column packings were prepared on the silane-treated support Gas-Chrom Z in the manner described by Bryce, Martin-Smith, Osske, Schreiber & Subramanian (1966).

Pharmacology

In view of the difficulty in securing individual components of the tannin fractions in pure form, the pharmacological examination of *H. odora* and *H. stricta* necessarily had to be made on the crude fractions. Hence, although nitrogenous compounds were established as being absent, tests were included for local anaesthetic action, ability to mimic or antagonize acetylcholine on various preparations, ability to mimic or antagonize adrenaline and ability to antagonize histamine, barium chloride and dimethylphenylpiperazine iodide to ascertain that compounds other than tannins were not contributing to the activity—comparison of the overall results with the known pharmacological properties of tannins (e.g. Ware, 1926) making definite conclusion possible.

CONSTIPATORY EFFECT

The number of stools passed by healthy adult rats fed solutions (2 ml) of the tannin fraction (50 mg/ml) by stomach tube was compared statistically, using Student's *t*-test, with that produced by control animals given equal volumes of water,

GANGLION BLOCKING ACTIVITY

Sympathetic. The effect of previously (30 sec) administered aqueous solution of tannin fraction (10 mg/kg) via the external jugular vein on electrically-induced (12 V, 2 msec, 800/sec) contractions of the cat nictitating membrane elicited by supramaximal stimulation of the preganglionic sympathetic chain was compared with that of hexamethonium bromide (0·25 mg/kg).

Parasympathetic. The effect of adding a solution (0·16 mg/ml) of the tannin fraction 30 sec before the next stimulus to inhibit the peristaltic reflex in the guinea-pig ileum (Trendelenburg's experiment, Feldberg & Lin, 1949) was compared with that of hexamethonium bromide (0·05 mg/ml).

EFFECTS ON NON-VASCULAR INTESTINAL SMOOTH MUSCLE

Guinea-pig ileum. Reproducible submaximal contractions to the agonists acetylcholine chloride (0·1 μg/ml), histamine acid phosphate (0·5 μg/ml), barium chloride (100 μg/ml), and dimethylphenylpiperazine iodide (1·4 μg/ml), each acting for 30 sec were recorded. Solutions of total tannin fraction (0·5–5 mg/ml) were added 30 sec before the next addition of agonist.

Rat uterus. Reproducible submaximal contractions to acetylcholine chloride (0·1 μg/ml) acting for 30 sec were obtained. Solutions of tannin fraction (0·5 and 1·0 mg/ml) were added 30 sec before the next dose of agonist.

Rabbit duodenum. The effect of addition of tannin (0·3 mg/ml) solution on the spontaneous activity of the rabbit duodenum suspended in Locke's solution was observed and compared with that of papaverine hydrochloride (5 μg/ml).

VASCULAR SMOOTH MUSCLE

The isolated rat hindquarters preparation was perfused using the method of Burn (1952). Adrenaline hydrogen tartrate (2·5 μg) was injected into the abdominal aorta until a reproducible response was obtained. A solution of the tannin fraction (10 mg) was injected before the next dose of adrenaline hydrogen tartrate and the effect on the outflow recorded.

THE BLOOD PRESSURE OF THE ANAESTHETIZED CAT

The blood pressure of cats anaesthetized by intraperitoneal injection of sodium pentobarbitone (50–60 mg/kg) was recorded from a common carotid artery. The effect of a solution of tannin fraction (25 mg/kg) injected via the external jugular vein was recorded.

ISOLATED PERFUSED RABBIT HEART (Langendorff's method, 1895)

The effects of a solution of tannin fraction (8 mg) injected into the aorta, on heart rate, coronary flow and amplitude of contraction were noted.

FROG GASTROCNEMIUS MUSCLE-SCIATIC NERVE PREPARATION

The effect on contractions of the gastrocnemius muscle of the frog, elicited by indirect supramaximal electrical stimulation (4 V, 1–2 msec, 12/min) via the sciatic nerve, obtained by addition of a solution of the tannin fraction (2 ml of 50 mg/ml solution) to the bath was noted and compared with the inhibition of twitch height achieved by the addition of procaine hydrochloride (10 mg/ml) under identical conditions.

Results and discussion

The results of the alkane analyses of *H. bollonsii* and *H. corriganii* are summarized in Table 1 and in Fig. 1 which also shows earlier (Eglinton & others, 1962a) results obtained with the twigs and leaves of *H. odora*, *H. parviflora*, *H. diosmifolia* and *H. stricta* for comparison. It is to be noted that none of the species contain more than traces of branched alkanes (none being detected in *H. odora*, *H. corriganii* or *H. bollonsii*). It is of interest that *H. corriganii* and *H. bollonsii* possess very similar alkane distribution patterns, which might imply that the utility of plant alkane analysis as a method of "fingerprinting" individual species for chemotaxonomic and pharmacognostical purposes may prove to be more limited than had been originally hoped (Eglinton & others, 1962a, b, c).

TABLE 1. n-ALKANES FROM DIFFERENT *Hebe* SPECIES*

Plant	Total alkane fraction present (based on dry weight of leaves), %	Number of carbon atoms in alkane, %								
		C_{25}	C_{26}	C_{27}	C_{28}	C_{29}	C_{30}	C_{31}	C_{32}	C_{33}
Hebe corriganii ..	0·054	2	1	14	2	34	2	41	2	2
Hebe bollonsii ..	0·074	3	2	15	2	32	5	32	3	6

* The quantities of the individual alkanes are expressed as a percentage of the total alkanes isolated.

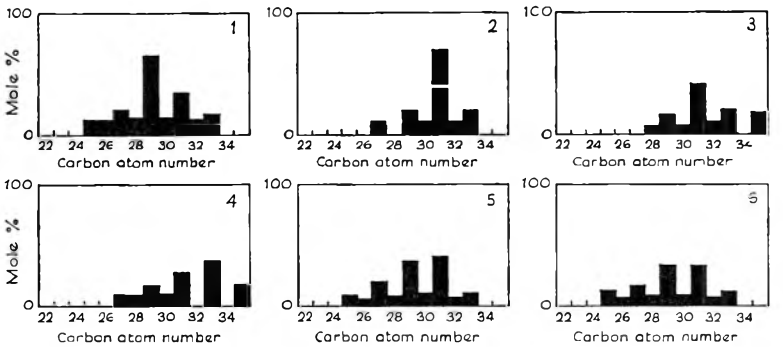

FIG. 1. Distribution in mole % of n-alkanes in six *Hebe* species. Twigs and leaves: 1. *H. odora*, 2. *H. parviflora*, 3. *H. diosmifolia*, 4. *H. stricta*. Leaves: 5. *H. corriganii*, 6. *H. bollonsii*.

The results of the fatty acid and n-alkanol analyses as shown in Tables 2 and 3 reveal little potential application for such analyses in chemotaxonomic differentiation of the genus *Hebe*. The total number of representatives in each series in no case exceeds five, with decan-1-ol the predominant alcohol in all cases and lauric acid the major acid except in *H. odora*.

TABLE 2. FATTY ACIDS FROM DIFFERENT *Hebe* SPECIES*

Plant	Total acid fraction present (based on dry weight of leaves), %	Capric (decanoic), %	Lauric (dodecanoic), %	Myristic (tetradecanoic), %	Palmitic (hexadecanoic), %	Stearic (octadecanoic), %
Hebe odora ..	—	32	28	22	12	8
Hebe corriganii	0·93	26	42	31	11	2
Hebe bollonsii ..	1·12	1	49	29	21	

* The quantities of the individual acids are expressed as a percentage of the total acids isolated.

TABLE 3. ALCOHOLS FROM DIFFERENT *Hebe* SPECIES*

Plant	Total alcohol fraction present (based on dry weight of leaves), %	Octan-1-ol %	Decan-1-ol %	Dodecan-1-ol %	Hexadecan-1-ol %
Hebe odora ..	—	24	46	19	14
Hebe corriganii	0·68	29	43	17	12
Hebe bollonsii ..	0·64	30	48	22	

* The quantities of the individual alcohols are expressed as a percentage of the total alcohols isolated.

Consistent results were obtained on repeat analyses to within ±2%. No unsaturated acids could be detected. All peaks on the gas-liquid chromatographic traces obtained with the mixed methyl esters fell on the one straight line when log retention time was plotted against carbon atom

number indicating that all resulted from the one (saturated) homologous series (James, 1960).

The short chain lengths of the acids (C_{10}–C_{18}) and alcohols (C_8–C_{16}) as compared to the chain lengths of the alkanes (C_{25}–C_{33}) are as to be expected in terms of current biogenetic theory in which it is considered (Eglinton & Hamilton, 1963) that at least one route leading to the formation of n-alkanes involves the coupling of two molecules of fatty acid before decarboxylation and reduction to the alkane, much as in the biogenesis of corynomycolic acid (Gastambide-Odier & Lederer, 1959).

In accord with previous observations (Chibnall, Piper, Pollard, Williams & Sahai, 1934; Waldron, Gowers, Chibnall & Piper, 1961; Eglinton & others, 1962b) in all the *Hebe* species, n-alkanes with an odd number of carbon atoms form the major components whilst the acids and alcohol appear restricted only to those with an even number of carbon atoms.

In keeping with the established astringent properties of tannins (Ware, 1926) the crude total tannin fractions from *H. odora* and *H. stricta* were without appreciable activity against acetylcholine or histamine on the guinea-pig ileum, but at a concentration of 0·625 mg/ml solutions of the crude fractions completely inhibited the effect of barium chloride (0·125 mg/ml) thus exhibiting a direct effect upon the intestinal musculature. This was also seen on the rabbit duodenum where an aqueous solution of the tannins (3 mg/ml) produced a slow strong relaxation without inhibiting spontaneous activity—an effect equivalent to that produced by 50 μg of papaverine.

The total crude tannins showed neither sympathetic nor parasympathetic ganglion blocking properties and had no adrenaline-like action on the isolated rabbit heart. Again these results are as to be expected for tannins. The apparent antagonism of the effect of acetylcholine on the rat uterus can also be ascribed to the expected direct action of tannins. Absence of inhibition of the contraction in the frog rectus abdominis muscle induced by acetylcholine and failure to block contraction in the frog gastrocnemius muscle sciatic nerve-preparation indicated the absence of any neuromuscular-blocking activity or local anaesthetic activity in the tannin fractions.

No statistically valid difference between the number of stools passed by healthy rats fed with solutions of the condensed tannins by stomach tube and the number of stools passed by control rats fed equal volumes of water could be discerned, but this test is not necessarily comparable to the loose bowel conditions for which extracts of *H. stricta* are efficacious. A further complication here was introduced by the necessity to oven-dry the plant material for purposes of preservation and transport and this heat process, coupled with the later hot solvent extraction of the ground dried plant, may have induced polymerization of simpler tannins (cf. Haslam, 1966). Indeed there have been conflicting reports over the efficacy of various extracts from *H. stricta* prepared by different methods (Baber, 1886; Bell, 1890; Gardner, 1923) and these would certainly be explicable in terms of the polymerization of the tannins present.

In view of the total pharmacological results, especially the absence of

any effect against nervously controlled intestinal movements and the marked potency against muscle spasm induced by barium chloride solution, the constipatory properties of *H. stricta* and *H. odora* can be explained in terms of their tannin content.

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An experimental approach to long-lasting hypotensive eledoisin-like peptides

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A new series of eledoisin-like peptides was synthesized with the object of obtaining long-lasting hypotensive drugs. Acyl residues were introduced into the molecule of the peptide

R
|
H–Lys–Phe–Ile–Gly–Leu–Met–NH_2

They were dissolved in some polar solvents (water, diacetin, triacetin, dimethylsulphoxide), and injected intramuscularly into either anaesthetized or unanaesthetized dogs. Comparison was made with eledoisin dissolved both in water and diacetin. Some of the peptides, mainly the butyryl and valeryl derivatives, in diacetin had a significantly longer-lasting action. The intensity of the hypotension could be also reduced, to avoid side-effects in unanaesthetized dogs, but it was not possible to make it gradual.

PREVIOUS reports (Bergamaschi & Glässer, 1963, 1964; Erspamer & Glässer, 1963; Fregnan & Glässer, 1966) have strongly supported the view that eledoisin exerts its hypotensive action in the dog by a direct vasodilating mechanism on the vascular smooth muscle. Clinical trials (Sicuteri, Fanciullacci, Franchi & Michelacci, 1963; Gersmeyer, Castenholz & Nicolay, 1965) were also successful in indicating that the endecapeptide is a powerful hypotensive vasodilator even in man. Unfortunately its action is short-lasting and abrupt when administered either intramuscularly or intravenously. Synthetic eledoisin-like peptides behaved similarly (Bernardi, Bosisio, Chillemi & others, 1964, 1965). Stürmer & Fanchamps (1965) were able to prolong the hypotension due to eledoisin by the use of a suitable vehicle (Depot-Präparat). We now report on the possibility of prolonging the hypotensive action of some eledoisin-like peptides and making it gradual in onset and disappearance. We felt that by suitable introduction of hydrophobic residues into the molecule of these peptides their distribution between hydrophylic and hydrophobic media could be varied and accordingly the absorption through the tissues could be modified.

For this purpose the hexapeptide Lys-Phe-Ile-Gly-Leu-Met-NH_2 was chosen because it was found to be almost as active as eledoisin and it had two free amine groups suitable for substitution. Lipophilic acyl substituents of different size were introduced into the ω-amino-group of lysine. The α-amino-group of lysine was kept free to provide a hydrophilic residue in the same molecule to influence, almost at will, the lipophilic-hydrophilic properties and solubility of these peptides.

The activity of the new peptides has been compared on a weight basis with that of eledoisin either in diacetin or water.

Experimental

CHEMICAL (de Castiglione)

The appropriate *N*α-t-butyloxycarbonyl-*N*ϵ-acyl-L-lysine was converted into the corresponding *p*-nitrophenyl ester and condensed with

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the pentapeptide L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methionin-amide (de Castiglione, 1965) to afford the protected *N*ε-acyl-hexapeptide. This was treated with hydrogen chloride in glacial acetic acid to eliminate the *N*α-t-butyloxycarbonyl group, and the free base was obtained by exchange with Amberlite IRA-410 (OH cycle).

N*α-t-Butyloxycarbonyl*-N*ε-acyl*-L-*lysine* (Table 1). To an ice-cold solution of *N*α-t-butyloxycarbonyl-L-lysine (Schwyzer, Costopanagiotis

TABLE 1. *N*α-t-BUTYLOXYCARBONYL-*N*ε-ACYL-L-LYSINE

Acyl group	Yield (%)	m.p. °C	$[\alpha]_D^{23}$	Formula	Found C	Found H	Found N	Required C	Required H	Required N
Butyryl ..	45	99–100	−16·4*	$C_{15}H_{28}N_2O_5$	56·7	8·9	8·9	56·9	8·9	8·8
Valeryl ..	40	86– 90	− 3·5†	$C_{16}H_{30}N_2O_5$	58·0	9·0	8·5	58·2	9·2	8·5
Caproyl ..	49	82– 84	−14·4*	$C_{17}H_{32}N_2O_5$	59·2	9·3	8·2	59·3	9·4	8·1
Cyclopentyl-propionyl ..	75	118–119	− 2·9†	$C_{19}H_{34}N_2O_5$	61·7	9·35	7·5	61·6	9·2	7·6
Cinnamyl ..	38	106–107	−12·6*	$C_{20}H_{28}N_2O_5$	63·7	7·4	7·7	63·8	7·5	7·4
Benzoyl ..	69	114–116	− 2·8†	$C_{18}H_{26}N_2O_5$	62·0	7·50	8·0	61·7	7·5	8·0
Nicotinyl ..	18	138–141	−15·5*	$C_{17}H_{25}N_3O_5$	57·9	7·45	11·8	58·1	7·2	12·0
1-Adamantane-carbonyl ..	71‡	187–188‡	+ 3*‡	$C_{22}H_{36}N_2O_5.C_{12}H_{23}N$	69·1	9·8	7·0	69·2	10·1	7·1

* *c*, 1 in *NN*-dimethylformamide.
† *c*, 1 in methanol.
‡ As dicyclohexylamine salt.

& Sieber, 1963) (10 mmole) in N sodium hydroxide (10 mmole) and water (80 ml) were added dropwise and separately, with vigorous stirring, a solution of acyl chloride (10 mmole) in anhydrous tetrahydrofuran (10 ml) and N sodium hydroxide (10 ml) over 20 min. The stirring was continued for 20 min at room temperature. The reaction mixture was then saturated with sodium chloride, cooled below 0° and acidified at pH ~1 with 2N hydrochloric acid. The product was extracted with ethyl acetate and the combined extracts were washed with saturated sodium chloride solution, dried over anhydrous sodium sulphate, and the solvent evaporated *in vacuo* at 40°. Crystallization from ether-light petroleum (b.p. 40–60°) or acetone-light petroleum afforded the pure material.

For the nicotinyl derivative, a solution of *N*α-t-butyloxycarbonyl-L-lysine (15 mmole) in N sodium hydroxide (45 ml) was vigorously shaken with a mixture of crushed ice and nicotinyl chloride (15 mmole). After 1 hr at room temperature the reaction mixture was saturated with sodium chloride and treated with N hydrogen chloride (15 ml). The product was then worked up as before.

p-*Nitrophenyl* N*α-t-butyloxycarbonyl*-N*ε-acyl*-L-*lysinate* (Table 2). *NN*-Dicyclohexylcarbodiimide (5 mmole) was added to an ice-cold solution of *N*α-t-butyloxycarbonyl-*N*ε-acyl-L-lysine (5 mmole) and *p*-nitrophenol (5·5 mmole) in ethyl acetate (15 ml). The reaction mixture was allowed to stand 1 hr at 0° and 2 hr at room temperature. The dicyclohexylurea was filtered after cooling and washed with ethyl acetate. The filtrate was successively washed with N hydrochloric acid (at a temperature below 0°), 5% sodium bicarbonate and saturated sodium chloride solutions. After drying over anhydrous sodium sulphate the

TABLE 2. *p*-NITROPHENYL-*N*α-t-BUTYLOXYCARBONYL-*N*ε-ACYL-L-LYSINATE

Acyl group	Yield (%)	m.p. °C	$[\alpha]_D^{23}$	Formula	Found C	Found H	Found N	Required C	Required H	Required N
Butyryl ..	66	119–120	−29·8*	$C_{21}H_{31}N_3O_7$	57·6	7·1	9·6	57·6	7·1	9·6
Valeryl ..	80	119–120	−30·8†	$C_{22}H_{33}N_3O_7$	58·3	7·4	9·2	58·5	7·4	9·3
Caproyl ..	78	119–121	−26·8*	$C_{23}H_{35}N_3O_7$	58·9	7·6	9·0	59·3	7·6	9·0
Cyclopentyl-propionyl ..	77	150	−28†	$C_{25}H_{37}N_3O_7$	61·1	7·6	8·5	61·1	7·6	8·5
Cinnamyl ..	70	159–160	−22*	$C_{26}H_{31}N_3O_7$	62·4	6·5	8·5	62·8	6·3	8·4
Benzoyl ..	75	140–142	−27†	$C_{24}H_{29}N_3O_7$	61·3	6·3	8·8	61·1	6·2	8·9
Nicotinyl ..	41	109–111	−26·3*	$C_{23}H_{28}N_4O_7$	58·4	6·1	11·9	58·5	6·0	11·9

* *c*, 1 in *NN*-dimethylformamide.
† *c*, 1 in methanol.

solvent was evaporated *in vacuo* and the residue crystallized from acetone-light petroleum (b.p. 40–60°).

For the nicotinyl derivative, *NN*-dimethylformamide was used as solvent instead of ethyl acetate, and no acidic washings were made. The 1-adamantanecarbonyl derivative was isolated as an oil and used as such in the next reaction.

TABLE 3. *N*α-t-BUTYLOXYCARBONYL-*N*ε-ACYL-L-LYSYL-L-PHENYLALANYL-L-ISO-LEUCYL-GLYCYL-L-LEUCYL-L-METHIONINAMIDE

Acyl group	Yield (%)	m.p. °C	$[\alpha]_D^{23}$*	Formula	Found C	Found H	Found N	Found O	Required C	Required H	Required N	Required O
Butyryl ..	92	257–259	−29·5	$C_{53}H_{73}N_9O_9S.\frac{1}{2}H_2O$	58·5	8·2	12·5	17·3	58·3	8·3	12·6	17·1
Valeryl ..	85	~260	−29·3	$C_{54}H_{74}N_9O_9S$	59·4	8·5	12·3		59·4	8·3	12·5	
Caproyl ..	74	258–259	−28·7	$C_{55}H_{76}N_9O_9S$	59·5	8·6	12·3		59·6	8·5	12·4	
Cyclopentyl-propionyl ..	72	263–266	−27·2	$C_{57}H_{76}N_9O_9S$	60·9	8·6	12·0		60·6	8·4	12·0	
Cinnamyl ..	68	~260	−28·6	$C_{58}H_{72}N_9O_9S$	61·7	8·0	11·7		61·5	7·7	12·0	
Benzoyl ..	77	256–258	−29·3	$C_{56}H_{70}N_9O_9S$	60·9	8·1	11·9		60·6	7·7	12·3	
Nicotinyl ..	55	258–260	−28	$C_{55}H_{69}N_{10}O_9S.\frac{1}{2}H_2O$	58·7	7·8	13·8		58·7	7·7	13·7	
1-Adamantane-carbonyl ..	69	252–254	−25·5	$C_{60}H_{80}N_9O_9S.\frac{1}{2}H_2O$	61·6	8·3	11·3	15·8	61·4	8·4	11·4	15·5

* *c*, 1 in *NN*-dimethylformamide.

N*α-t-Butyloxycarbonyl*-N*ε-acyl*-L-*lysyl*-L-*phenylalanyl*-L-*isoleucyl-glycyl*-L-*leucyl*-L-*methioninamide* (Table 3). A solution of *p*-nitrophenyl-*N*α t-butyloxycarbonyl-*N*ε-acyl-L-lysinate (1 mmole) and L-phenylalanyl-L-isoleucyl-glycyl-L-leucyl-L-methioninamide (1 mmole) in anhydrous *NN*-dimethylformamide (10 ml) was allowed to stand 5 days at 35°. The solution was then concentrated *in vacuo* and taken up with water. The product was filtered, dried, washed with ether and recrystallized from methanol.

N*ε-Acyl*-L-*lysyl*-L-*phenylalanyl*-L-*isoleucyl-glycyl*-L-*leucyl*-L-*methionin-amide*. *N*α-t-Butyloxycarbonyl-*N*ε-acyl-L-lysyl-L-phenylalanyl-L-iso-leucyl-L-glycyl-L-leucyl-L-methioninamide (0·4 mmole) was treated for 20 min at 25° with 1·33 N dry hydrogen chloride solution in glacial acetic acid (15 ml). The solvent was evaporated *in vacuo* and the residue was treated with anhydrous ether. The hydrochloride salt was dissolved in dioxane–water (1 : 1), and the solution was applied to an Amberlite IRA-410 column (OH cycle) which was eluted with dioxane–water

(1 : 1). The product, obtained after evaporation *in vacuo* at 40°, was dissolved in methanol and precipitated by addition of ether.

The physical constants of these products have already been reported (compare Bernardi & others, 1965). The new 1-adamantanecarbonyl derivative (95% yield) has m.p. 222–223°; $[\alpha]_D^{23}$ –17° (*c* 1, in acetic acid 95%); $E_{1,9} = 0{\cdot}42$ Leu. Required for $C_{45}H_{72}N_8O_7S{\cdot}H_2O$: C, 61·2; H, 8·4; N, 12·4; O, 14·7. Found: C, 60·9; H, 8·4; N, 12·6; O, 14·4.

PHARMACOLOGICAL (Fregnan and Glässer)

Beagle dogs of either sex (10–15 kg), were anaesthetized intravenously with 35 mg/kg of pentobarbitone sodium. Anaesthesia was maintained with additional doses as needed. After endotracheal intubation the animals were maintained under artificial respiration by a Starling Ideal pump.

Arterial blood pressure was measured from a cannulated carotid artery and recorded on a kymograph either by a mercury manometer or by an Elema electromanometer connected to a galvanometer. The pressure variations were expressed in mm Hg as per cent variation over the control values.

A few experiments were also run on unanaesthetized dogs in which the blood pressure was measured through a cannula chronically implanted into the caudal artery.

All drugs were dissolved, whenever possible, both in distilled water and diacetin, and injected into the medial group of the muscles of the thigh. Some drugs were also dissolved in triacetin and in dimethylsulphoxide (DMSO). Each dose was tested on at least 4 animals and always checked against eledoisin (10 μg i.m. in water).

Results

PHARMACOLOGICAL ASSAY

Table 4 summarizes the pharmacological results. Eledoisin dissolved either in water or in diacetin always caused an abrupt systemic hypotension with a peak effect proportional to the dose but with almost the same duration within the dose range studied. However, the solutions of eledoisin in diacetin were better than those in water, because they caused a weaker but somewhat longer-lasting hypotension in either anaesthetized or unanaesthetized dogs. The vascular effect due to the parent unacylated hexapeptide (soluble in water and not in diacetin) was identical in intensity to that of eledoisin. All the acyl derivatives (more soluble in diacetin than in water) caused a less marked but longer-lasting fall in blood pressure. The compounds containing the fatty acyl residues, mainly the butyryl and valeryl derivatives, were the best ones when injected into either anaesthetized or unanaesthetized dogs. As can be seen in Table 4 and in Figs 1–3, the two substances acted longer when dissolved in diacetin than in water. In fact, in diacetin the decrease in blood pressure was less marked (from −25 to −29 mm Hg against −60 mm Hg) but the hypotension lasted at least twice as long,

TABLE 4. PHARMACOLOGICAL RESULTS

Drugs	Vehicle	Dose μg/kg i.m.	Hypotension in dogs: Anaesthetized: % change mean arterial pressure: mmHg	Anaesthetized: % change mean arterial pressure: After min	Anaesthetized: Average duration min: 50% recovery	Anaesthetized: Average duration min: 100% recovery	Unanaesthetized: % change mean arterial pressure: mmHg	Unanaesthetized: % change mean arterial pressure: After min	Unanaesthetized: Average duration min: 50% recovery	Unanaesthetized: Average duration min: 100% recovery	Side-effects
Pyr-Pro-Ser-Lys-Asp(OH)-Ala-Phe-Ile-Gly-Leu-Met-NH_2 (Eledoisin)	Water	10	−36	3	32	70	−28	10	40	75	None
	"	30	−80	10	35	90	−60	15	60	110	Vomiting, diarrhoea, tachycardia
	Diacetin	10	−20	3	70	105					
	"	20	−55	12	50	100					
	"	30	−65	12	35	>90					
R | H–Lys–Phe–Ile–Gly–Leu–Met–NH_2											
R = hydrogen	Water*	10	−35	2	10	30					
R = butyryl	Water	1	−10	3	7	15					
	"	10	−60	2	30	80					
	Diacetin	30	−25	6	92	>200	−24	15	65	150	None
	"	60	−32	10	>90						
R = valeryl	Water	10	−60	2	27	70					
	Diacetin	10	−9	3	30	55					
	"	30	−29	4	75	115†	−25	15	65	140	None
	Triacetin	30	−54	3	21	60					
	DMSO	30	−57	2	25	70					
R = caproyl	Diacetin	30	−16	10	33	75	−15	15	80	145	None
	"	60	−28	3	63	128					
R = cyclopentyl-propionyl	Diacetin	30	Inactive								
	"	60	−20	2	8	25	Inactive	120			None
	"	200	−40	2	23	45					
	DMSO	30	−24	5	44	100					
R = cinnamyl	Diacetin	30	−12	1	3	5					
	"	60	−20	4	63	100	Inactive	120			None
R = benzoyl	Diacetin	30	Inactive								
	"	60	−25	2	50	120					
R = nicotinyl	Diacetin	30	−37	5	12	60‡	−50	4	45	110	Vomiting, diarrhoea, tachycardia
R = 1-adamantane-carbonyl	Diacetin	30	−19	5	10	60					

* This compound was not soluble in diacetin at the concentrations used and it could not be tested in this solvent.

† The blood pressure did not completely recover in 2 dogs after 130 min when the recording was discontinued.

‡ The blood pressure did not completely recover in 1 dog after 230 min when the recording was discontinued.

The activity of the peptides is compared on a weight basis.

and no substantial side-effects were evoked in unanaesthetized dogs. By doubling the dose of the butyryl derivative the maximal fall in arterial pressure of anaesthetized dogs did not vary too much, being −32 mm Hg for 60 μg/kg against −25 mm Hg for 30μg/kg. Other solvents beside water and diacetin were also tried, e.g. the valeryl derivative was dissolved in triacetin and DMSO. In this instance, these two vehicles did

not behave differently from water. But the cyclopentylpropionyl derivative in DMSO seemed to be more suitable than in diacetin: while in diacetin the compound was almost inactive and short-lasting, in DMSO it caused a moderate fall in arterial pressure (−24 mm Hg) which lasted for about 100 min.

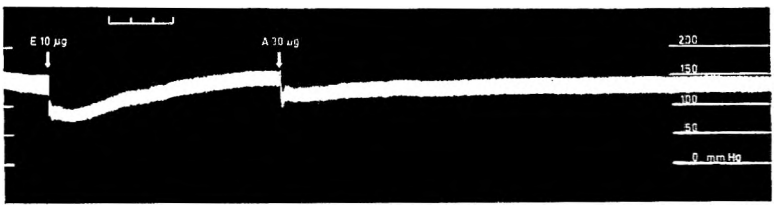

FIG. 1. Carotid blood pressure in an anaesthetized dog measured by a mercury manometer. Hypotension following intramuscular injection of eledoisin in water (E: 10 µg/kg) and butyryl derivative in diacetin (A: 30 µg/kg). Time intervals = 10 min.

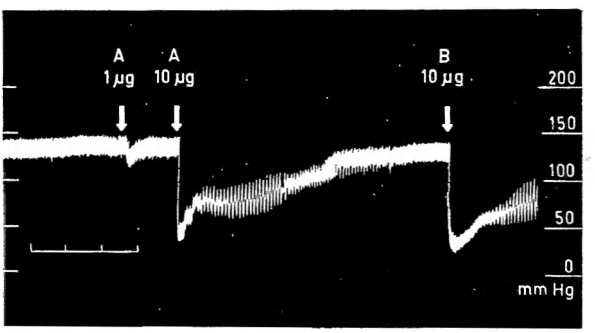

FIG. 2. Carotid blood pressure in an anaesthetized dog measured by a mercury manometer. Hypotension following intramuscular injections of the butyryl (A) and valeryl (B) derivatives in water. Doses in µg/kg. Time intervals = 10 min.

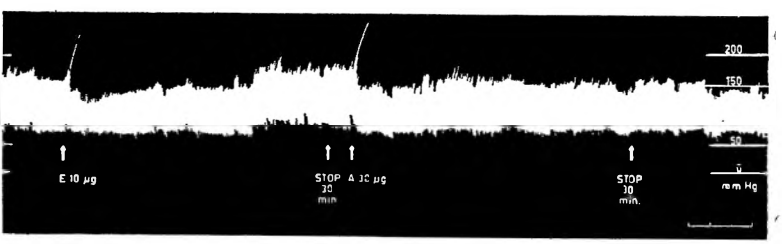

FIG. 3. Blood pressure measured from the caudal artery of an unanaesthetized dog by an electromanometer connected to a galvanometer. Hypotension following intramuscular injections of eledoisin (E) and butyryl derivative (A). Doses in µg/kg. Time intervals = 10 min.

Discussion

Eledoisin and all the eledoisin-like peptides so far known cause a sudden fall in arterial pressure which is short-lasting, possibly because of their rapid metabolism. On the other hand, the period of hypotension

cannot be prolonged to make it useful clinically, by simply increasing the dose. At higher doses the fall in blood pressure becomes more and more marked while the duration of this effect is not always proportional to the dose. In addition, abrupt and strong hypotensions stimulate the reflex mechanisms to restore normal conditions, and they might also evoke numerous side-effects (vomiting, diarrhoea, and tachycardia) particularly evident in unanaesthetized animals.

The problem is how to make the hypotension caused by eledoisin and eledoisin-like peptides gradual in onset and of sufficient duration without evoking side-effects.

Sturmer & Fanchamps (1965) claimed to have prolonged the depressor effect of eledoisin (Depot-Präparat) and to have made it gradual. We also found that it was possible to reduce the intensity of the hypotension and to prolong it, both by suitable changes in the molecule of the peptides and by the use of an appropriate solvent. Among the compounds tested the butyryl and valeryl derivatives dissolved in diacetin gave the best performance. The choice of an appropriate solvent for a given peptide seemed also to be important. The valeryl derivative was dissolved in water, diacetin, triacetin, and DMSO to study the influence of the vehicle. The results showed that diacetin was the best solvent for this drug; the blood pressure did not fall too much and the hypotension lasted for several hours. Triacetin and DMSO were no better than water. Diacetin also prolonged the action of eledoisin but did not significantly reduce the intensity of the hypotension to make it useful for therapeutic trials. In contrast, diacetin was useless for the cyclopentylpropionyl derivatives from a pharmacological point of view. In this instance, DMSO was more suitable.

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Synthesis and antiprotozoal activity of some nitro(nitroaryl)imidazoles

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A series of 5(4)-nitro-4(5)-nitroarylimidazoles has been synthesized and their *in vitro* antifungal and antiprotozoal activities have been studied. The compounds may be prepared by dinitration of an arylimidazole or by mononitration of either an arylnitroimidazole or a (nitroaryl)imidazole. In some of the compounds the ring nitrogen atom has been substituted. Several compounds exhibit high *in vitro* antitrichomonal activity against *Trichomonas vaginalis* and *T. foetus*, and a moderate activity against *Histomonas meleagridis* and *Entamoeba histolytica*. The chemical structure and antimicrobial activity of these and other imidazoles previously studied are briefly discussed.

In earlier papers we have shown that some arylimidazoles possess high *in vitro* antimicrobial action (Ellis, Epstein, Fitzmaurice, Golberg & Lord, 1964a,b); several compounds were fungicidal towards pathogenic organisms but introduction of a nitro-group into the imidazole ring largely destroyed this activity and simultaneously bestowed on some of the compounds very high antitrichomonal properties. The work is now extended to examine arylimidazoles containing two nitro-groups, one in each ring. Few representatives of this type of compound are known; the first such compound to be described was 4(5)-nitro-5(4)-*p*-nitrophenylimidazole (Grant & Pyman, 1921). Its two isomeric 1-methyl derivatives were prepared later (Hazeldine, Pyman & Winchester, 1924).

Most of the dinitro-compounds now described were prepared (see Fig. 1) from either the un-nitrated or mononitrated imidazoles but introduction of a substituent on the ring nitrogen atom of a nitro(nitroaryl)-imidazole also provided a useful route in some instances, for example, by reaction with an alkyl halide or sulphate or by an addition reaction with an activated alkene. Some *N*-2-cyanoethyl and *N*-pyrid-2-ylethyl compounds prepared by this method were described earlier (Ellis & others, 1964a). The reactions of but-1-en-3-one (methyl vinyl ketone) and 2-vinylpyridine with nitro(nitroaryl)imidazoles are described but no attempt was made to find optimum conditions for these reactions. Mononitration of an arylimidazole usually gives a mixture of nitroaryl- and nitro-imidazole derivatives (Ellis & others, 1964b); further nitration of either of these produces the same dinitro-compound, which usually has a lower water-solubility than its precursors.

The orientation of the nitro-group in the benzene ring of the 4-chloro-2- and -3-nitrophenyl compounds was determined by oxidation of the dinitro-compound with alkaline potassium permanganate to the known substituted benzoic acid. This was unnecessary for 4-(4,5-dichloro-2-nitrophenyl)-1-methyl-5-nitroimidazole, since the constitution of 4-(4,5-

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dichloro-2-nitrophenyl)-1-methylimidazole was established (Ellis & others, 1964a) by synthesis from 4,5-dichloro-2-nitroacetophenone (Keneford & Simpson, 1947).

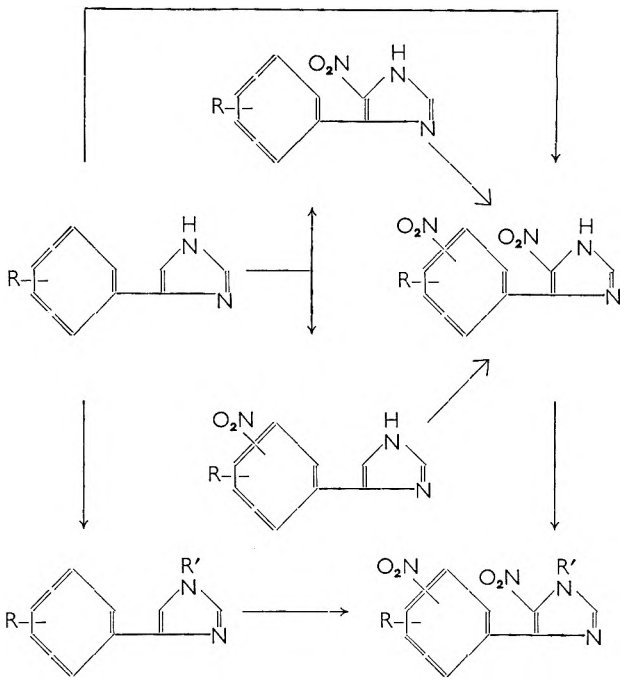


FIG. 1. The synthesis of nitro(nitroaryl)imidazoles.

Experimental

CHEMICAL

General methods. Two methods of nitration (Ellis & others, 1964b) were used: in *method A* the nitrate salt of the imidazole was treated with sulphuric acid; in *method B* the imidazole was heated for up to 2 hr in concentrated sulphuric acid with finely powdered sodium nitrate. The reaction mixture was poured into ice water, and the precipitate was filtered off and recrystallized from a suitable solvent.

4(5)-(4-*Chloro*-3-*nitrophenyl*)-5(4)-*nitroimidazole.* (*a*) 4(5)-*p*-Chlorophenyl-5(4)-nitroimidazole (10 g) (Ellis & others, 1964b) was nitrated by method B to give the colourless *dinitro-compound* (10 g), m.p. 223° (from acetone).

(*b*) Nitration of 4(5)-(4-chloro-3-nitrophenyl)imidazole (0·7 g) by method B gave the *dinitro-compound* (0·6 g), m.p. and mixed m.p. with sample from (*a*), 222° (from acetone).

4-(4-*Chloro*-3-*nitrophenyl*)-1-*methyl*-5-*nitroimidazole.* (*a*) 4-*p*-Chlorophenyl-1-methyl-5-nitroimidazole (11·9 g) on nitration by method B gave

very pale yellow needles (12 g), m.p. 122–125°, of the *dinitro-methylimidazole*; this, on oxidation with aqueous alkaline potassium permanganate, yielded 4-chloro-3-nitrobenzoic acid which did not depress the m.p. of an authentic sample.

(*b*) 4(5)-(4-Chloro-3-nitrophenyl)-5(4)-nitroimidazole (8·4 g) was heated at 100° for 30 min with dimethyl sulphate (3 ml). The reaction mixture was treated with aqueous sodium carbonate solution and extracted with chloroform (2 × 100 ml). The extract was dried and evaporated to quarter bulk. Addition of light petroleum (b.p. 40–60°) (200 ml) precipitated the *dinitro-methylimidazole* (4 g), m.p. and mixed m.p. with the product obtained in (*a*), 122–123° (from ethanol).

4(5)-(4-*Chloro*-2-*nitrophenyl*)-5(4)-*nitroimidazole*. 4(5)-(4-Chloro-2-nitrophenyl)imidazole (20 g) was nitrated by method A to give the yellow *dinitro-compound* (16·2 g), m.p. 241–243° (from ethanol). Oxidation of a sample with alkaline potassium permanganate gave 4-chloro-2-nitrobenzoic acid, which did not depress the m.p. of an authentic sample.

4(5)-(4,5-*Dichloro*-2-*nitrophenyl*)-5(4)-*nitroimidazole*. (*a*) 4(5)-(4,5-Dichloro-2-nitrophenyl)imidazole (1·8 g) gave, on nitration by method A, the *dinitro-compound* as yellow crystals (1·5 g), m.p. 220–221° (from aqueous ethanol).

(*b*) 4(5)-(3,4-Dichlorophenyl)-5(4)-nitroimidazole (6·8 g) by similar treatment yielded the *dinitro-compound* (6 g), m.p. and mixed m.p. with the product from (*a*), 219°.

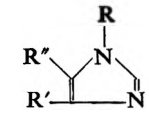
4-(4,5-*Dichloro*-2-*nitrophenyl*)-1-*methyl*-5-*nitroimidazole*. (*a*) 4(5)-(4,5-Dichlorophenyl)-1-methylimidazole (9·6 g), when nitrated with a large excess of sodium nitrate (7·5 g) by method B, gave the *dinitro-methylimidazole* (3·7 g) as almost colourless crystals, m.p. 148° (from ethanol).

(*b*) 4(5)-(4,5-Dichloro-2-nitrophenyl)-5(4)-nitroimidazole (7·7 g) was heated at 100° for 0·5 hr with dimethyl sulphate (2·4 ml). Hot sodium carbonate solution was added to the reaction mixture, which was then extracted with chloroform (2 × 100 ml). The extract was dried, evaporated to 50 ml and light petroleum (b.p. 40–60°) (200 ml) was added, whereupon an oil separated. When this was treated with hot ethanol (30 ml) and cooled, crystals of the *dinitro-methylimidazole* (2 g), m.p. and mixed m.p. with sample from (*a*), 148° (from ethanol), were deposited.

(*c*) 4-(3,4-Dichlorophenyl)-1-methyl-5-nitroimidazole (4·1 g) was nitrated by method B to give the *dinitro-methylimidazole* (2 g), m.p. and mixed m.p. with sample from (*a*), 148°.

Ethylation of 4(5)-(4,5-*dichloro*-2-*nitrophenyl*)-5(4)-*nitroimidazole*. The title compound (10 g) was refluxed in acetone (250 ml) for 20 hr with ethyl iodide (5·1 g) and potassium carbonate (2·2 g). The reaction mixture was cooled, filtered and evaporated to dryness. Fractional crystallization of the residue from ethyl acetate gave 4-(4,5-dichloro-2-nitrophenyl)-1-ethyl-5-nitroimidazole (2·2 g), m.p. and mixed m.p. with product of the

TABLE 1. NITRO(NITROARYL)IMIDAZOLES



Compound No.	R	R′	R″	m.p. °C	Formula	Analysis							
						Found				Required			
						C	H	Cl	N	C	H	Cl	N
1	H[a]	NO_2	4-Chloro-2-nitrophenyl	241–243	$C_9H_6ClN_4O_4$	40·3	1·9	13·4	21·1	40·2	1·9	13·2	20·8
2	H[a]	NO_2	4-Chloro-2-nitrophenyl	225	$C_9H_6ClN_4O_4$	40·4	2·0	13·4	20·9	40·2	1·9	13·2	20·8
3	H[a]	NO_2	4,5-Dichloro-2-nitrophenyl	221	$C_9H_5Cl_2N_4O_4$	36·0	1·5	24·0	19·0	35·6	1·3	23·4	18·5
4	H[a]	NO_2	3,4,6-Trichloro-2-nitrophenyl	224–225	$C_9H_4Cl_3N_4O_4$	32·3	1·0	32·2	—	32·0	0·9	31·6	—
5	H[a]	NO_2	2,3,4-Trichloro-6-nitrophenyl	168–169	$C_9H_4Cl_3N_4O_4$	32·4	0·9	32·3	—	32·0	0·9	31·6	—
6*b*	Me	4-Nitrophenyl	NO_2	215–216	$C_{10}H_8N_4O_4$	48·2	3·4	—	22·5	48·4	3·2	—	22·6
7	Me	4-Chloro-3-nitrophenyl	NO_2	122–125	$C_{10}H_7ClN_4O_4$	42·5	2·6	12·4	19·3	42·5	2·5	12·5	19·8
8	Me	4,5-Dichloro-2-nitrophenyl	NO_2	148	$C_{10}H_6Cl_2N_4O_4$	38·0	1·8	—	17·4	37·8	1·9	—	17·6
9	Et	NO_2	4,5-Dichloro-2-nitrophenyl	188–189	$C_{11}H_8Cl_2N_4O_4$	40·3	3·0	—	17·4	39·9	2·4	—	16·9
10	Et	4,5-Dichloro-2-nitrophenyl	NO_2	158–159	$C_{11}H_8Cl_2N_4O_4$	40·6	2·5	—	16·8	39·9	2·4	—	16·9
11	Pr	4,5-Dichloro-2-nitrophenyl	NO_2	121–123	$C_{12}H_{10}Cl_2N_4O_4$	41·8	3·0	—	16·4	41·7	2·9	—	16·2
12	Bu	4,5-Dichloro-2-nitrophenyl	NO_2	96	$C_{13}H_{12}Cl_2N_4O_4$	43·8	3·2	—	15·4	43·5	3·3	—	15·6
13	$MeCO{\cdot}CH_2{\cdot}CH_2$	NO_2	4,5-Dichloro-2-nitrophenyl	163–164	$C_{12}H_{10}Cl_2N_4O_5$	41·0	2·9	—	15·0	41·8	2·7	—	15·0
14	2-(pyrid-2-yl)-ethyl	NO_2	4,5-Dichloro-2-nitrophenyl	143	$C_{18}H_{13}Cl_2N_6O_4$	47·8	2·6	—	16·9	47·0	2·7	—	17·1
15	$CN{\cdot}CH_2{\cdot}CH_2$	NO_2	4,5-Dichloro-2-nitrophenyl	241–242	$C_{12}H_7Cl_2N_5O_4$	40·4	2·0	—	19·8	40·4	2·0	—	19·7
16	$CO_2H{\cdot}CH_2$	4,5-Dichloro-2-nitrophenyl	NO_2	283–284	$C_{11}H_6Cl_2N_4O_6$	36·6	1·8	—	15·1	36·6	1·7	—	15·5

a Tautomeric compounds. *b* See Hazeldine, Pyman & Winchester (1924).

nitration (method B) of 4-(3,4-dichlorophenyl)-1-ethylimidazole, 158–159°, and 5-(4,5-dichloro-2-nitrophenyl)-1-ethyl-4-nitroimidazole (2·0 g) m.p. 188–189°.

5-(4,5-*Dichloro*-2-*nitrophenyl*)-4-*nitro*-1-[2-(*pyrid*-2-*yl*)*ethyl*]*imidazole*. 4(5)-(4,5-Dichloro-2-nitrophenyl)-5(4)-nitroimidazole (3 g) was warmed with 2-vinylpyridine (5 ml) at 100° for 1 hr, treated with Triton B solution (5 drops) and warmed for a further hour. Trituration with ethanol gave needles (2·5 g) of the *pyridylethyl compound*, m.p. 143° (from ethanol).

5-(4,5-*Dichloro*-2-*nitrophenyl*)-4-*nitro*-1-(3-*oxobutyl*)-*imidazole*. 4(5)-(4,5-Dichloro-2-nitrophenyl)-5(4)-nitroimidazole (10 g) was warmed on a steam-bath with methyl vinyl ketone (25 ml). Triton B (1 ml) was added and after an hour on the steam-bath the volatile materials were removed, leaving a thick oil which solidified on trituration with ether. The *methyl ketone*, m.p. 163° (from ethyl acetate), crystallized as yellow needles (6 g).

BIOLOGICAL METHODS

The compounds were assayed *in vitro* against the following organisms obtained from the sources stated: *Trichomonas vaginalis* (T 70), Liverpool Public Health Laboratory; *T. foetus* (T 69, Belfast strain), Agricultural Research Council, Weybridge; *Histomonas meleagridis* (Joyner strain, Agricultural Research Council, M.A.F.F., Weybridge; *Entamoeba histolytica* (Strain DC), Liverpool School of Tropical Medicine. The methods used were identical with those described previously (Ellis & others, 1964a,b).

Results

Table 2 lists the activity of those compounds which possess anti-trichomonal action at a concentration of less than 3 μg/ml. The dinitro-derivatives showed no *in vitro* antifungal activity but several compounds inhibited the growth of trichomonads (see Table 2) at very low concentrations. Compounds 7, 8 and 10 showed good activity against the other two protozoa.

TABLE 2. MICROBIOLOGICAL ACTIVITY OF IMIDAZOLE DERIVATIVES

Compound No.	Minimum cidal concentration, μg/ml			M.I.C., μg/ml
	Trichomonas	Histomonas	Entamoeba	Trichophyton
2	2			50
3	2			50
5	1·5			50
6	0·1			100
7	0·1	1·5	2·5	50
8	2·5	2·5		50
10	0·25	2	2·5	50
11	1			20
Metronidazole	0·3	2·5	2·5	
Acinitrazole	1	1		
*Nithiazide	1	3		
†Dimetridazole	0·2	0·5	2	

* 1-Ethyl-3-(5-nitrothiazol-2-yl)urea.
† 1,2-Dimethyl-5-nitroimidazole.

Discussion

Comparison of the *in vitro* antimicrobial activity of un-nitrated aryl-imidazoles with that of their mono- and di-nitro-derivatives shows a decrease in antifungal activity and an increase in antiprotozoal properties, although the presence of more than one nitro-group has little effect on the latter type of activity. In our earlier paper (1964b) a significant difference in trichomonacidal potency was found to exist between a 4- and a 5-nitro-1-substituted imidazole, the 5-nitro-compound being several times more active than its isomer. A fivefold difference in activity was also found between the isomeric dinitro-compounds (compounds 9 and 10). Moreover, alkylation of the ring nitrogen atom in the dinitro-compounds as in the mononitro-compounds, improves activity only if the alkyl group is a small one.

The majority of the compounds synthesized in this work have contained one or more halogen atoms in the benzene ring but it is significant that high *in vitro* antitrichomonal activity has also been shown by mono- and dinitro-compounds which contained no halogen atom, for example, compound 6 in Table 1 and 4-*p*-acetamidophenyl-1-methyl-5-nitroimidazole (Ellis & others, 1946b). Fungicidal potency, on the other hand appears to require two or more halogen atoms in the aryl group.

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The mechanism of inhibition by salicylate of the pentose phosphate pathway in the human red cell

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Salicylate inhibits the pentose phosphate pathway of glucose metabolism in human red cell haemolysates. The site of inhibition is concerned with glucose 6-phosphate and 6-phosphogluconate dehydrogenases and the mechanism of inhibition involves competition with NADP.

SALICYLATE exerts multiple effects on glucose metabolism in the human red cell. The drug interferes with glycolytic reactions and with the pentose phosphate pathway, the latter route of glucose catabolism being the more sensitive (Sturman & Smith, 1966). One possible explanation of these findings is that salicylate inhibits the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases by a mechanism involving competition with the pyridine nucleotide coenzyme, NADP. It has been shown that salicylate inhibits the activities of several other dehydrogenases, including malate, lactate and isocitrate, by competing with either NAD or NADP (Hines & Smith, 1964) and that the overall activity of the pentose phosphate pathway is largely governed by the availability of NADP (Couri & Racker, 1959).

The present work is concerned with studies of the effects of salicylate on a haemolysate of human red cells, incubated under conditions in which the supply of NADP was not a limiting factor in determining the proportion of glucose metabolized by the pentose phosphate pathway. In addition the effects of salicylate on glucose 6-phosphate and 6-phosphogluconate dehydrogenase preparations from human red cells were investigated with special reference to the mechanism of inhibition.

Experimental

RED CELL HAEMOLYSATES

Preparation. Venous blood was collected from healthy adults and heparin used as anticoagulant. The white cells were removed by filtering through cotton wool and washing three times with 0·15M sodium chloride (Buchanan, 1960). The red cells were haemolysed by adding an equal volume of water and then rapidly freezing and thawing the mixture.

OPTIMUM CONCENTRATION OF NADP

Incubations were carried out at 37° in stoppered Warburg flasks, the side arms of which contained 0·5 ml of 50% (w/v) trichloroacetic acid. The centre well contained 0·2 ml of 20% (w/v) potassium hydroxide and the main compartment 2 ml of 0·1M glycylglycine buffer, pH 7·4, containing [$^{14}C_1$] glucose (specific activity 1·39 μc/mg), ATP, NAD, Mg^{2+} and inorganic phosphate ions to give final concentrations of 1 mM, 1 mM, 0·3 mM, 1·5 mM and 2 mM respectively (Chapman, Hennessey, Walters-dorph & others, 1962) and NADP in final concentrations ranging from 0·01

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to 5 mM. The reaction was started by the addition of 2 ml of haemolysate. After an incubation period of 1 hr, the trichloroacetic acid was added from the side arm and the incubation continued for a further hour to ensure that all $^{14}CO_2$ was absorbed in the centre well. The suspension in the main compartment was removed, centrifuged at 2000 *g* for 30 min to separate the cell debris, and the supernatant used for the determinations. Glucose was separated from lactate and phosphate compounds by chromatography on Whatman No. 4 paper using butanol–propionic acid–water (3:2:2), and located by a Nuclear-Chicago Actigraph scanner. The amounts of glucose initially present were measured in corresponding mixtures to which the trichloroacetic acid was added at zero time. Portions of the solution in the centre well were dried on Whatman No. 4 paper. All radioactive counting was performed directly on paper with a Packard Tri-Carb Liquid Scintillation Spectrometer, using as phosphor 15 ml of 0·4% 2,5-diphenyloxazole and 0·01% 1,4-bis-2-(4-methyl-5-phenyloxazolyl) benzene in toluene.

EFFECTS OF SALICYLATE ON [$^{14}C_1$] GLUCOSE METABOLISM IN HAEMOLYSATES

Similar experiments were made in the presence of salicylate, added to the reaction mixtures to give final concentrations ranging from 5 to 500 mM except that the final concentration of NADP present was left constant at 1 mM.

DEHYDROGENASE EXPERIMENTS

Preparation. To 20 ml of red cell haemolysate was added 20 ml of 8% w/v DEAE cellulose (1 m-equiv./g) suspension and the mixture stirred for 20 min at 4°. The supernatant, principally haemoglobin, was removed by centrifuging for 15 min at 1900 *g* and any residual haemoglobin was removed by washing the adsorbent 5 times with a total of 1 litre of 0·003 M potassium phosphate buffer, pH 7·0. The enzyme proteins were desorbed from the cellulose with 10 ml of 0·5M potassium chloride and stirred for 1 hr in an ice bath. The supernatant was removed by centrifuging for 15 min at 1900 *g*. Serum albumin in a final concentration of 10 mg/ml was added immediately to this supernatant in order to stabilize the dilute enzymes. This procedure was repeated once more with the adsorbent and the supernatants combined. The resulting solution was used as the enzyme preparation in all subsequent work.

Effects of salicylate on dehydrogenase activities. Glucose 6-phosphate and 6-phosphogluconate dehydrogenase activities were determined by the techniques described by Kornberg & Horecker (1955) and Horecker & Smyrniotis (1955). The assays were carried out in 0·05M glycylglycine buffer, pH 7·4, at 25° and followed by measuring the increase in optical density at 365 mμ (reduction of NADP to $NADPH_2$), in a Unicam SP800 recording spectrophotometer. Measurements of extinction were made at 365 mμ, and not at 340 mμ, to avoid interference caused by the absorption of the salicylate at the lower wavelength. The initial rates, determined from the tracings obtained with an external recorder, were obtained

in either the absence or the presence of 20 or 50 mM salicylate and with NADP concentrations ranging from 0·01 to 1 mM.

Results

The results, given in Fig. 1, clearly show that maximum activity of the pentose phosphate pathway, assessed by the amount of $^{14}CO_2$ produced and by the proportion of the [$^{14}C_1$] glucose converted to $^{14}CO_2$, occurred

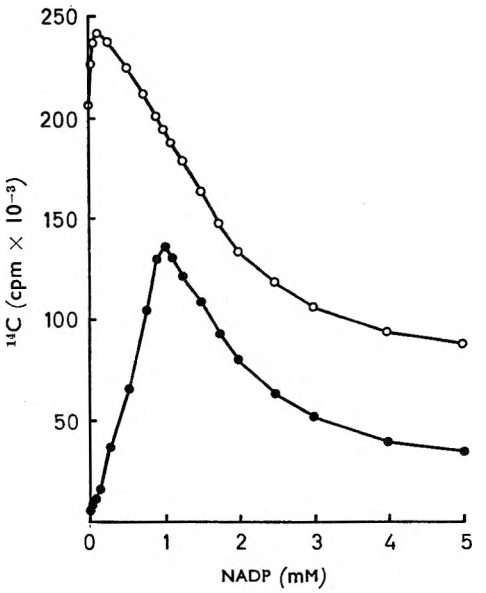

FIG. 1. Effects of NADP concentration on [$^{14}C_1$] glucose utilization and $^{14}CO_2$ production by human red cell haemolysates. ○, [$^{14}C_1$] glucose utilized; ●, $^{14}CO_2$ produced.

after the addition of 1 mM NADP. This concentration of NADP was therefore added to all the subsequent reaction mixtures used to study the effects of salicylate on glucose metabolism in the red cell haemolysates.

Table 1 shows that the addition of increasing concentrations of salicylate reduce both the utilization of the [$^{14}C_1$] glucose and the production of

TABLE 1. EFFECTS OF SALICYLATE ON THE UTILISATION OF [$^{14}C_1$] GLUCOSE AND PRODUCTION OF $^{14}CO_2$ BY HUMAN RED CELL HAEMOLYSATES. The results represent the means of duplicate experiments. Radioactivity is expressed as disintegrations per min (dpm) × 10^{-3}

Salicylate concentration (mM)	[$^{14}C_1$] Glucose utilized	$^{14}CO_2$ produced	% [$^{14}C_1$] Glucose converted to $^{14}CO_2$
0	421	281	66·8
5	386	272	70·5
10	368	267	72·5
20	325	252	77·4
50	191	158	82·8
100	115	103	90·0
200	17	16	92·9
500	2	2	98·7

$^{14}CO_2$ by the haemolysates. However, the percentage of [$^{14}C_1$] glucose converted to $^{14}CO_2$ rose as the salicylate concentration was increased. Thus in the presence of the 1 mM NADP, the pentose phosphate pathway is less sensitive to the inhibitory action of salicylate than the glycolytic pathway.

TABLE 2. EFFECTS OF SALICYLATE ON THE GLUCOSE 6-PHOSPHATE DEHYDROGENASE ACTIVITY OF HUMAN RED CELL HAEMOLYSATES. The results represent the means of duplicate experiments and are expressed as percentage inhibitions

	Salicylate	
NADP concentration (mM)	20 mM	50 mM
0·01	41·2	65·2
0·025	25·7	48·5
0·05	18·8	31·3
0·075	14·2	30·8
0·10	13·1	28·2
0·25	9·0	18·7
0·50	5·5	12·4
0·75	4·0	9·4
1·00	0·0	7·2

TABLE 3. EFFECTS OF SALICYLATE ON THE 6-PHOSPHOGLUCONATE DEHYDROGENASE ACTIVITY OF HUMAN RED CELL HAEMOLYSATES. The results represent the means of duplicate experiments and are expressed as percentage inhibitions.

NADP concentration (mM)	Salicylate (50 mM)
0·01	20·8
0·025	14·6
0·05	9·3
0·075	7·1
0·10	5·5

The results in Tables 2 and 3 show that salicylate inhibits the activities of both glucose 6-phosphate and of 6-phosphogluconate dehydrogenases in the red cell extract and that the degree of inhibition is reduced by the presence of increasing concentrations of NADP. These data suggested that the mechanism of inhibition of the dehydrogenases by salicylate involved a reversible competition with the coenzyme. More acceptable proof that this is the correct mechanism was obtained by graphically representing the data by the method of Hunter & Downs (1945). The resulting plots (Figs 2 and 3) revealed a straight line relationship dependent on NADP concentration. This is the accepted criterion for a reversible competitive inhibition (Dixon & Webb, 1964).

Discussion

The present work shows that the supply of NADP influences the pattern inhibition of glucose metabolism by salicylate. When the coenzyme is present in limited amounts then salicylate inhibits the pentose phosphate pathway to a greater extent than the glycolytic pathway (Sturman & Smith, 1966) whereas the reverse occurs when the supply of NADP is no longer limiting (Table 1). These observations suggested that salicylate interfered

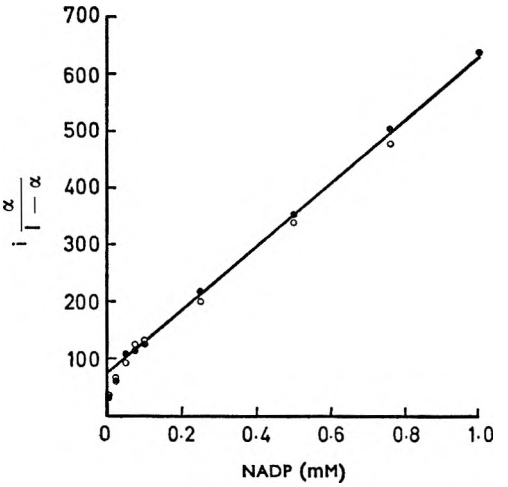

FIG. 2. Inhibition of glucose 6-phosphate dehydrogenase of human red cell haemolysates by salicylate plotted by the method of Hunter & Downs (1945). ○, data obtained in presence of 20 mM salicylate; ●, data obtained in presence of 50 mM salicylate. Values of kinetic constants from plot: K_I = 70 mM, K_m = 0·12 mM.

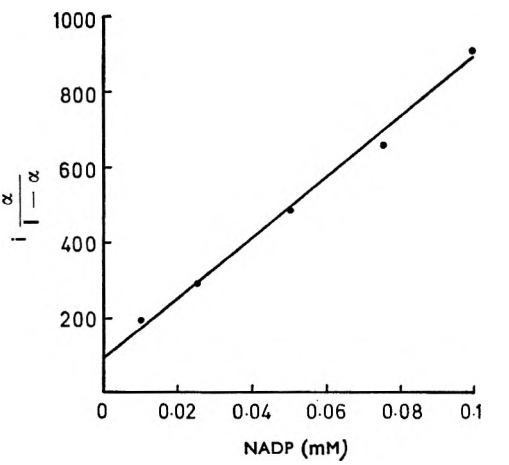

FIG. 3. Inhibition of 6-phosphogluconate dehydrogenase of human red cell haemolysates by salicylate plotted by the method of Hunter & Downs (1945). Data obtained in presence of 50 mM salicylate. Values of kinetic constants from plot: K_I = 100 mM, K_m = 0·011 mM.

with the NADP-dependent dehydrogenases concerned with the production of $^{14}CO_2$ from [$^{14}C_1$] glucose. The results in Tables 2 and 3 show that salicylate inhibits the activities of glucose 6-phosphate and 6-phosphogluconate dehydrogenases in extracts prepared from the red cell haemolysates. In addition the degree of inhibition produced by a given salicylate concentration varied with the NADP concentration. From the experimental data were prepared graphical plots (Figs 2 and 3) which showed

that the mechanism of inhibition of the two dehydrogenases by salicylate involved a reversible competition with NADP.

The inhibitor constants (K_I) for both enzymes were also determined from the plots. They indicate that salicylate is a stronger inhibitor (K_I = 70 mM) of glucose 6-phosphate dehydrogenase than of 6-phosphogluconate dehydrogenase (K_I = 100 mM). The K_I values show that salicylate is a relatively weak inhibitor of both dehydrogenase activities *in vitro*. This does not necessarily mean that salicylate, in the concentration ranges observed either during therapy (2 to 3 mM) or during acute poisoning (3 to 10 mM) will not affect the dehydrogenase activities, and hence carbohydrate metabolism, in circulating human red cells. There is experimental evidence that 5 mM salicylate preferentially inhibits the pentose phosphate pathway in human erythrocyte suspensions (Sturman & Smith, 1966). It is also possible to calculate, from the data in Figs 2 and 3, that while 2 to 10 mM salicylate would produce little, if any, inhibition of 6-phosphogluconate *in vitro* these salicylate concentrations would inhibit the glucose 6-phosphate dehydrogenase activity between 5 and 20%. As the mechanism of inhibition of the dehydrogenases by salicylate involves a reversible competition with the coenzyme, the degree of inhibition *in vivo* would depend, at least in part, on the intra-erythrocytic concentrations of NADP and these are relatively low, a range of 0·013 to 0·017 mM having been found (Bartlett, 1959).

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Probable conformations of the analgesically active basic anilide *N*-[(2-benzylmethylamino)propyl]-propionanilide

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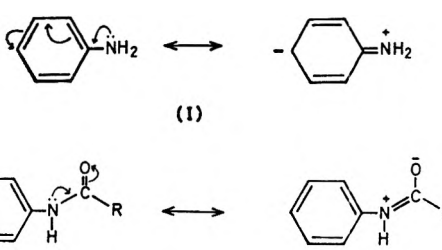
A spectroscopic (ultraviolet, infrared and nuclear magnetic resonance) study of *N*-[(2-benzylmethylamino)propyl]propionanilide hydrochloride is reported and probable conformations of this molecule (in which the phenyl group and amide function are non-planar, the protonated basic centre lies close to the amido-nitrogen atom, and the secondary methyl group is removed from the region above the aromatic plane) proposed on the basis of the spectroscopic results. Differences in analgesic stereospecificity and basic group structure between enantiomorphs of methadone and *N*-[(2-benzylmethylamino)propyl]propionanilide are discussed in terms of the probable conformations of the two analgesics.

RECENTLY attention was drawn to structural differences in the basic group of 3-amino-1,1-diphenylpropane analgesics such as methadone, and *N*-(2-aminopropyl)propionanilide analgesics such as diampromid, and to the different stereospecificity exhibited by the analgesic receptor towards enantiomorphs of the two classes (Casy & Hassan, 1967). Since both types of analgesic possess the system Ph–X–C–C–N< (where X is a

non-hydrogen bearing atom), it seems likely (especially in view of differences in stereospecificity) that the relative orientation of the components of this system differ in the two classes of compound, with the probable result that their modes of binding to the analgesic receptor are dissimilar. The present spectroscopic examination was made to obtain evidence about the conformation of the molecules in question, and to ascertain whether any marked difference in the orientation of the system specified above does in fact exist in the two classes.

ULTRAVIOLET SPECTROSCOPY

Aniline has three absorption bands above 200 mμ; those at 208 and 280 mμ are associated with local excitation (L.E.) transitions of the aromatic π-electrons, and that at 230 mμ to the $\rho \rightarrow \pi$ electron transfer (E.T.) symbolized in (I) (Scott, 1964, and references there cited). Acetanilide and propionanilide also have three absorption bands above 200 mμ,



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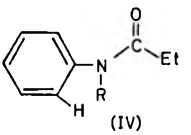
associated with transitions of a similar nature (Table 1). The intensity of the E.T. band of propionanilide is reduced as the solvent pH is lowered but is still relatively high even in N hydrochloric acid-ethanol [in contrast, the E.T. band of aniline is completely absent in 0·1N HCl (Forbes, Ralph & Gosine, 1958)]. In anilides the electron transfer may be represented by (II → III),* structure (III) being analogous to the styrene chromophore [λ_{max} 248 mμ ϵ 14,000 in ethanol (Scott, 1964)].

When hydrogen in the anilide (II; R = Et) is replaced by methyl,



(IV)

R = (a), Me; (b) Et; (c) iso C_3H_7;
(d) CH_2·CH(Me)·N(CH_2·Ph)Me;
(e) CH_2·CH(Me)·NMe_2

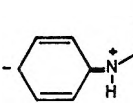
the E.T. band suffers a blue shift and an intensity decrease. These effects are larger in the *N*-ethyl anilide (IVb), while in the *N*-isopropyl anilide (IVc) the E.T. band is only just apparent (Table 1). The chromophore present in (III) is only effective when the phenyl and amide entities

TABLE 1. ULTRAVIOLET SPECTROSCOPIC CHARACTERISTICS OF SOME PROPIONANILIDE [PhN(COEt)R] DERIVATIVES

R	Solvent	λ_{max} mμ (ϵ)	
		L.E. band	E.T. band
Aniline	EtOH	208 (3860)[1]	236 (6050)
H[2]	EtOH	208 (11,600)[3]	244 (15,500)
H	0·01N HCl–EtOH–H_2O	209 (6300)	244 (13,000)
H	0·1N HCl–EtOH–H_2O	209 (5860)	244 (12,000)
H	N HCl–EtOH–H_2O	209 (6600)	244 (12,600)
Me	EtOH	211 (8000)	228 (6040)
Et	EtOH	211 (8700)	225[4] (6000)
iso Pr	EtOH	210 (8400)	225[4] (4200)
$CH_2CHMeN(CH_2Ph)Me$	EtOH	212 (17,400)[6]	225[6] (8700)
"	H_2O[7]	205 (18,000)	225[6] (5200)
$CH_2CHMeNMe_2$	EtOH	210 (9400)	225[4] (5000)

[1] Also displays L.E. band at 287 mμ (ϵ 2200). [2] Acetanilide has λ_{max} 242 mμ (ϵ 14,400) (Ungnade, 1954). [3] Also displays L.E. band at 281 mμ (ϵ 500). [4] Inflection. [6] The higher intensity of this band is due to the additional phenyl group in the molecule. [6] Change of slope. [7] Hydrochloride.

are coplanar. Such conformations are progressively less favoured as the size of the *N*-substituent (R) increases because of non-bonded interactions between R and an *ortho*-hydrogen of the phenyl ring (evidence of models). Hence the changes in the E.T. band of the anilides, described above, are attributed to steric inhibition of resonance, an effect also demonstrated in *o*-alkylacetanilides (Ungnade, 1954). The E.T. bands of the basic anilides (IVd and e) are suppressed in a similar manner to that of the

* The alternative (II ⟶ ), proposed by Picard & McKay (1953), appears less likely because the amide carbonyl group is known to be highly polarized as in (III) (infrared evidence).

isopropyl derivative (IVc). The E.T. band is also suppressed in the hydrochloride salt of the anilide (IVd), examined in water.

It is concluded, therefore, that the more favoured conformations of the basic anilides (IVd and e) possess *N*-phenyl and amide moieties that do not lie in the same plane.

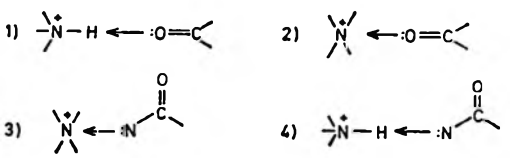
INFRARED SPECTROSCOPY

The carbonyl stretching frequency ($\nu_{C=O}$) of the *N*-ethyl propionanilide (IVb) falls in the normal range of 1670–1630 cm^{-1} quoted for tertiary amides (Rao, 1963) and is little altered when *N*-ethyl is substituted by a β-amino-group, as in the basic anilide (IVd) (Table 2, Nos 1 and 2).

TABLE 2. CARBONYL STRETCHING FREQUENCIES OF SOME PROPIONANILIDE [PhN(COEt)R] DERIVATIVES

No.	R	Form	Solvent (conc.)	$\nu_{C=O}$ cm^{-1}
1	Et	—	(film)	1661–2
	"	—	CS_2 (10%)	1662
	"	—	$CHCl_3$ (10%)	1649
2	$CH_2CHMeNMe(CH_2Ph)$	base	(film)	1663
	"	"	CS_2 (10%)	1666
	"	"	$CHCl_3$ (10%)	1649
3	"	HCl	$CHCl_3$ (0·1)	1659
	"	"	" (0·3)	1659·5
	"	"	" (0·5, 1·0, 3·0, 5·0)	1658·5
	"	"	$CHCl_3$ (10%)	1665
	"	"	" (20%)	1658–1668·5 (broad band)
	"	"	(Nujol mull)	1670
4	"	MeI	$CHCl_3$ (10%)	1662
			(Nujol mull)	1658
5	$CH_2CHMeNMe_2$	base	(film)	1663
	"	"	$CHCl_3$ (10%)	1644·5
	"	HNO_3	$CHCl_3$ (10%)	1661·5
	"	"	(Nujol mull)	1665

There is no infrared evidence, therefore, for an interaction between the amide and amino-functions of the basic anilide (IVd) in the non-protonated state. Solvent effects of similar degree are observed in both anilides, the $\nu_{C=O}$ shift of 13–17 cm^{-1} to lower wave numbers ($CS_2 \rightarrow CHCl_3$) being attributed to hydrogen bonding of the type $Cl_3CH \cdots O = C\lessdot$. $\nu_{C=O}$ for the anilide (IVd) hydrochloride is significantly *greater* (10–21 cm^{-1}) than that of the free base, the difference being a maximum when the salt is examined as a mull (Table 2, Nos 2 and 3). Elevation of $\nu_{C=O}$ is also seen in the methiodide of (IVd) and these results show that some interaction between the amide group and the positively charged basic centre occurs in protonated and quaternary salts of the basic anilide (IVd). Four types of interaction (intra and/or intermolecular) may be postulated, namely:



(the arrows denote the direction of electron flow)

Types 1 and 2 would weaken the C=O bond and hence lead to *lower* $\nu_{C=O}$ values. Interactions of types 3 and 4 reduce the availability of the nitrogen lone-pair of the amide function and would be expected to lead to a diminution of the ionic character ($\succ\overset{+}{N}=C\prec^{O}$) of the amide, with the result that the amide $\nu_{C=O}$ value is *raised.* This effect is analogous to the base-weakening influence of protonated nitrogen upon the second basic centre of a diamine.*

Operation of the interactions 1 and 4 require an acidic proton and hence would also be expected to influence the $\overset{+}{N}$—H stretching frequency. However, ν^{+}_{N-H} values for hydrochloride salts of the anilide (IVd) (2550 cm^{-1}, as mull) and benzylethylmethylamine (2500 cm^{-1}, as mull), an analogue of (IVd) lacking the amide function, show no significant difference and, on these grounds, it is considered that the acidic proton does not play an important role in these interactions. Types 2 and 3 do not require an acidic proton and may thus operate in the case of quaternary salts.

Since elevated, rather than reduced $\nu_{C=O}$ stretching frequencies are observed in both the hydrochloride and quaternary salts of (IVd), an interaction of type 3 is indicated. The data available suggest that the effect may be both inter- and intramolecular. The carbonyl stretching frequency is constant over the concentration range 5-0·1%. This behaviour is indicative of an intramolecular interaction, while higher values are observed in more concentrated solutions which is indicative of an intermolecular influence also operating (Table 2, No. 3). Both influences are liable to be optimum in the solid state, the highest $\nu_{C=O}$ value being seen when the anilide hydrochloride is examined as a mull. The $\nu_{C=O}$ infrared properties of the two anilides (IVd and e) are also alike, $\nu_{C=O}$ of the of the dimethylamino derivative (IVe) being elevated to a similar degree when the base is protonated [$\nu_{C=O}$ salt — $\nu_{C=O}$ base = 17 cm^{-1} for 10% solutions in chloroform (Table 2, No. 5)].

These results show that the preferred conformations of the anilide (IVd) hydrochloride in dilute solution (where intramolecular effects alone are assumed) are such that the protonated nitrogen centre lies in the vicinity of the amido-nitrogen atom (as in interaction 3).

NUCLEAR MAGNETIC RESONANCE SPECTROSCOPY

The nmr characteristics in $CDCl_3$ of *N*-[(2-benzylmethylamino)propyl]-propionanilide (IVd) as free base, hydrochloride and methiodide, and of the related compound (IVe), are given in Table 3. The following points of interest are to be noted:

(1) In the anilide hydrochloride (IVd), both the *N*-methyl and s-methyl signals are duplicated, each appearing as a doublet of doublets (Fig. 1). (The two *N*-methyl doublets overlap and a triplet is observed.) These results show that the hydrochloride exists as a mixture of *N*-epimers in

* In the monoprotonated salt of 1,2-ethanediamine, for example, charged nitrogen reduces the availability of the lone-pair electrons of the second basic centre, as seen from pK_a values [pK_a (H^+) 10·09; pK_a ($2H^+$) 7·00] (Albert & Serjeant, 1962).

TABLE 3. NUCLEAR MAGNETIC RESONANCE CHARACTERISTICS OF SOME *N*-(2-AMINOPROPYL) PROPIONANILIDES [PhN(COEt)R]

			nmr signals[1]					
R	Form	Solvent	Aromatic	N–Me	N-CH_2Ph	s-Me[4]	$COCH_2Me$[5]	Others
CH_2CHMe–$NMe(CH_2Ph)$	base	$CDCl_3$	437[3]	127[2]	216·5, 211[3]	59 (J7)	62 (J7)	230 (C–CH_3)[6]
		$CDCl_3$–CF_3CO_2H	450[7]	174[2]	266, 258[3]	81 (J6·5)	60·5 (J7)	124[8] (J7) ($COCH_2Me$)
,,	HCl	$CDCl_3$	463, 438[7]	171[4] (J6) 165[4] (J6)	very complex	92 (J7) 82·5 (J6)	62 (J7)	126[9] (broad) ($COCH_2Me$)
		$CDCl_3$–D_2O	462, 440[7]	171·5[2]	282, 269, 260, 247[3] (J_{AB}13)	85 (J7)	60·5 (J7)	127[8] (broad) ($COCH_2Me$)
,,	CH_3I	$CDCl_3$	447[2]	192[2]	247[3] (broad)	93[8]	59 (J7)	124[9] (broad) ($COCH_2Me$)
CH_2CHMe–NMe_2	base	$CDCl_3$	440[2]	129[2]	—	51 (J7)	59 (J7)	118[8] (J7) ($COCH_2Me$)
,,	HNO_3	$CDCl_3$	447, 445[9]	179[2]	—	80 (J6)	60 (J7)	127[9] (broad) ($COCH_2Me$)

[1] Chemical shifts in c/sec from TMS (operating frequency 60 Mc); coupling constants in c/sec. Concentrations approx. 10%. [2] Singlet or near-singlet. [3] Peaks of AB signal (outer peaks may be lost in noise). [4] Doublet. [5] Triplet. [6] Centre of multiplet. [7] Main peak(s) of multiplet. [8] Centre of deformed doublet. [9] Quartet.

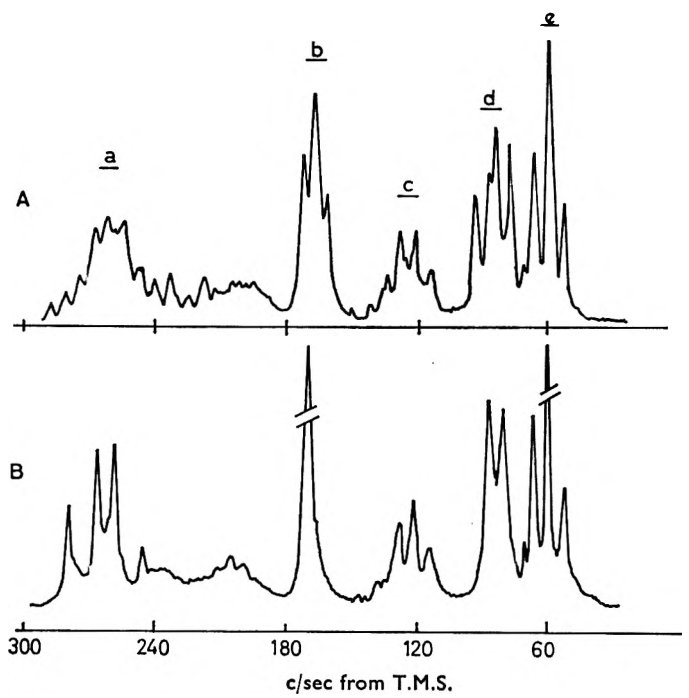
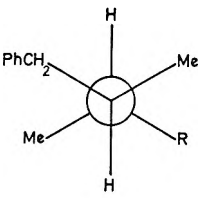
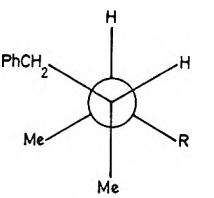


FIG. 1. Part of the nmr spectrum of *N*-[(2-benzylmethylamino)propyl] propionanilide hydrochloride: (A) in $CDCl_3$; (B) in $CDCl_3$–D_2O. Signals: a, NCH_2Ph; b, N·Me; c, $CO{\cdot}CH_2{\cdot}Me$; d, s-Me; e, $CO{\cdot}CH_2{\cdot}Me$.

$CDCl_3$, depicted in the Newman diagrams (V and VI), which arise as a result of the two possible modes of proton addition to the basic centre. (The assignment of configurations to these and related epimers will be discussed elsewhere.) In these conformations, the relative environments



(V)



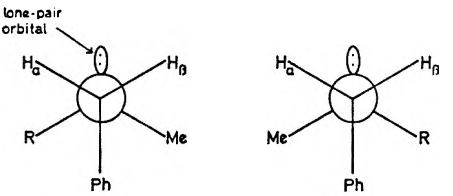
(VI)

R = CH_2·N(COEt)Ph
(Front atom is nitrogen)

of both the *N*-methyl and s-methyl groups differ, hence the two configurations of the salt exhibit different signals for these two groups. Additional multiplicity is observed in the *N*-methyl signal because of spin-spin coupling between *N*-methyl and the acidic proton on nitrogen. The nmr signal of the *N*-benzylmethylene (NCH_2Ph) protons, whose environments also differ in the two epimers, is discussed below. The sharp nature of the two *N*-methyl and s-methyl doublets indicates that the rate of proton exchange between epimers (resulting in their interconversion) must be relatively slow in $CDCl_3$. When proton exchange is accelerated by the addition of D_2O, the *N*-methyl signal becomes a singlet and the s-methyl, a doublet. Rapid proton exchange must also occur in the presence of trifluoroacetic acid and its anion since *N*- and s-methyl doublet of doublets were not observed when the anilide was examined in $CDCl_3$ — CF_3CO_2H (Table 3).

In the methiodide of (IVd), the *N*-methyl, $N\text{-}CH_2Ph$, and s-methyl signals were not duplicated, an expected result because the basic centre in this case is symmetrical.

(2) In the free base of the anilide (IVd), the *N*-benzylmethylene (NCH_2Ph) signal formed an AB pattern indicating the non-equivalence of the protons in this group. This pattern was most clearly visible when the anilide hydrochloride was examined in $CDCl_3$–D_2O (in the free base, the less intense, outer, peaks were obscured somewhat by the noise level)—the signal of the salt in dry $CDCl_3$ was more complex as a result of spin-spin coupling involving the acidic proton on nitrogen. Evidence for the non-equivalence of *N*-benzylmethylene protons was also obtained in the case of the compounds *N*-[2(benzylmethylamino)propyl]aniline and 1-benzylmethylamino-propan-2-ol (unpublished results). In acyclic tertiary amines, non-equivalence of the type depicted in (VII and VIII) may only arise if inversion of the nitrogen lone-pair (resulting in the interconversion of VII and VIII) is restricted, and has been demonstrated at low temperatures, when slow inversion rates obtain (Griffith & Roberts, 1965). No examples of a non-equivalent open-chain $-CH_2N<$ system have, to

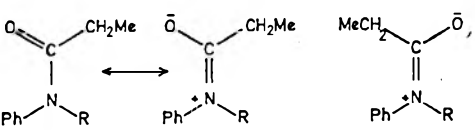


(VII) (VIII)

(Front atom is carbon, rear is nitrogen)

our knowledge, been previously reported at room temperature and the reasons for slow inversion rates in the present cases are being investigated.*

(3) The $COCH_2Me$ signal forms a broad, rather than a sharp quartet, a result probably due to the slow interconvertibility of the conformers (IX) and (X) through restricted rotation about the N–C bond which has



(IX) (X)

double bond character. The methylene protons environments differ in the two forms, while the methyl protons (which form a relatively sharp triplet) differ less in this respect because they are further removed from the *N*-substituents.

(4) The environment of s-methyl in the anilide (IVd) is similar to that of the same group in the diphenylpropane derivatives (XI; R = H and CN), as seen from the comparative chemical shift values.

$Me_2N{\cdot}CH(Me){\cdot}CH_2{\cdot}CPh_2R$

(XI)

secondary chemical shift c/sec from TMS (in $CDCl_3$)

R = H, 55 (base); 84 (HCl)
R = CN, 54 (base); 82 (HCl)
Anilide (IVd) 59 (base); 85 (HCl)

In the latter compounds there is evidence that the s-methyl groups are not especially shielded by the aromatic groups (Casy, 1967) [when aromatic shielding occurs the s-methyl signal shows a significant upfield shift, as in methadone (base, 29 c/sec; HCl, 42 c/sec)] and on these grounds it is concluded that the same holds true for s-methyl in the anilide (IVd).

A probable conformation of *N*-[(2-benzylmethylamino)propyl]propionanilide, based upon the described spectroscopic evidence, is shown in Fig. 2. It is consistent with the spectroscopic data in the following respects:

* A non-equivalent $-CF_2N<$ system has recently been described (Banks, Barlow, Haszeldine & McCreath, 1965).

(1) the phenyl group and amide function are non-planar (ultraviolet evidence);

(2) the protonated basic centre is close to the amido-nitrogen atom (infrared evidence) (the conformation in which carbonyl oxygen and ethyl are reversed is also probable);

(3) the s-methyl group does not lie above the plane of the aromatic ring (as it does in probable conformations of methadone—see later) and is judged to fall approximately on the boundary of the aromatic dia- and paramagnetic screening zones (nmr evidence shows that the s-methyl protons are not significantly shielded by the aromatic group).

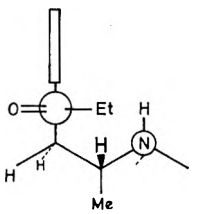


FIG. 2. Representation of a probable conformation of *N*-[(2-benzylmethylamino)-propyl]propionanilide hydrochloride. Note: 1. End-on view of aromatic ring is shown. 2. Amido-carbonyl carbon eclipses amido-nitrogen. 3. H and Me on C-2 may be interchanged. 4. For clarity, N·Me and ·CH_2·Ph substituents have been omitted.

The *N*-arylalkyl substituent of the basic anilide appears to have a minor influence upon the conformation of the molecule since the nmr spectral characteristics of the benzylmethyl- and dimethylamino-anilides (IVd and e) are similar with respect to common proton groups (Table 3).

A probable conformation of methadone hydrochloride, based upon crystallographic (Hanson & Ahmed, 1958) and spectroscopic evidence (Casy, 1967), is shown in Fig. 3. The major point of difference between

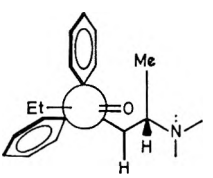


FIG. 3. Representation of a probable conformation of methadone hydrochloride (N·Me groups are omitted).

probable methadone and basic anilide conformations lies in the relative orientations of phenyl, s-methyl and basic nitrogen in the two molecules. In the methadone conformation (Fig. 3), the s-methyl group lies close to, and directly above, the plane of one of the aromatic rings (in consequence it falls well within the aromatic screening zone and has an abnormally high chemical shift on this account). In contrast, s-methyl in the basic anilide conformation (Fig. 2) is further removed from the aromatic ring

and is not situated above its plane. This difference arises from the fact that, while C-4 of methadone is tetrahedral, the equivalent atom of the anilide (the anilo-nitrogen) is trigonal, hence the relative orientations of the β-aminoethyl side-chain to the aromatic group must be dissimilar in the two compounds.

If it is assumed that the conformations shown in Figs 2 and 3 are likely to resemble those adopted by the analgesic at the receptor site, and further, that the phenyl-s-methyl-basic centre orientation of the methadone conformation (Fig. 3) is particularly conducive to drug-receptor association, a possible reason may be advanced for the differing stereochemical and basic group features of methadone and basic anilide analgesics. Although the spatial arrangement of the three groups specified above (as in Fig. 3) is not favoured in the basic anilide, this compound is nevertheless an active analgesic and it is therefore probable that its mode of binding to the receptor differs markedly from that of methadone. Hence, (1) the stereospecificity of the receptor towards enantiomorphic forms of the anilide is not necessarily the same as that which it exhibits towards methadone isomers, and (2) binding sites, additional to those operating in the case of the methadone-receptor association, may be required for the effective uptake of basic anilide molecules upon the receptor surface—such sites could possibly be provided by the arylalkyl *N*-substituent of basic anilides (absent in methadone and related compounds). It is significant, in this respect, that the dimethylamino anilide (IVe) has a low order of analgesic potency (Casy & Hassan, 1967).

In the *N*-arylalkylmethyl basic anilides, a second asymmetric centre is created when the basic nitrogen accepts a proton (nmr evidence) and it may well be that one particular configuration of the basic centre is preferred for drug-receptor association. Under conditions of rapid proton exchange the two epimers will be rapidly interconverting and that of the preferred configuration will readily be derived from the unfavoured epimer. However, if the interconversion rate in the vicinity of the receptor is slow [as would result if the physiological pH near the receptor was lower than the generally accepted figure of 7·2–7·4 (certain enzymes, for example, behave as though acting in environments some two pH units lower than the bulk (Weiss, 1963)], then the relative population of the two epimers may have an important influence upon the drug-receptor combination.

Experimental

N-*Methylpropionanilide*. Sodium hydride (1·44 g, 50% in oil) was added to a hot solution of propionanilide (4·5 g) in dry toluene (40 ml) and the mixture stirred and heated at 110–120° for 3 hr. Methyl iodide (4·29 g) in toluene (10 ml) was added to the resultant suspension of sodium propionanilide and the mixture stirred and heated for 6 hr. The cooled product was filtered and the filtrate evaporated under reduced pressure. The residue was distilled to give N-*methylpropionanilide* (4·5 g), b.p. 94–96°/0·1 mm, m.p. 57–59° (from light petroleum b.p. 60–80°). Found:

C, 73·4; H, 7·9; N, 8·2. $C_{10}H_{13}NO$ requires C, 73·6; H, 8·0; N, 8·5%. N-*Ethylpropionanilide*, b.p. 82–84°/0·6 mm (found: C, 74·5; H, 8·8; N, 8·0. $C_{11}H_{15}NO$ requires C, 74·5; H, 8·5; N, 7·9%) and N-*isopropylpropionanilide*, b.p. 90–91°/0·05 mm, m.p. 39–40° (from light petroleum b.p. 60–80°) (found: C, 75·3; H, 8·9; N, 7·3. $C_{12}H_{17}NO$ requires C, 75·3; H, 9·0; N, 7·3%) were similarly prepared using the appropriate alkyl halide.

The ultraviolet spectra were recorded on a Unicam S.P. 800, and the infrared spectra on a Unicam S.P. 100 spectrophotometer. In the infrared work, calibration was accurate to ± 1 cm^{-1} over the region 1630–1700 cm^{-1}, and cells of path length 0·05 mm (10 and 20%), 0·5 mm (0·5–3%) and 2 mm (0·1 and 0·3%) were used for the solution studies. The nmr spectra were recorded on a Varian A-60 spectrophotometer using deuterochloroform as solvent and TMS as internal standard.

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Bronchodilator actions of papaverine in the anaesthetized guinea-pig

SIR,—It has been observed that intravenous injection of papaverine reduced the temporary increase in bronchial resistance produced by histamine in the anaesthetized guinea-pig. This fact accords with the well-known spasmolytic action of the drug. However we found, as did Dr G. James, that the spasmolytic action of papaverine on bronchial smooth muscle *in vivo* is reduced by the prior administration of pronethalol, a β-adrenergic blocking agent. We now describe the elucidation of the mechanism of the bronchodilator action of papaverine in the anaesthetized guinea-pig. The methods employed have been described in detail elsewhere (Farmer & Lehrer, 1966).

Papaverine (0·5 μg/ml) and isoprenaline (5ng/ml) produced relaxation in approximately equal magnitude of the tracheal chain preparation (Castillo & de Beer, 1940; Akçasu, 1959). When pronethalol and phentolamine were added to the bath, each at a concentration of 2·5 μg/ml, the response of the tissue to isoprenaline was abolished whilst that to papaverine remained unchanged. This evidence eliminated the possibility of an action of papaverine on adrenoceptive receptors. For the assessment of the activity of papaverine hydrochloride against histamine-induced bronchospasm in the anaesthetized guinea-pig (Konzett & Rossler, 1940), at time zero, 10 mg/kg of papaverine hydrochloride was injected intravenously and the response of the animal at various time intervals to intravenous injection of histamine was assessed. This procedure was performed in further guinea-pigs after pronethalol (10 mg/kg i.m.), bilateral adrenalectomy, total destruction of the central nervous system, and bilateral denervation of carotid sinuses (sinuses removed). The results (means of at least 4 guinea-pigs) showed that each procedure much reduced the duration of action of papaverine against histamine bronchospasm. The time for 50% recovery of the response to histamine, after injection of papaverine, was reduced from approximately 55 min to 14-22 min (depending on procedure). There was also some reduction in the absolute potency but the design of the experiment does not allow interpretation of this latter effect. Intravenous injection of papaverine, 10 mg/kg, in the anaesthetized guinea-pig produced a fall in arterial blood pressure of 58 $\pm$ 4·6 (5) mm Hg (maximum) and the time taken for 50% recovery was 16 $\pm$ 6·9 (5) min.

These experiments show that the antagonism by papaverine of histamine bronchospasm in the anaesthetized guinea-pig is mediated by at least two mechanisms. Firstly there is a direct action of papaverine on bronchial smooth muscle and secondly an action due to a release of catecholamines. The catecholamines originate mainly from the adrenal medulla and the effective stimulus for such a secretion is a fall in mean arterial blood pressure which results in a reflexly mediated increase in sympathetic outflow.

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Histamine release by *OO'*-diethylbebeerine in the isolated rat diaphragm

SIR,—Histamine release by (+)-tubocurarine in the rat isolated diaphragm was found by Rocha e Silva & Schild (1949) to be enzymatic in character. Moussatché & Prouvost-Danon (1958), Yamasaki, Muraoka & Endo (1960), Rothschild, Vugman & Rocha e Silva (1961), Uvnäs & Diamant (1961) among others, have shown that tissue responsiveness to histamine-releasing stimuli requires metabolic cellular activity. The present note shows that the same is true for *OO'*-diethylbebeerine, a semisynthetic curarizing drug, having about 10 times more histamine-releasing potency than (+)-tubocurarine in the rat (Rothschild & Corrado, 1963).

TABLE 1. EFFECT OF 2,4-DINITROPHENOL (DNP) ON HISTAMINE RELEASE FROM THE ISOLATED RAT DIAPHRAGM BY *OO'*-DIETHYLBEBEERINE

Treatment of the tissue during pre-incubation*	Histamine released (% of total)	Inhibition (%)
—	15·6 ± 2·8	—
Glucose 4·5 mM	16·5 ± 2·6	—
DNP, 0·3 mM	2·4 ± 1·2	85
Glucose + DNP	13·3 ± 3·8	19

* 20 min at 37° in Krebs–Ringer phosphate buffer. After preincubation, 200 μg/ml of *OO'*-diethylbebeerine was added to all tubes and incubations continued for another 20 min. Released and residual (tissue bound) histamine was estimated by bioassay on the guinea-pig isolated ileum by standard techniques. Each result represents the average of 4 experiments from which the contribution of spontaneously released histamine has been deducted.

Table 1 shows the effect of *OO'*-diethylbebeerine on histamine release from the rat isolated diaphragm. The effect takes place both in complete and in glucose-free media, but is extensively inhibited by 2,4-dinitrophenol in the glucose-free medium. In the presence of glucose the inhibitor is ineffective. Dinitrophenol is an uncoupler of aerobic high-energy phosphate bond synthesis which, however, does not block the initial anaerobic steps of glucose consumption by mammalian cells. It is probable therefore, that the metabolites required for the histamine-releasing response of rat mast cells to *OO'*-diethylbebeerine can arise through the anaerobic metabolism of glucose.

A dependence on cell metabolism similar to that shown here for the histamine-releasing activity of a curarizing drug, has been previously demonstrated for certain other basic "chemical" histamine releasing compounds like 48/80 and its analogues, (+)-tubocurarine (Rothschild, 1966) and sinomenine (Yamasaki & Saeki, 1966). Such a dependence however, does not seem to be a requirement for the action of all simple basic histamine releasers active on rat tissues, since it could be shown (Rothschild, 1962, 1966), that histamine release from isolated peritoneal fluid rat mast cells by ring chlorinated analogues of catecholamines (dichloroisoprenaline, dichloroadrenaline and dichloronoradrenaline) proceeds unhindered in cells treated with 2,4-dinitrophenol in a glucose-free medium.

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Enlargement of liver in rats after chronic administration of flumedroxone acetate

SIR,—The synthetic steroid flumedroxone acetate (17-acetoxy-6α-trifluoromethylpregn-4-ene-3,20-dione; WG537; Demigran) has been used as a prophylactic treatment for migraine (summarized in Lundberg, 1966). The experiments reported here followed the observation that 100 mg/kg of flumedroxone acetate produced in the laboratory rat, after chronic intraperitoneal treatment, a liver weight increase. This steroid drug, together with 17-acetoxy-3β(β-carboxypropionyloxy)-6-trifluoromethylpregn-5-ene-20-one (VD682) was synthesized and provided by Leo Pharmaceutical Products Ltd., Ballerup, Denmark.

Female albino Porton rats weighing between 100–200 g, maintained on standard laboratory chow with water *ad libitum*, were used. Each steroid was suspended in water (1 ml) using compound tragacanth powder and introduced by gastric intubation. Control and treated animals were killed 24 hr after the last treatment and body and liver weights determined.

Table 1 shows that rats treated with flumedroxone acetate and VD682 have an increased liver weight when compared to control animals, or animals treated

TABLE 1. LIVER WEIGHT OF RATS AFTER TREATMENT WITH FLUMEDROXONE AND VD682

Drug	Dose mg/kg (No. days)	Animal weight range g	Mean body weight g (No. animals)	Liver weight g/100 body weight (range, g)
Flumedroxone acetate	10 (7)	100–149	130·4 (3)	5·7 (6·9–8·3)
	20 (7)		145·2 (2)	6·3 (7·6–8·9)
	50 (7)		141·5 (4)	6·3 (7·9–9·6)
	100 (7)		145·2 (4)	7·3 (9·4–11·8)
Flumedroxone acetate	100 (3)	100–200	152·5 (3)	5·7 (8·2–9·2)
	100 (5)		150·4 (2)	6·0 (8·8–10·6)
	100 (7)		145·2 (4)	7·3 (9·4–11·8)
	100 (14)		195·0 (2)	8·2 (15·0–16·7)
VD682	50 (7)	100–149	137·2 (3)	7·1 (8·4–10·8)
	100 (14)	150–200	185·4 (4)	10·5 (18·6–20·1)
Compound tragacanth powder	700 (14)	100–149	137·2 (6)	3·9 (4·3–6·0)
	700 (14)	150–200	161·4 (5)	4·1 (4·9–7·9)
None	—	100–149	138·1 (8)	3·8 (4·4–7·5)
	—	150–200	176·2 (9)	3·9 (5·3–8·2)

TABLE 2. LIVER WEIGHT OF RATS AFTER TREATMENT WITH STEROID COMPOUNDS FOR 9 DAYS

Drug	Dose mg/kg	Mean body weight g (No. of rats)	Liver weight g/100g body weight (range, g)
Progesterone	25	160·2 (5)	3·8 (5·4–6·8)
	50	152·5 (6)	5·0 (7·2–7·9)
	100	163·3 (4)	5·2 (7·2–8·1)
Flumedroxone acetate	50	138·2 (4)	7·2 (8·7–10·6)
VD682	50	136·7 (4)	7·8 (9·4–12·1)
None	—	164·1 (10)	3·8 (4·2–7·1)

with compound tragacanth powder. The relationship between dose level and duration of treatment was not completely elucidated by these experiments, although 50 mg/kg for a period of 7 days always gave an appreciable liver weight increase. Progesterone at 50 or 100 mg/kg gave a much smaller increase in liver weight than either of the synthetic steroids, Table 2. Other experiments have shown that the intraperitoneal route can reduce the treatment time or the dose level needed to achieve a liver weight increase comparable to that obtained when using the oral route. This held true for all drugs tested. An isolated experiment involved a pregnant rat on progesterone treatment in which the liver grew at striking rate: the rat had a final weight of 150 g; after progesterone 20 mg/kg for 6 days, the animal had a liver weight of 10·65 g or 7·1 g/100 g body weight: this increase is comparable to the liver weight reached after treatment with 50–100 mg/kg of flumedroxone acetate for 7–9 days.

These increases in liver weight have been shown to be accompanied by a change in the esterase electropherogram of serum and of liver tissue (Pantelouris & Hines, 1966). Chlorpromazine, phenylbutazone, SKF525-A and benzydamine can each produce in mice, after weekly treatment, a liver weight increase, a reduction in hexobarbitone sleeping time and a decrease in the retention of blood serum phosphatase (Silvestrini, Catanese & Del Basso, 1966). These drugs have also been implicated in the induction of microsomal enzymes, which in turn activates the breakdown of the inducer, or of quite different compounds (Conney & Burns, 1962; Remmer, 1964).

Phenobarbitone sodium can produce in mice an increased liver weight and a proliferation of the endoplasmic reticulum (Hart & Fouts, 1965). Probably of more significance to the present study is the report of enhanced development of pituitary tumours and the occurrence of some hepatomas in virgin female mice following repeated oral administration of synthetic progestins, including mestranol (Poel, 1966).

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Thiol inhibition of the effects of bradykinin upon vasopressin in the toad bladder

SIR,—It seems that the antagonism of vasopressin and oxytocin by thiols is specific and that at least in part the thiol acts by directly affecting intracellular processes rather than solely by chemical reduction of the hormones (Handler & Orloff, 1964; Martin & Schild, 1965). Martin & Schild (1965) also presented evidence that the antagonism between thiols and these disulphide polypeptides is not typically competitive and may be caused by a reversible inactivation of essential disulphide groups in the receptor. Handler & Orloff (1964) have shown in addition that in the toad bladder preparation cysteine inhibits the response to theophylline (which does not contain a disulphide bond).

In view of our recent finding (Furtado, 1966; Furtado & Machado, 1966) that bradykinin inhibits the effects of vasopressin and oxytocin in the toad bladder, apparently acting as a specific and competitive antagonist, and considering the observation of Cirstea (1965) that at least some effects of bradykinin are potentiated by thiol compounds, we decided to further investigate the issue to see whether there was an interaction between bradykinin and thiols in the toad bladder.

Both bradykinin (Furtado & Machado, 1966) and the thiol compounds (Bentley, 1964; Handler & Orloff, 1964) are known to inhibit the increases due to vasopressin in the movement of water as well as the net flux of sodium across the wall of the toad bladder. The interaction of these drugs was thus examined in the isolated urinary bladder of the toad, *Bufo marinus*, using the preparation of Bentley (1958). Each of the sacs of the bilobed bladder was mounted in a chamber with Bentley-Ringer solution bathing the serosal surface, and ½-dilute Ringer within the bladder. The preparation was kept at 25 ± 1°, and at pH 7·4–7·6. The osmotic flow of water was estimated gravimetrically at 20 min intervals. The test substances were added to the serosal Ringer bath. Various doses of cysteine (cysteine hydrochloride, Eastman Kodak) (Cys) were combined with 5·0 mU/ml of vasopressin (Pitressin, Parke, Davis) (V); a single dose (1·0 μg/ml) of bradykinin (synthetic bradykinin, Sandoz) (BK) was also used. The dosage scheme can be diagrammatically represented as follows:

	Group I		Group II	
	Side A	Side B	Side A	Side B
Period I	V	V	V	V
Period II	V	V + BK (or Cys)	V + Cys	V + BK
Period III	V	V + BK + Cys	V + Cys + BK	V + BK + Cys

The control periods (side A in the group I) were used to correct for the percentage calculations because of the customary increase in response observed in applying a neurohypophysial hormone repeatedly to the bladder.

As previously observed (Furtado, 1966; Furtado & Machado, 1966) bradykinin at the dose of 1 μg/ml (1·0 mM) caused about a 50% inhibition of vasopressin (5·0 mU/ml = approximately 0·01 μM). Cysteine alone against vasopressin (0·01 μM) was ineffective at 0·5 mM; it was not until the concentration was increased to 8·0 mM that the effect of vasopressin was statistically significantly inhibited (40% inhibition). Finally, a dose of 12·0 mM cysteine promoted a 50% inhibition of the effects of vasopressin, equalling the degree of antagonism of 1 μg/ml of bradykinin (Table 1).

Fig. 1 shows that in the dose range of cysteine used there was neither summation nor potentiation of the inhibiting effects of bradykinin and cysteine upon vasopressin as far as its action on the permeability to water of the toad bladder is

TABLE 1. EFFECTS OF BRADYKININ, CYSTEINE, AND BRADYKININ + CYSTEINE ON WATER TRANSFER EVOKED BY VASOPRESSIN ACROSS THE TOAD BLADDER

V‡ + Cys (0·5)†	V + BK	V + BK + Cys (0·5)	V + Cys (1)	V + BK	V + BK + Cys (1)	V + Cys (4)	V + BK	V + BK + Cys (4)	V + Cys (8)	V + BK	V + BK + Cys (8)	V + Cys (12)	V + BK	V + BK + Cys (12)
100* (3)	55 (2)	25 (5)	95 (3)	50 (3)	85 (6)	89 (3)	60 (2)	90 (5)	61 (3)	55 (3)	60 (5)	45 (3)	40 (2)	45 (5)

‡ V = vasopressin 5·0 mU/ml;
† Cys = cysteine, dose in mM in parentheses;
BK = bradykinin 1·0 mM;
* The results presented in this horizontal line express the remaining activity of vasopressin in the presence of the drugs (Cys and/or BK) as the percentage of the response obtained with vasopressin alone in the corresponding period.
Number of experiments in parentheses.

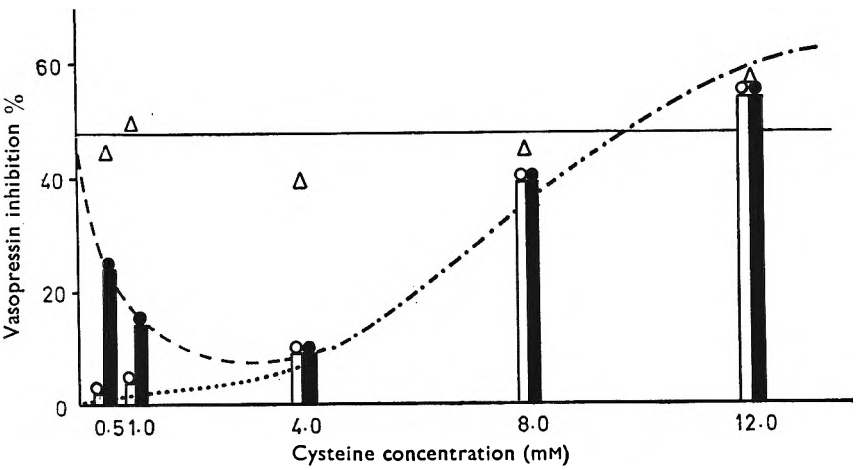

FIG. 1. Inhibition of the effects of vasopressin (5·0 mU/ml) on the permeability to water of the toad bladder by bradykinin, 1·0 mM. (△—△), cysteine in various doses (○.....○, white columns), and bradykinin + cysteine (●---●, black columns). Each point represents the mean of the experimental observations.

concerned. On the contrary, it shows that there was an inhibition of the effects of bradykinin upon vasopressin in the presence of cysteine, and this inhibition was increased by augmenting the concentration of cysteine up to 4·0 mM. By increasing the dose of thiol to 8·0 and 12·0 mM it was possible to exceed even the 50% degree of inhibition that was achieved when bradykinin was used alone against vasopressin.

In view of the present observation of inhibition of the effects of bradykinin by cysteine, both drugs being "agonists" in their having an antagonistic action upon vasopressin in the toad bladder, one may interpret the whole picture as a "competition" for a common site in the receptor. Fig. 1 shows that at low concentrations (0·5 and 1·0 mM) cysteine alone was practically ineffective but was sufficient, in a comparable dosage, to block part of the response to bradykinin, possibly by competing for a common binding site. In the presence of higher doses (4·0–12·0 mM) of cysteine the access of bradykinin to receptor sites was probably completely blocked by the thiol, and the degree of inhibition thus obtained conceivably reflected only the response due to cysteine alone (open columns with the same height as solid columns in Fig. 1).

As previously suggested (Furtado, 1966), bradykinin seems to compete with neurohypophysial hormones for the receptor site through ionic, hydrogen, and hydrophobic bonds rather than by breaking the S—S bridge of the hormones. If bradykinin and cysteine are actually competing for a common site in the receptor this fact makes it probable that thiols might also directly act at a receptor level rather than by solely reducing the hormones.

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Decrease of homovanillic and 5-hydroxyindoleacetic acids in the brain after hypothalamic lesions

SIR,—Destruction of the medial forebrain bundle (MFB) in the lateral hypothalamus decreases the 5-hydroxytryptamine (5-HT), noradrenaline and dopamine content in various parts of the rat brain (Heller & Moore, 1965; Andén, Dahlström, Fuxe & others, 1966). These changes occur not only in areas directly innervated by the axons of the MFB, e.g. the hypothalamus and limbic forebrain, but also in regions not directly connected with the MFB, possibly as a consequence of lesions of other fibres, e.g. nigro-striatal (Andén & others, 1966). The decrease of the cerebral monoamines might be due to different mechanisms, for example diminution of the storage or inhibition of the synthesis of the amines. The finding of a diminished activity of decarboxylase of aromatic amino-acids after lesion of the MFB reported by Heller, Seiden, Porcher & Moore (1965) does not necessarily indicate an impaired synthesis of catecholamines and 5-HT, since decarboxylation of 3,4-dihydroxyphenylalanine and 5-hydroxytryptophan do not seem to be a limiting step in the biosynthesis of these amines (Hess, Connamacher, Ozaki & Udenfriend, 1961). By influencing the cerebral content of homovanillic and 5-hydroxyindoleacetic acids, the major metabolites of dopamine and 5-HT respectively, further information might be gained on the mechanism which leads to a decrease of the aromatic monoamines. Therefore these acids, as well as the chlorpromazine-induced increase of homovanillic acid, were measured in the basal parts of each brain side after unilateral lesions of the MFB. Chlorpromazine was chosen since the drug enhances the hydroxylation of tyrosine *in vivo* and increases the formation of homovanillic acid in the brain possibly as a consequence of a primary blockade of dopaminergic receptors (Andén, Roos & Werdinius, 1964; Burkard, Gey & Pletscher, 1967).

TABLE 1. EFFECT OF ELECTROLYTIC LESIONS OF THE MEDIAL FOREBRAIN BUNDLE (MFB) ON THE CONTENT OF MONOAMINES AND METABOLITES AS WELL AS ON THE CHLORPROMAZINE-INDUCED RISE OF HOMOVANILLIC ACID (HVA) IN THE BASAL BRAIN PARTS OF RATS

Monoamine or metabolite	Control side	Side with lesion	
	Absolute values (μg/g)	Absolute value (μg/g)	value %
Dopamine	2·56 ± 0·19	0·82 ± 0·35	31 ± 11 (I)
Homovanillic acid	0·11 ± 0·01	0·05 ± 0·01	48 ± 4 (II)
Homovanillic acid after chlorpromazine	0·38 ± 0·06	0·11 ± 0·01	32 ± 7 (III)
Noradrenaline	0·78 ± 0·19	0·46 ± 0·06	62 ± 4 (IV)
5-Hydroxytryptamine	1·03 ± 0·12	0·72 ± 0·08	71 ± 2 (V)
5-Hydroxyindoleacetic acid	0·53 ± 0·01	0·43 ± 0·03	81 ± 3 (VI)

The determinations were made 15 days after the lesion. 10 mg/kg chlorpromazine were administered i.p. 2 hr before death. Each figure represents an average ± s.e. of 3–6 experiments. The percentage values were calculated for each individual experiment, the side without lesion serving as the control.

Significance: I, II, III, IV, V, VI $P<0.01$
I: IV $0.01<P<0.05$
I: V $P<0.01$
II: VI $P<0.01$

In male albino rats, unilateral (right side) electrolytic lesions were made according to Heller, Harvey & Moore (1962). The left side was sham-operated performing all the surgical procedures of the right side, but introducing the electrode in the brain above the target area without delivering current. Since most of the MFB fibres are uncrossed (Guillery, 1957), the left side was used as a control for the biochemical determinations. The animals were killed 15 days later after a fasting period of 16 hr. Histological controls indicated that the electrolytic lesions were located in the region of the MFB. Partial damage of the nigro-striatal fibres can, however, not be excluded.

In addition, in the basal parts (basal ganglia including limbic structures, thalamus and hypothalamus) of each brain side, dopamine, homovanillic acid, 5-HT, 5-hydroxyindoleacetic acid and noradrenaline were measured by spectrophotofluorimetric procedures (ref. Pletscher, Bartholini, Bruderer & others, 1964). Furthermore, estimations of homovanillic acid were made 2 hr after intraperitoneal injection of 10 mg/kg chlorpromazine, the animals being kept in an environment of 31° to prevent the development of hypothermia.

The present results (Table 1) confirm earlier reports (Heller & Moore, 1965; Andén & others, 1966) by demonstrating that unilateral lesions of the MFB cause a decrease of dopamine, noradrenaline and 5-HT. The dopamine is more markedly affected than 5-HT and noradrenaline. Our findings furthermore show that, together with the diminution of the amines, a significant decrease of homovanillic and to a lesser extent also of 5-hydroxyindoleacetic acid occurs on the injured side. In addition, the chlorpromazine-induced increase of homovanillic acid as observed on the control side is markedly attenuated on the side with the lesion.

These findings indicate that by lesions of the MFB the synthesis of dopamine, 5-HT and possibly noradrenaline in the basal brain parts might be impaired.

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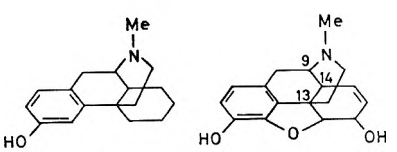
Optical rotatory dispersion characteristics of (−)-3-hydroxy-*N*-methylmorphinan and (−)-morphine

SIR,—The relative configurations of (−)-3-hydroxy-*N*-methylmorphinan (I, levorphanol) and (−)-morphine (II) are of interest in view of the established stereoselectivity of the analgesic receptor towards many classes of analgesics (Beckett & Casy, 1965). In the absence of direct chemical methods, the use of optical rotatory dispersion (o.r.d.) data (Crabbé, 1965) offers the most promising physical method for the stereochemical correlation of (−)-I and (−)-II. The following results were obtained using a Polarmatic 62 photo-electric spectropolarimeter. The o.r.d. curve of (−)-morphine base (*c*, 0·02% in ethanol, 0·05 dm cell) showed a negative Cotton effect, $[\phi]^{25}_{340}-1499$, $[\phi]^{25}_{303}-4123$, $[\phi]^{25}_{291}-5620$ (trough), $[\phi]^{25}_{281}+4120$ (inflection), $[\phi]^{25}_{256}+13490$ (peak), $[\phi]^{25}_{244}-30240$ (limit of measurement); the curve for (−)-morphine hydrochloride was similar with trough characteristics, $[\phi]^{25}_{290}-5223$. Bobbitt, Weiss & Henessian (1959), using a less sensitive spectropolarimeter, recorded the o.r.d. curves of morphine, codeine and dihydrocodeine in dioxane and observed negative Cotton effects with troughs near 300 mμ in each case; values beyond 298 mμ could not be obtained. The o.r.d. curve of levorphanol base (*c*, 0·01% in ethanol, 0·05 dm cell) also showed a negative Cotton effect, $[\phi]^{25}_{400}-3375$, $[\phi]^{25}_{313}-15520$, $[\phi]^{25}_{290}-40460$ (trough), $[\phi]^{25}_{288}-33050$ and $[\phi]^{25}_{278}-35780$ (fine structure, absent in the salt), $[\phi]^{25}_{268}+16190$ (peak); in 0·1N hydrochloric acid-ethanol, trough and peak characteristics were $[\phi]^{25}_{291}-45320$ and $[\phi]^{25}_{270}+18880$ respectively.



The negative Cotton effects of (−)-I and (−)-II are attributed to the optically active phenolic chromophore because the Cotton effect mid-points (near 286 mμ for morphine and 280 mμ for levorphanol) are close to the phenolic ultraviolet absorption maxima of the two compounds [morphine λ_{max} 285 (base), 288 mμ (salt); levorphanol λ_{max} 284 (base), 283 mμ (salt) in ethanol]. Differences in the o.r.d. curves of I and II, viz. characteristics at wavelengths below 280 mμ and the lower $[\phi]^{25}_{29}$ (trough) value for (−)-II, probably arise as a result of the presence in (−)-II of optically active chromophores (additional to the phenolic function) that are absent in (−)-I, a compound of simpler structure.

Since the sign of a Cotton effect is governed chiefly by the stereochemical environment of the responsible optically active chromophore (Crabbé & others, 1965), the identity of sign noted for Cotton effects in (−)-I and (−)-II provides strong evidence for the configurations of the C-9, 13 and 14 asymmetric centres of (−)-morphine being the same as those of the corresponding centres of levorphanol. Portoghese (1966, quoting unpublished data) reports that the o.r.d. curves of some (−)-phenolic benzomorphan derivatives exhibit Cotton effects which also have the same sign as that of morphine. Hence, the results of o.r.d. studies substantiate previous conclusions of configuration based upon work involving stereoselective adsorbents (Beckett & Anderson, 1960).

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Note added in proof. Weiss & Rull (1965) report Cotton effects (by circular dichroism measurements) of similar sign near 260μ) for (—)-3-methoxy-*N*-methylmorphinan, dihydrodesoxycodeine and tetrahydrodesoxycodeine, results which provide further evidence of configuration in morphinan derivatives.

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Excretion of a glucuronide conjugate of 3-hydroxyphenyltrimethylammonium

Sir,—We have found that, after intramuscular injection of neostigmine in the rat, about 50% of the dose is excreted in the urine unchanged, a high proportion of the remainder being metabolized in the liver and excreted in the urine as 3-hydroxyphenyltrimethylammonium (Roberts, Thomas & Wilson, 1963, 1965a,b). We anticipated that some of this phenolic metabolite might be excreted as a conjugated product (described by Williams, 1959), but the paper electrophoresis technique we used did not enable us to establish this point.

Further investigation using [^{14}C]3-hydroxyphenyltrimethylammonium by intramuscular injection into rats showed that when the urine was examined by a modification of the electrophoresis procedure (Veronal buffer pH 7·0, 0·05M), two peaks of radioactivity were obtained. One of these peaks corresponded with a concurrently run authentic sample of 3-hydroxyphenyltrimethylammonium, the other was tentatively assumed to be the glucuronide conjugate of this compound. This assumption was confirmed by incubating samples of urine with β-glucuronidase ("Ketodase", Warner & Co.) and when these were subjected to paper electrophoresis, only the peak for 3-hydroxyphenyltrimethylammonium was identified.

Using this procedure urine was collected from rats for periods up to 24 hr after intramuscular injection of [^{14}C]3-hydroxyphenyltrimethylammonium. Fig. 1

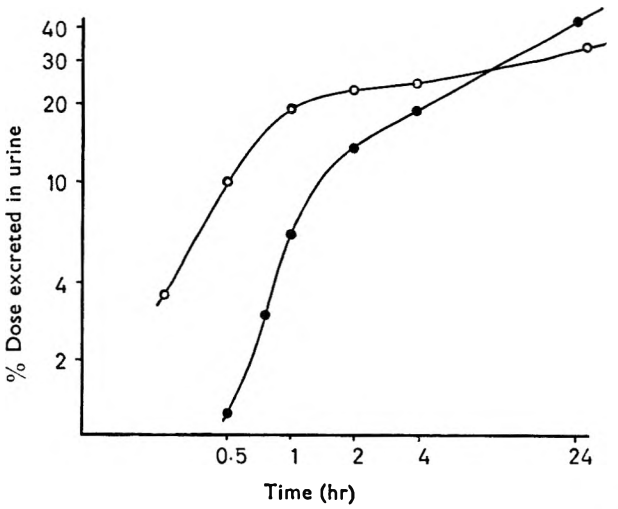

FIG. 1. Excretion in rat urine of 3-hydroxyphenyltrimethylammonium (○——○) and its glucuronide conjugate (●——●) after intramuscular injection of 100 μg of [^{14}C]3-hydroxyphenyltrimethylammonium. Each point is the mean of 3 experiments.

shows that more than half the dose was excreted as the glucuronide in 24 hr. Additional experiments using [^{14}C]neostigmine by intramuscular injection have shown that during 24 hr approximately 50% of the phenolic metabolite is excreted as the glucuronide conjugate.

The relevance of this mechanism for the metabolism and excretion of neostigmine by patients with myasthenia gravis is currently under investigation.

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The *N*-oxidation of chlorpromazine *in vitro*—the major metabolic route using rat liver microsomes

SIR,—Incubation of chlorpromazine with suitably fortified liver homogenates has been shown to result in sulphoxidation (Salzmann & Brodie, 1956; Gillette & Kamm, 1960), demethylation (Young, Ross & Maass, 1959; Ross, Flanagan & Maass, 1962), and hydroxylation (Robinson & Beaven, 1964) of the drug molecule.

Recently (Curry, 1965; Robinson, 1966), attempts were made to obtain a comprehensive picture of *in vitro* chlorpromazine metabolism after incubation

with fortified rat liver microsomes and soluble fraction; ether was used as solvent to extract chlorpromazine and its metabolites. Extraction was made from alkaline solution and the extract analysed by thin-layer chromatography. The expected products were found, namely chlorpromazine, chlorpromazine sulphoxide, demethylchlorpromazine, didemethylchlorpromazine and 7-hydroxychlorpromazine. Demethylhydroxychlorpromazine (Robinson, 1966) was also detected.

We have found, however, that *N*-oxidation is the major route in the biotransformation of chlorpromazine by rat liver microsomes, the previously described metabolic routes being relatively minor. The failure by previous workers to detect the formation of chlorpromazine *N*-oxide may be partially explained by the fact that ether is unsatisfactory as the sole extracting solvent for all chlorpromazine metabolites. For instance, when an incubation mixture comprising [^{35}S]chlorpromazine hydrochloride and rat liver microsomes fortified with nicotinamide adenine dinucleotide phosphate (NADP), glucose 6-phosphate,

TABLE 1. THIN-LAYER CHROMATOGRAPHY OF CHLORPROMAZINE, ITS METABOLITES, AND REFERENCE COMPOUNDS ON SILICA-GEL PLATES

Compound	Rf values and solvent systems[1] A Methanol–Diethylamine–Benzene (15:10:75)	B[2] Chloroform–Acetone–Diethylamine (88:2:10)	C Dioxan–Diethylamine–Water–Benzene (17·5:7·5:1:70)	Reaction with 50% v/v H_2SO_4 spray	Metabolite indicated
Combined ether and n-butanol extracts of alkalinized incubation mixture					
Chlorpromazine ..	0·83	0·79	0·70	Pink	
Metabolite A	0·75	0·66	0·54	Pink	Demethylchlorpromazine
„ B	0·73	0·70	0·46	Pink[3]	Chlorpromazine sulphoxide
„ C	0·58	0·31	0·24	Purple	7-Hydroxychlorpromazine
„ D	0·48	0·45	0·11	Pink[3]	Demethylchlorpromazine sulphoxide
„ E	0·18	0·08	on base line	Pink	Chlorpromazine* *N*-oxide[4]
„ F	0·10	on base line	on base line	Pink[3]	Chlorpromazine *N*-oxide sulphoxide
Chlorpromazine ..	0·84	0·79	0·70	Pink	
Demethylchlorpromazine	0·75	0·66	0·54	Pink	
Chlorpromazine sulphoxide ..	0·75	0·71	0·46	Pink[3]	
7-Hydroxychlorpromazine ..	0·58	0·33	0·26	Purple	
Demethylchlorpromazine sulphoxide ..	0·48	0·45	0·12	Pink[3]	
Chlorpromazine *N*-oxide	0·20	0·08	on base line	Pink	
Chlorpromazine *N*-oxide sulphoxide	0·11	on base line	on base line	Pink[3]	
Didemethylchlorpromazine ..	0·75	0·78	0·60 (streaks)	Pink	
7-Hydroxychlorpromazine sulphoxide ..	0·19	on base line	on base line	Purple	

[1] Thickness of silica gel 250 μ; distance of solvent development about 18 cm; measurements for Rf values made from leading edge of spot; temperature 21° ± 1°.
[2] Fishmann & Goldenberg, 1965.
[3] Colour development only on heating.
[4] Traces of 7-hydroxychlorpromazine sulphoxide are possible.
*Further confirmed by reduction with zinc and dilute hydrochloric acid to yield chlorpromazine.

magnesium chloride, nicotinamide, and glucose 6-phosphate dehydrogenase, was made alkaline with ammonia and extracted with two volumes of ether per extraction, 53, 37, 27, 25 and 22% of the initial radioactivity remained respectively after each successive extraction. Two ether extractions followed by two n-butanol extractions resulted in only 0·5% of the initial radioactivity being left in the aqueous phase.

Combined ether and n-butanol extracts were concentrated by distillation under vacuum at about 70° and examined by thin-layer chromatography; the systems and results are shown in Table 1.

The metabolic extract contained chlorpromazine, chlorpromazine sulphoxide, demethylchlorpromazine, demethylchlorpromazine sulphoxide, 7-hydroxychlorpromazine, chlorpromazine *N*-oxide, and chlorpromazine *N*-oxide sulphoxide. Didemethylchlorpromazine was found to be absent using two-dimensional thin-layer chromatography with systems B and C (see Table 1).

N-Oxidation (34%), demethylation (12%), hydroxylation (2%) plus the formation of 10% of chlorpromazine sulphoxide occurred when 2·5 μM of [^{35}S] chlorpromazine hydrochloride were incubated with male rat liver microsomes (equivalent to 5·8 mg of protein) fortified with optimal amounts of NADP, glucose 6-phosphate, magnesium chloride, nicotinamide and glucose 6-phosphate dehydrogenase, in a total volume of 4 ml. Measurements were made by scintillation counting of the spots removed from the plates.

Preliminary co-factor requirement experiments indicate that the N-oxidation, demethylation and hydroxylation reactions are NADP-dependant, whereas chlorpromazine sulphoxide formation is only partially NADP-dependant. Using the procedures outlined above, over half the chlorpromazine sulphoxide was found to be produced non-enzymatically, indicating that care should be taken in the interpretation of results where the assay of chlorpromazine sulphoxide is used as a measure of drug metabolism.

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Letters to the Editor

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