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The polymorphism of phenobarbitone

R. J. MESLEY, R. L. CLEMENTS, B. FLAHERTY AND K. GOODHEAD

The polymorphism of phenobarbitone has been investigated using infrared spectroscopy, X-ray diffraction and differential scanning calorimetry. Eight crystalline modifications have been isolated and shown to have distinguishable infrared absorption spectra and X-ray powder diffraction patterns. Other forms were found to be present in mixed crystals and molecular compounds obtained from mixtures of phenobarbitone with other barbiturates. Differential thermograms of three forms of thialbarbitone are also given.

THE polymorphic modifications of phenobarbitone have been investigated by several authors using various techniques and it is not easy to decide exactly how many forms have already been reported. Huang (1951a), using microsublimation, prepared four forms which he designated I–IV, and which were characterized by hot-stage microscopy and by X-ray diffraction patterns. He also reported (Huang, 1951b) a form IVa, obtained by crystallization from a melt, and subsequently (Huang, 1951c) a form IVb, though the latter was encountered only in the presence of other barbiturates.

TABLE 1. KNOWN POLYMORPHIC FORMS OF PHENOBARBITONE

Form	Melting point °C (a)	Melting point °Abs (b)	Remarks
I	176	448	
II	174	446	
III	167	440	
IV	163	435	Huang's IVa probably mixture of this with XII
V	160		Probably Huang's IVb
VI	157		Huang's form IV
VII	153		Not encountered in this work
VIII	141		Not encountered in this work
IX	133		Encountered only in mixed barbiturates
X	126		Encountered only in mixed barbiturates
XI	112		Not encountered in this work
XII	not known		New form not previously reported
XIII	not known		Form V of Cleverley & Williams

(a) Taken from Brandstätter-Kuhnert & Aepkers (1962).
(b) Onset temperature of melting transitions observed by differential scanning calorimetry.

Cleverley & Williams (1959), working mainly on materials recovered from solutions, obtained Huang's forms I, II and III and a new form which they designated V. In addition to X-ray diffraction patterns they recorded infra-red spectra of these forms, though they reported that forms I and II gave identical spectra. They also noted that form V differed from the other forms in showing absorption bands attributable to OH groups, and they suggested that it existed in the enol rather than the normal keto-form.

Brandstätter-Kuhnert & Aepkers (1961, 1962), using only hot-stage microscopy, reported a total of eleven forms, though six of these were stable only in the presence of other barbiturates. Of the stable forms, I, II and III correspond to those of Huang, whilst from their melting points it is apparent that Brandstätter-Kuhnert's form IV is Huang's form IVa, and her form VI is Huang's form IV.

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In the course of the present work eight relatively stable forms of phenobarbitone have been prepared, and in trying to correlate these with the unstable forms of Brandstätter-Kuhnert, we have produced two more forms in the presence of other barbiturates, one of which is probably stable on its own. All of these ten forms were found to be distinguishable by means of their infrared spectra and of their X-ray diffraction patterns. In the account which follows, the numbering of Brandstätter-Kuhnert is used where possible, and the additional forms have been given new numbers. A list of all known forms is given in Table 1.

Experimental and results

MATERIALS

Commercial phenobarbitone was recrystallized from aqueous ethanol. It was thus obtained in form II and its melting point, as determined by normal capillary tube technique, was 174–177° C.

INFRARED SPECTROSCOPY

Infrared absorption spectra of all forms obtained were recorded as mulls in liquid paraffin using a Grubb Parsons GS2 grating spectrometer, which has a linear wavelength scale (see Figs 1–4). They were also recorded on a Perkin-Elmer 237 grating spectrometer with a linear wave-number scale. With each instrument spectra of all forms were clearly distinguishable from each other.

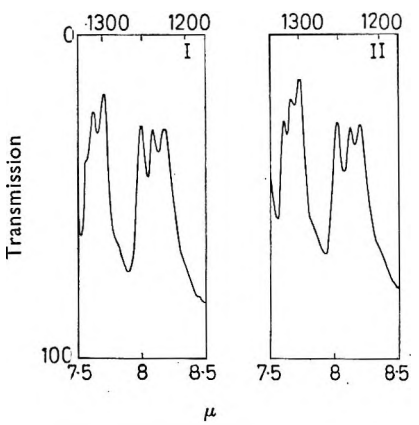

FIG. 1. Comparison of infrared spectra of forms I and II of phenobarbitone.

X-RAY DIFFRACTION

X-ray powder diffraction patterns were recorded photographically using a Unicam 9 cm camera and vanadium-filtered chromium Kα radiation. Measurements of I/I_0 were made with a Joyce recording microdensitometer. Patterns of forms I, II, III and VI agreed with those given by Huang (1951a) as forms I–IV. Patterns of forms IV, V, XII and XIII are given in Table 2 (that for form XIII agrees with the

pattern published by Cleverley & Williams (1959) as form V, but is included here for comparison with form XII, with which it is similar).

DIFFERENTIAL SCANNING CALORIMETRY

For the thermal analysis a Perkin-Elmer DSC-1B apparatus was used. The carrier gas was dry nitrogen at 20 ml/min, and the rate of heating employed throughout was 8°/min. Samples were hermetically sealed in aluminium pans to reduce the loss of material by sublimation, and sample weights varied from 1–10 mg. The temperatures quoted (° Abs.) represent the onset temperatures of transitions and are accurate to $\pm 1°$, indium being used for calibration. Melting points so obtained are on average one degree lower than those quoted by Brandstätter-Kuhnert & Aepkers (1962). Thermal analysis was carried out in two ways:

TABLE 2. X-RAY DIFFRACTION PATTERNS OF PHENOBARBITONE FORMS

Form IV		Form V		Form XII		Form XIII	
d(A)	I/Io	d(A)	I/Io	d(A)	I/Io	d(A)	I/Io
15·6	50	16·3	14	15·1	67	15·6	100
11·24	33	13·57	21	11·13	4	7·88	30
9·15	41	12·80	96	8·92	8	6·94	3
7·81	5	11·24	17	7·76	16	6·49	7
6·37	50	6·77	60	7·13	7	5·95	80
5·97	10	6·34	100	6·88	15	5·50	31
5·71	100	6·00	55	6·44	18	5·37	33
5·48	20	5·70	40	5·94	29	5·25	11
5·10	25	5·20	30	5·79	30	4·79	6
4·61	32	4·73	32	5·67	32	4·53	3
4·51	19	4·57	21	5·32	90	4·34	3
4·28	12	4·36	21	5·15	14	4·19	4
4·14	7	4·19	8	4·81	4	3·94	100
3·91	5	4·04	14	4·64	6	3·77	5
3·73	62	3·87	8	4·20	25	3·59	11
3·54	9	3·71	50	3·93	100	3·41	7
3·38	8	3·49	8	3·73	8	3·38	5
3·24	40	3·38	10	3·45	9	3·24	5
3·01	6	3·28	14	3·35	10	3·12	9
2·91	12	3·16	10	3·22	14	3·01	14
2·84	17	2·84	55	3·14	16	2·89	10
		2·70	14	3·07	10	2·81	31
		2·20	17	2·97	8	2·71	4
		2·01	32	2·95	11	2·63	10
				2·79	21	2·60	5
				2·71	4	2·50	4
				2·66	4	2·36	4
				2·63	6	2·24	5
				2·57	6	2·18	3
				2·35	6	1·94	8

Procedure A: a freshly prepared powdered sample was heated at 8°/min from ambient temperature to 10° above the melting point of the most stable form, i.e., 460° Abs.

Procedure B: following procedure A, the sample was held at 460° Abs for 2–3 min, then cooled quickly by means of the manual control on the instrument to 310–350° Abs, maintained at this temperature for 10–15 min and then re-heated as before.

PRODUCTION AND IDENTIFICATION OF DIFFERENT FORMS

Form I was obtained by heating all other forms at temperatures above 150°. A sample which was stored for two months at room temperature reverted to form II.

Form II is the form in which commercial samples are normally obtained, and was produced by heating all forms except I at 130–140° C. It is apparently the most stable form at room temperature. The infrared spectra of forms I and II are very similar, but may be distinguished by their absorptions near 1300 cm^{-1} (Fig. 1).

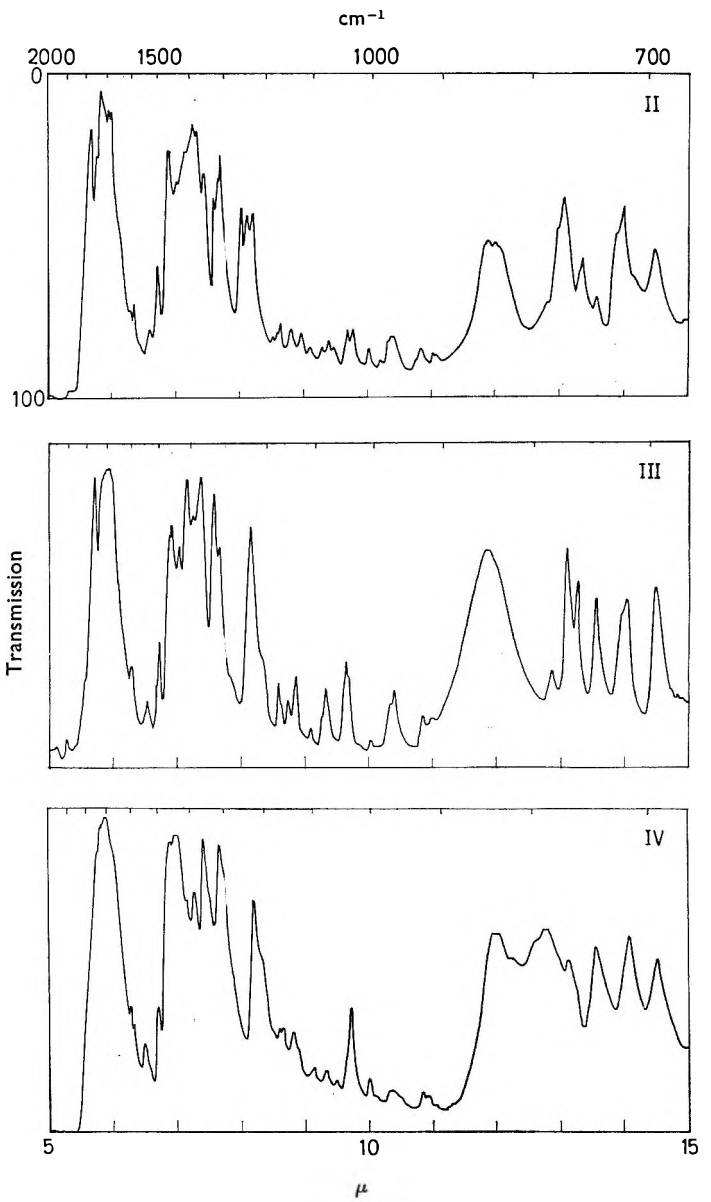

FIG. 2. Infrared spectra of phenobarbitone forms II, III and IV.

Form III was obtained by heating most other forms at 100–120° C. On heating above 120° C, or on long standing at room temperature it was converted to form II.

Brandstätter-Kuhnert & Aepkers (1961, 1962) reported that at temperatures between 100° and 140° C crystallization from a supercooled melt could produce forms II, IV and VI. The melting point quoted for form IV, 163° C, agrees with that given by Huang (1951b) for his form IVa which he prepared by crystallization between two cover slips. Using this technique we have on several occasions obtained a homogeneous

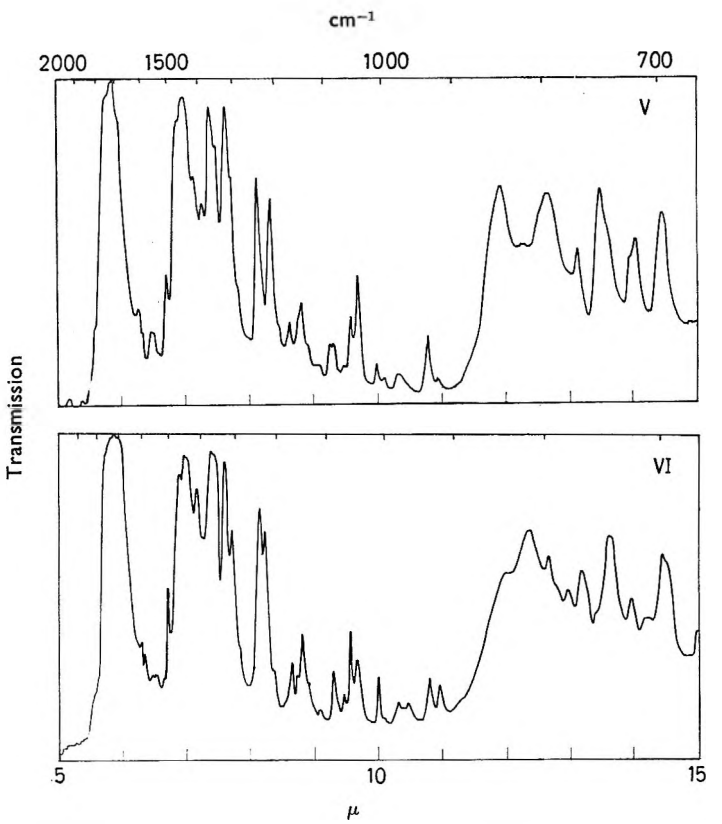

FIG. 3. Infrared spectra of phenobarbitone forms V and VI.

product giving an X-ray diffraction pattern which differs in several respects from that of Huang's form IVa. However, in his work on mixtures of barbiturates, Huang (1951c) includes patterns described as "similar to IVa" which closely resemble our pattern. If the original form IVa pattern is now compared with these, it is seen to include additional lines at 5·87, 5·44, 3·90 and 2·78 Å. These are in fact four of the strongest lines in the pattern given by Cleverley & Williams (1959) for their form V (here designated form XIII): corresponding lines also

occur in the form XII pattern. It therefore seems possible that Huang's form IVa was not a pure single form, and we believe that our product may be identified as Brandstätter-Kuhnert's form IV.

Form V was not obtained in a pure state by Brandstätter-Kuhnert & Aepkers, but was said to be stable in the presence of as little as $2\frac{1}{2}\%$ of Rutonal. These authors also stated that it was structurally similar to form IV and showed reciprocal seeding properties with this. In the present work a form was frequently obtained which has an infrared spectrum similar to that of form IV, and which was often contaminated

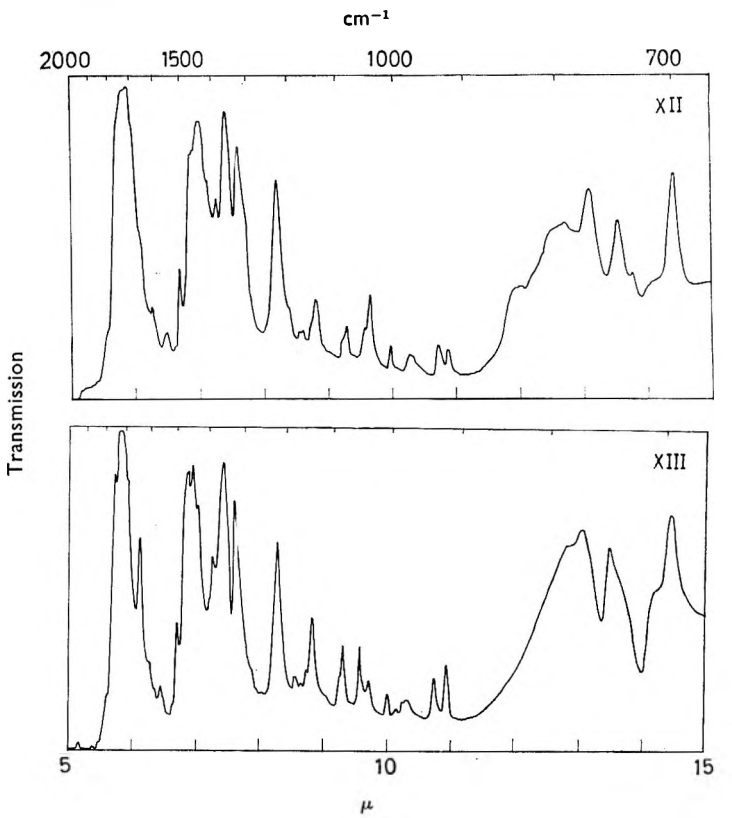

FIG. 4. Infrared spectra of phenobarbitone forms XII and XIII.

with form IV. Ether solutions of either of these forms when evaporated to dryness usually gave a mixture of the two forms. This new form, although obtained several times by crystallization from a melt of pure phenobarbitone, was most often encountered in melts containing other barbiturates, particularly pentobarbitone and amylobarbitone, and there seems little doubt that it is Brandstätter-Kuhnert's form V. The X-ray diffraction pattern shows a general resemblance to that of Huang's form IVb, obtained in the presence of 10% hexobarbitone, and that of a similar form obtained in the presence of 10% barbitone (Huang, 1951c).

Brandstätter-Kuhnert's form VI has the same melting point (157°) as Huang's form IV, and they are therefore presumably the same. A product was sometimes obtained by crystallization from a melt maintained at about 90° C which gave an X-ray diffraction pattern corresponding to that of Huang's form IV, so this is presumably form VI.

It should be mentioned that no consistent method was found for preparing forms IV, V and VI. Crystallization from melts maintained at temperatures near 100° C, either between two glass cover slips or on a single glass plate, gave variously forms II, III, IV, V and VI and frequently two different forms crystallized simultaneously. Seeding with material previously prepared usually gave a uniform product.

Two other forms of phenobarbitone were not obtained by crystallization from melts, and it seems likely that they are not included in the eleven forms listed by Brandstätter-Kuhnert & Aepkers. The less stable form, corresponding to form V of Cleverley & Williams (1959) is here designated form XIII and was obtained as a precipitate by addition of hydrochloric acid to an alkaline solution of phenobarbitone. It could also be prepared by grinding form II in water, and this suggests that it may be hydrated; this possibility is considered below. It is difficult to remove surface moisture from this form without causing a change to another state. Cleverley & Williams found that the transition to form III began to occur at 40° C, and even at room temperature some change occurs within 24 hr.

Attempts to remove surface moisture from form XIII by storage for a week in a vacuum desiccator over concentrated sulphuric acid or phosphorus pentoxide caused complete conversion to form XII, which also appears to be an enol form, and which has a very similar X-ray diffraction pattern to form XIII. Form XII is also very unstable on heating and at as low a temperature as 60° C was completely converted to either form III or form II in 1 hr. The former is probably the normal behaviour, as the material which gave form II may have contained a trace of this modification which acted as a seed for the remainder.

Some work has been done on mixtures of phenobarbitone with other barbiturates, as a result of which two more forms were identified, apparently corresponding to Brandstätter-Kuhnert's forms IX and X. Form IX was stated (Brandstätter-Kuhnert & Aepkers, 1963) to be isomorphous with several other barbiturates, including amylobarbitone II and the stable form I of cyclobarbitone. In the present work, crystals obtained from a mixed melt containing approximately equal proportions of phenobarbitone and amylobarbitone gave a spectrum similar in certain respects to that of material obtained by seeding a phenobarbitone melt with cyclobarbitone, and both of these were attributed to the presence of form IX. In the latter instance other forms of phenobarbitone were also observed, but the proportion of form IX was quite high even in parts of the crystal mass well separated from the cyclobarbitone seed, and it seems likely that form IX is capable of independent existence in pure phenobarbitone.

Form X is also isomorphous with several other barbiturates, including amylobarbitone I and pentobarbitone I. Mixed melts containing two

parts of phenobarbitone and one part of either amylobarbitone or pentobarbitone were seeded with a crystal of the appropriate barbiturate, giving products with almost identical infrared spectra. These and the X-ray diffraction patterns showed a marked resemblance to those of amylobarbitone I and pentobarbitone I and were presumably mixed crystals of phenobarbitone X with the other barbiturates.

In the course of the work with mixtures of phenobarbitone and cyclobarbitone a product was obtained on two occasions from mixed melts which had an infrared spectrum similar to phenobarbitone IX, yet clearly distinguishable from this. The X-ray diffraction pattern of this mixture was generally similar to those of forms IX and X, and also to the two patterns given by Huang (1951c) for phenobarbitone-cyclobarbitone mixtures. Huang attributed these to a molecular compound which he

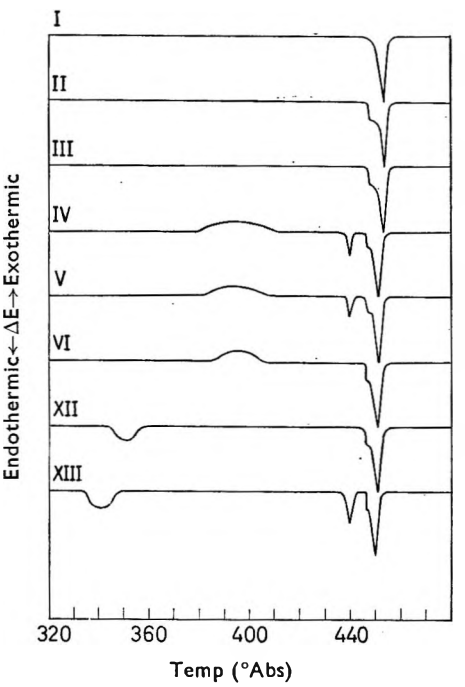

FIG. 5. Differential thermograms of eight forms of phenobarbitone (Procedure A).

thought might exist in two polymorphic modifications. Brandstätter-Kuhnert & Aepkers (1962) subsequently showed that an equimolecular mixture of these two substances could exist as either (*a*) a molecular compound between phenobarbitone IX and cyclobarbitone I—this molecular compound was isomorphous with phenobarbitone V and could be obtained by seeding a mixed melt with phenobarbitone IV; or (*b*) mixed crystals containing phenobarbitone X and the unstable cyclobarbitone II. The product obtained in the present work was indeed produced on one occasion by seeding with phenobarbitone IV, and its

infrared spectrum showed clear differences from phenobarbitone X, so it may be assumed that this was in fact the molecular compound.

DIFFERENTIAL SCANNING CALORIMETRY

Procedure A. The thermograms of eight forms of phenobarbitone are shown in Fig. 5. Thermal analysis shows that all forms absorb approximately the same amount of energy on melting, the final transition corresponding to the melting of form I at 448° Abs.

Forms I and II show sharp melting (endothermic) transitions at 448° Abs and no other transitions are discernible. The other forms investigated all show one or two small, additional endothermic transitions at 440° and 446° Abs, corresponding to the melting of forms III and II respectively. In addition, the two enol forms, XII and XIII, show a large, broad endothermic transition in the region 330–350° Abs, the significance of which is discussed below. Forms IV, V and VI, all of which were prepared by crystallization from melts, show a small, broad (exothermic) transition in the region 390–400° Abs. These transitions are possibly due to crystallization of residual super-cooled liquid occluded in the crystals.

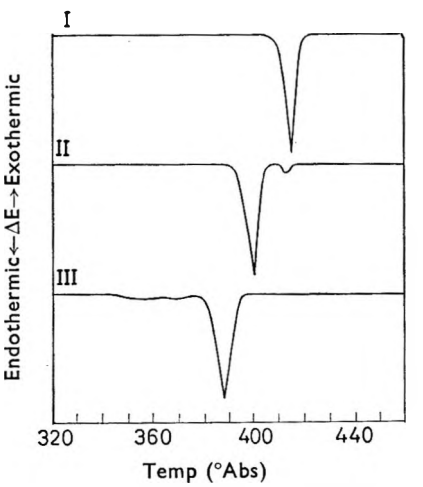

FIG. 6. Differential thermograms of three forms of thialbarbitone (Procedure A).

From Fig. 5 it is apparent that samples of, for example, forms IV, V and XIII, the homogeneity of which has been demonstrated by infrared spectroscopy, can produce transitions corresponding to forms III, II and I, and this sequence of polymorphic changes is thus confirmed. No information is given, however, regarding the possible transitions between the less stable modifications.

Thermograms are shown in Fig. 6 for the three forms of thialbarbitone. When heated in sealed pans, these give rise to distinctive thermograms, the melting transitions of the three forms being readily discernible at

approximately 378°, 393°, and 407° Abs (a commercial sample of thialbarbitone form II, when heated in a capillary tube, showed partial melting at 124° C and melted completely at 138° C). In addition a very broad endothermic transition is observed in form III commencing at about 345° Abs. As this form apparently has the enol structure (Cleverley & Williams, 1959) there is thus an analogy with the transitions in this region in the two enol forms of phenobarbitone. When thialbarbitone form III was heated in a pan which was not completely sealed, the transition at 378° Abs was not observed and the resulting thermogram was barely distinguishable from that of form II.

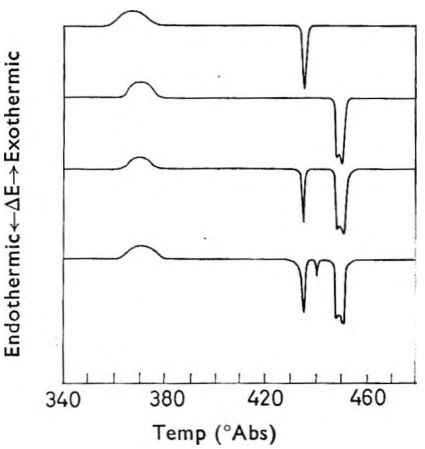

FIG. 7. Typical differential thermograms obtained from supercooled melts of phenobarbitone (Procedure B).

Procedure B. By this procedure, melted samples were cooled rapidly to 310–350° Abs (samples removed at this stage were found to be supercooled melts; when scratched they all gave form III) and then re-heated at 8°/min. The resulting thermograms showed considerable variation, typical results being shown in Fig. 7. These differ from all thermograms obtained by procedure A in two respects: firstly there is a broad exothermic transition of variable geometry in the region 360–390° Abs, which represents crystallization of the super-cooled liquid; and secondly there is a strong endothermic transition at 435° Abs in most of the thermograms. This temperature corresponds to the melting of form IV, and the presence of this was confirmed by removing some samples at about 400° Abs, after the appearance of the crystallization peak, and determining their infrared spectra. Three such samples were found to be form IV, whilst on a fourth occasion form VI was obtained.

It is significant that when powdered form IV was examined by procedure A, the transition at 435° Abs was not observed. The transition from form IV to form III, which takes place rapidly in the finely divided powder, is obviously retarded in the compact mass obtained by crystallization from the melt to such an extent that some of the form IV remains

unchanged up to its melting point. The thermograms of the various forms of phenobarbitone as determined by procedure A, although showing certain fairly consistent features, are thus not independent of particle size, so that any attempt to identify the different forms by this means alone should be treated with caution.

Discussion

Of the many crystalline forms of phenobarbitone, two stand out from the remainder in their infrared spectra and differential thermograms, and also in their method of preparation, namely forms XII and XIII. The infrared spectra are characterized by the presence of absorptions near 3500 cm^{-1}, apparently due to OH groups, and the absence of a strong band near 840 cm^{-1}, assigned by Cleverley & Williams (1959) to the N–H out-of-plane bending vibration. These characteristics are also shown by thialbarbitone form III, and there is little doubt that they are due to enolization. Form XII of phenobarbitone differs from form XIII and from thialbarbitone form III in the absence of the peak near 1620 cm^{-1}, which could reasonably be assigned to a conjugated C=N group in the enolized structure. Also in this form the O–H stretching absorptions are relatively weaker than the N–H stretching bands, suggesting that it has less enolic character than form XIII. It seems unlikely that the two forms can be represented simply as mono- and di-enols, since the C=O stretching absorption in form XIII still appears to represent more than one carbonyl group. However, the presence of small, non-stoichiometric amounts of keto-groups in a predominantly enol structure, and vice versa, has already been suggested for certain crystalline forms of oestradiol (Smakula, Gori & Wotiz, 1957), ethinyloestradiol and spironolactone (Mesley, 1966).

An alternative explanation of the 1620 cm^{-1} band and of the stronger OH absorptions in form XIII could be the presence of water of crystallization. This would be quite possible, since form XIII is only obtained in the presence of water, whilst the transition to form XII takes place under conditions conducive to dehydration. On the other hand, the 1620 cm^{-1} band in thialbarbitone form III is not due to water, since this form has been obtained from a melt, and the thermogram for phenobarbitone form XIII is not apparently consistent with loss of water. The strong endothermic transition at about 340° Abs shows a superficial resemblance to those obtained by Carless, Moustafa & Rapson (1966) for the hydrated forms of cortisone acetate. However, in the latter, the transitions were observed at temperatures above the boiling point of water, whereas in the case of phenobarbitone the transition is completed well below 100° C, with no further change occurring before the melting of form III. There is a further analogy with the work of Carless, Moustafa & Rapson in that they reported that the anhydrous form III of cortisone acetate could only be prepared in the presence of water.

It must therefore be concluded that the low-temperature transitions in phenobarbitone forms XII and XIII and thialbarbitone form III are

primarily associated with the enol-keto tautomeric change, though it is possible that a trace of water is necessary for the existence of form XIII. The greater energy absorption in form XIII relative to form XII would then be in accordance with the previous suggestion that it has a greater degree of enol character than form XII. The only other transitions observed in the thermal analysis of the various forms are those corresponding to melting, so it would appear that transitions involving only lattice rearrangements are not detectable by this technique.

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Infrared identification of barbiturates with particular reference to the occurrence of polymorphism

R. J. MESLEY AND R. L. CLEMENTS

Infrared spectra of twelve substituted barbituric acids, in a total of 34 polymorphic forms, have been compared. Comparison of a sample spectrum with that of an authentic specimen provides a reliable means of identification, provided that both are in the same crystalline form. To ensure consistent production of a single form a specific treatment is recommended for each substance exhibiting polymorphism.

IN a number of monographs in the British Pharmacopoeia and the British Pharmaceutical Codex an identification test is included in which the infrared absorption spectrum of the sample under examination is compared with that of an Authentic Specimen, supplied for this purpose. It is a necessary condition for this comparison that the two specimens should be in the same physical state. Where it is possible to record the spectra of the substances in solution this presents no difficulty, but if solid-state spectra are used polymorphism may be encountered, and it may then be necessary to specify treatments for individual substances to ensure the production of consistent spectra, as in such compounds as steroids (Mesley & Johnson, 1965) and sulphonamides (Mesley & Houghton, 1967).

The substituted barbituric acids are a class of compounds to which an infrared identification procedure is particularly suited, but they are also notorious for their polymorphism.

Infrared spectra of barbiturates, examined in the form of potassium bromide discs, were recommended for identification purposes by Manning & O'Brien (1958), no allowance being made for polymorphism; however, several of their spectra refer to salts, although described as the corresponding barbituric acids. Infrared spectra of derivatives have also been used, including copper-pyridine complexes (Levi & Hubley, 1956), *p*-nitrobenzoyl derivatives (Chatten & Levi, 1957), and dixanthyl derivatives (Flann & Cloutier, 1967). The effects of polymorphism on infrared spectra of barbiturates were noted by Cleverley & Williams (1959b) and by Paulig, Gansau & others (1963). Goenechea (1966) tabulated frequencies for N–H, C–H and C=O stretching absorptions of 21 barbiturates and suggested that these were suitable for the identification of the individual substances; he also noted that the greatest differences between the spectra of polymorphic modifications were found in the N–H and C–H stretching absorptions, and commented that for analytical purposes consideration must be given to the method of purification. The C=O stretching absorptions were used by Bouché, Coclers & Delahaut (1966) for the quantitative estimation of individual barbiturates, examined as potassium bromide discs; for this purpose the sample preparation procedure was rigorously controlled to ensure that the same crystalline form was reproducibly obtained.

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Spectra recorded in chloroform solution have been used (Umberger & Adams, 1952) but, owing to the limited solubility of many of the barbituric acids, the spectra obtained were of low intensity and not always clearly distinguishable from each other. In an attempt to overcome the effects of polymorphism on solid phase spectra, Cleverley (1960) used a technique in which the substance, incorporated into a potassium bromide disc, was heated in an oven at about 10° above its melting point, then cooled and the spectrum recorded. This method suffers from the disadvantages that each compound has to be heated to a different temperature (melting points quoted by Cleverley range between 86° and 229°), and that many of the spectra obtained correspond to the amorphous forms of the substances, which again are not always distinguishable from each other (e.g. amylobarbitone and butobarbitone).

In order to make use of the more characteristic spectra given by the crystalline solids, as many forms as possible were prepared from each substance and methods devised for converting them to a common form with a reproducible spectrum. For this purpose only the free acids were considered; if samples are obtained in the salt form they may be examined as such, but if spectral differences between the sample and the authentic specimen are observed then both should be converted to the corresponding acid and re-examined.

Experimental

MATERIALS

Most of the samples used were B.P. Authentic Specimens. In some instances commercial products were also used, after checking that their infrared spectra were identical with those of the appropriate Authentic Specimens. Samples of methohexitone, methylphenobarbitone and thialbarbitone were supplied by Mr. C. A. Johnson of the British Pharmacopoeia Commission. Solvents used were of B.P. or A.R. quality.

PRODUCTION OF DIFFERENT POLYMORPHS

The production of polymorphic forms by solvent treatments has previously been investigated for most of the substances concerned by Cleverley & Williams (1959b). Polymorphs obtained by microsublimation (Huang, 1951a,b,c) and by crystallization from melts (Brandstätter-Kuhnert & Aepkers, 1961, 1962) have also been described. The present work was therefore restricted to the preparation and interconversion of the various forms already reported.

INFRARED ABSORPTION SPECTRA

Samples were prepared for infrared examination both as mulls in liquid paraffin (Nujol) and as pressed potassium bromide discs using the technique previously described (Mesley & Johnson, 1965). Spectra were recorded using Grubb Parsons GS 2 and Perkin-Elmer 237 grating spectrometers.

Results

The barbiturates examined were those which are currently included in the British Pharmacopoeia and British Pharmaceutical Codex. These are listed in Table 1, which shows the number of crystalline forms encountered. The individual substances are considered below.

TABLE 1. INCIDENCE OF POLYMORPHISM IN THE BARBITURIC ACIDS EXAMINED

Substance	Substituents	No. of crystalline forms encountered
Amylobarbitone	5-ethyl-5-isoamyl	2
Barbitone	5,5-diethyl	4*
Butobarbitone	5-butyl-5-ethyl	3*
Cyclobarbitone	5-cyclohex-1′-enyl-5-ethyl	1*
Methohexitone	5-allyl-1-methyl-5-(1-methylpent-2-ynyl)	1
Methylphenobarbitone	5-ethyl-1-methyl-5-phenyl	1
Nealbarbitone	5-allyl-5-neopentyl	2
Pentobarbitone	5-ethyl-5-(1-methylbutyl)	4*
Phenobarbitone	5-ethyl-5-phenyl	10*
Quinalbarbitone	5-allyl-5-(1-methylbutyl)	1
Thialbarbitone	5-allyl-5-cyclohex-1′-enylthiobarbituric acid	3
Thiopentone	5-ethyl-5-(1-methylbutyl)thiobarbituric acid	2

* Additional forms have been reported elsewhere.

Amylobarbitone. Two forms were distinguished by Cleverley & Williams (1959b) and by Brandstätter-Kuhnert & Aepkers (1962). The recrystallization procedure prescribed in the British Pharmacopoeia consistently gives form II.

Barbitone. Huang (1951b) reported X-ray diffraction patterns of four distinct forms. Cleverley & Williams (1959b) published infrared spectra of forms I and II, and stated that the spectrum of form IV was identical to that of form II. In the present work all four of Huang's forms were obtained, and all were distinguishable by means of their infrared spectra; in particular, the spectrum of form IV was found to be quite different from that of form II. In addition to these four forms, Huang (1951c) has reported a fifth form present in mixed crystals with cyclobarbitone, and Brandstätter-Kuhnert & Aepkers (1962) have detected two such additional forms in barbiturate mixtures.

The procedure recommended below yields form II, which was found to give a consistent spectrum from either a Nujol mull or a potassium bromide disc, though Cleverley & Williams (1959a) have reported that this form is unstable on grinding.

Butobarbitone. Cleverley & Williams (1959b) described three forms and on occasions found infrared and X-ray evidence of a fourth form. In the present work only forms I–III were encountered. The recommended procedure gives form I, which should be examined as a Nujol mull, as this form may give a different spectrum after grinding with potassium bromide (Cleverley & Williams, 1959a).

Cyclobarbitone. It appears that only one crystalline form can be obtained from pure cyclobarbitone, though Brandstätter-Kuhnert & Aepkers (1962) have reported a second form in mixed crystals with phenobarbitone. Nevertheless, published infrared spectra and X-ray diffraction patterns show some variation. The spectrum published by

Manning & O'Brien (1958) is in fact that of the calcium salt, and other spectra, particularly when recorded from potassium bromide discs, probably show some evidence of amorphous material. Two portions of material recovered from different solvents showed slight spectral differences, but they both gave the same X-ray pattern, which corresponded to that of Huang (1951a); rather different patterns have been published by Penprase & Biles (1956) and by Williams (1959).

Methohexitone. No evidence of polymorphism was detected.

Methylphenobarbitone. No evidence of polymorphism was detected.

Nealbarbitone. This substance has not been investigated by previous workers. Two forms were encountered, one of which tended to contain residual solvent when recovered from chloroform solution. The recommended procedure gives a form with a consistent spectrum, whether recorded as a Nujol mull or as a potassium bromide disc.

Pentobarbitone. At least five forms of pentobarbitone have been reported. Cleverley & Williams (1959b) described four forms, of which I–III were said to have identical infrared spectra and closely similar X-ray diffraction patterns. Of these the pattern of form II was found to correspond to that already published by Huang & Jerslev (1951). Subsequently Jerslev & Ravn-Jonsen (1960) asserted that this latter pattern was not characteristic of pure pentobarbitone, as it corresponded to mixed crystals obtained from a melt containing about 15% of the isomeric 5-ethyl-5-(1-ethylpropyl)barbituric acid, whereas pure pentobarbitone crystallized as form I. This assertion may not be wholly justified since (*a*) the material of Huang & Jerslev was obtained by precipitation and not by crystallization from a melt, and (*b*) the published pattern in fact corresponds to a polymorphic form prepared from pure pentobarbitone by Cleverley & Williams. Jerslev & Ravn-Jonsen also gave X-ray data for forms I and II (corresponding to forms I and IV of Cleverley & Williams) and for a form IIb, which was obtained as a commercial sample and does not correspond to any of the four forms of Cleverley & Williams. Brandstätter-Kuhnert & Aepkers (1962) have reported the existence of three forms, but from the melting points alone it is not possible to correlate these with the forms reported elsewhere.

In the present work the four forms described by Cleverley & Williams were encountered and their infrared spectra recorded. Forms I–III certainly give similar spectra, and it is doubtful whether forms II and III can be distinguished, but consistent small differences were observed in the spectrum of form I. The recommended procedure normally gives form I, but occasionally some form II may be present; however the presence of a small amount of form II produces no significant change in the spectrum.

Phenobarbitone. Brandstätter-Kuhnert & Aepkers (1961, 1962) decribed eleven forms, some of which were found only in mixed crystals with other barbiturates. In the present work ten forms were obtained, including two not mentioned by Brandstätter-Kuhnert & Aepkers, and the individual forms have already been described (Mesley, Clements, Flaherty & Goodhead, 1968). The consistent conversion of all of these

to a single form presents some difficulty. No solvent treatment was found to be universally applicable, although recrystallization from aqueous ethanol usually gave form II. The only satisfactory technique was to heat the substance to such a temperature that only form I could remain, precautions being taken to prevent loss of the sample by sublimation.

Quinalbarbitone. Attempts to recover quinalbarbitone from preparations, in which it is normally present as the sodium salt, or from solutions in organic solvents, normally give a viscous oil which crystallizes only on long standing (usually 1–2 weeks). Oils from three different treatments all gave the same crystalline form, the infrared spectrum of which agreed with those published by Levi & Hubley (1956) and Chatten & Levi (1957), whilst the X-ray diffraction pattern agreed with that of Williams (1959). The spectrum published by Manning & O'Brien (1958) is quite different and refers to the sodium salt, but possible evidence of a second crystalline form is provided by a different X-ray pattern included in the Powder Diffraction File (No. 9–520) and also obtained by Penprase & Biles (1956).

Thialbarbitone. Cleverley & Williams (1959b) described three forms, and concluded from its infrared spectrum that form III was an enol form. The procedure given in the British Pharmaceutical Codex for the precipitation of thialbarbitone from a solution of its sodium salt initially yields form III, which is converted to form II on heating at 100°; the latter form, however, is much more soluble than the precipitated form III and may therefore dissolve in any water still present, so that a period of preliminary drying at 60° is necessary. A quicker procedure, given below as an alternative, yields form III.

Thiopentone. Cleverley & Williams (1959b) described two crystalline forms, but stated that their infrared spectra are identical. This has been confirmed, and no treatment is therefore recommended.

Discussion

Comparing the spectra of the 34 polymorphic modifications encountered, it is apparent that, although different forms of the same substance may not always be distinguishable from each other, there is no difficulty in distinguishing them from any of the other barbiturates examined. Because of their similarity, however, it is not always possible to detect the presence of a second barbiturate in a mixture, and this is particularly the case where the two substances may have forms which are isomorphous with each other, or where molecular compounds can exist. Spectra may then be obtained which do not correspond to the stable forms of either component of the mixture.

In general, however, when comparing a barbiturate sample with an authentic specimen of the same substance, in most instances it will be found that they give the same spectrum. If they do not, then the possibility of polymorphism must be considered. If both samples are subjected to the appropriate treatment indicated below, they should yield the

same crystalline form. These treatments were effective for all the forms encountered in this work, and although other forms may exist it is probable that the treatments would be effective for them as well. Since the publication of the earlier reports on steroids (Mesley & Johnson, 1965) and sulphonamides (Mesley & Houghton, 1967) several new polymorphic forms have been encountered, but all of these have proved amenable to the treatments previously recommended.

RECOMMENDED PROCEDURES

Amylobarbitone. Recrystallize from ethanol (25% v/v).

Barbitone. Dissolve in chloroform, evaporate the solution to dryness at room temperature in a current of air.

Butobarbitone. Dissolve in ether, evaporate solution to dryness on water bath, leave on water bath until material crystallizes.

Cyclobarbitone. No recommendation necessary.

Methohexitone. No recommendation necessary.

Methylphenobarbitone. No recommendation necessary.

Nealbarbitone. Recrystallize from ethanol (25% v/v).

Pentobarbitone. Recrystallize from ethanol (25% v/v).

Phenobarbitone. Heat in covered vessel for 1 hr at 150° or overnight at 140°.

Quinalbarbitone. If material is not crystalline, either (*a*) examine as chloroform solution, or (*b*) dissolve in the minimum of chloroform, place a drop or two on a rock salt plate, heat to 100° to remove solvent, place second heated plate on top of sample, allow to cool and record spectrum of resulting film.

Thialbarbitone. (*a*) Dissolve in minimum of dilute sodium hydroxide solution, acidify by dropwise addition of 0·1N hydrochloric acid until no further precipitation occurs, allow to stand until precipitate coagulates, filter, wash precipitate with water and dry for 2 hr at 60°, then for 2 hr at 100°, or (*b*) dissolve in ethanol, evaporate to dryness on water bath, allow to cool, add sufficient ethanol (25% v/v) to cover the glassy product, allow to crystallize, decant off the liquid and dry in a current of air.

Thiopentone. No recommendation necessary.

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Effect of electrolytes on coacervation of the systems gelatin–water–ethanol and gelatin–water–sodium sulphate

J. R. NIXON, SALEH A. H. KHALIL* AND J. E. CARLESS

In the ethanol system the electrolytes had a suppressive effect on the coacervation of isoionic gelatin, whilst at other pH values the phenomenon was favoured in the presence of polyvalent ions. In the sodium sulphate system the added electrolytes produced insignificant effects. The similarity between the systems studied and the complex coacervating system: gelatin$^{(+)}$–water–acacia$^{(-)}$ is discussed.

THE classification of the phenomenon of coacervation into simple and complex types by Bungenberg de Jong (1949) is based on the role played by the charges on the colloidal components. In simple coacervation it has been suggested that charge effects are negligible and phase separation occurs due to "desolvation," as in the systems: isoelectric gelatin–water–ethanol and isoelectric gelatin–water–sodium sulphate (Holleman, Bungenberg de Jong & Modderman, 1934). On the other hand complex coacervation occurs due to adequate charge opposition between the colloids involved, as in the systems gelatin$^{(+)}$–water–acacia$^{(-)}$ at pH 2·6–4·2. The extensive studies of Bungenberg de Jong and his co-workers (1949) have shown that complex coacervating systems are extremely sensitive to pH changes and electrolytic impurities. The effect of these factors on simple coacervating systems was not investigated in detail. Recently, Khalil, Nixon & Carless (1968) examined the role of pH on the coacervating systems: gelatin–water–ethanol and gelatin–water–sodium sulphate. For the ethanol system, coacervation was only possible at pH values in the vicinity of the isoionic point, whilst the sodium sulphate system showed a much wider pH coacervation range.

The present paper examines the effect of added electrolytes on the two systems previously studied by us and compares the results with data available on complex coacervating systems.

Experimental

MATERIALS

Gelatin. A lime-pretreated 240 Bloom sample as described by Khalil & others (1968) was used.

The gelatin was deionized by the mixed-bed ion-exchange technique of Janus, Kenchington & Ward (1951). *Coacervating agents: Absolute ethanol* and 20% w/w *sodium sulphate solution. Electrolytes:* All electrolytes, with the exception of aluminium chloride, were A.R. grade. The latter was a pure sample containing not less than 99% $AlCl_3$. *Glass distilled water* was used (pH 5·4, specific conductivity 4·8 μ mhos cm^{-1}).

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* This work forms a part of a thesis submitted by S.A.H.K. for the degree of Ph.D. in the University of London.

METHODS

Determination of the onset of coacervation, measurement of the coacervate volume and analysis of the phases. These techniques have been described by Nixon, Khalil & Carless (1966) and Khalil & others (1968).

Measurement of turbidity. An Evans Electroselenium Ltd. nephelometer was used. The coacervating agent was added to 3·0 ml portions of 0·6% w/v gelatin solution at 40° ± 0·05°. Readings were recorded after mixing and equilibrating the turbid mixture for 3 min at 40°. A gelatin solution of the same total concentration as in the test was used as a blank.

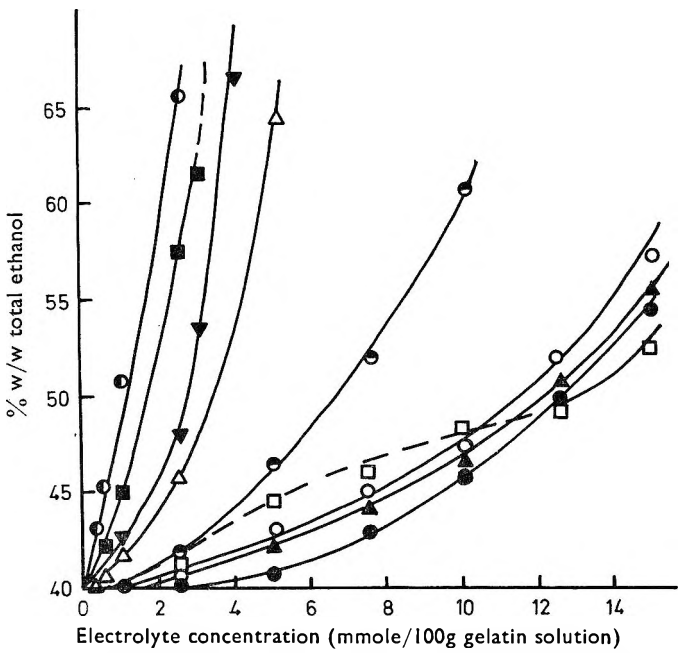

FIG. 1. Effect of electrolytes on the occurrence of coacervation in the system: gelatin–water–ethanol. Minimum ethanol concentrations (% w/w) required to produce coacervation plotted against electrolyte concentration. Initial gelatin concentration 5 % w/w. Temp. 40° ± 0·1°, pH 4·9 (pI). ◐——◐, $AlCl_3$. ■——■, $K_4[Fe(CN)_6]$. ▼——▼, $K_3[Fe(CN_6)]$. △——△, $CaCl_2$. ◓——◓, K_2SO_4. ○——○, KI. ▲——▲, KBr. ●——●, KCl. □- - -□, KF.

Fig. 1 shows the effect of electrolytes on the minimum ethanol concentrations required to produce coacervation in isoionic gelatin. The suppressive effect of the electrolytes followed the valency rule for both the anions and cations:

$Cl^- < SO_4^{2-} < [Fe(CN)_6]^{3-} < [Fe(CN)_6]^{4-}$ and $K^+ < Ca^{2+} < Al^{3+}$.

In the concentrations used, the lyotropic effect was not clearly shown. Nevertheless the arrangement of the potassium halides, in the order of peptizing power, was in accordance with the Hofmeister series: $(F^-) < Cl^- < Br^- < I^-$. At low concentrations potassium fluoride showed anomalous results which can be attributed to pH changes caused by its ionization. Aluminium chloride produced similar effects.

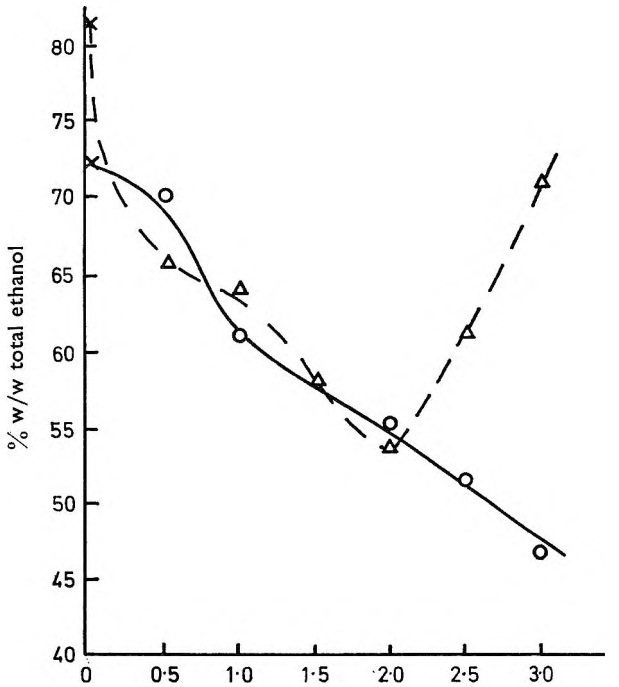

FIG. 2. Effect of calcium chloride (at pH 9·1) and potassium ferrocyanide (at pH 2·4) on coacervation in the system: gelatin–water–ethanol. Minimum ethanol concentrations required to produce a phase change plotted against electrolyte concentration. Initial gelatin concentration 5% w/w. Temp. 40° ± 0·05°. × flocculation, ○, △ coacervation.

At pH values away from the isoionic point polyvalent ions favoured coacervation in mixtures which showed flocculation in the absence of these ions. Typical results are shown in Fig. 2 for Ca^{+2} at pH 9·1 and $[Fe(CN)_6]^{4-}$ at pH 2·4.

TABLE 1. EFFECT OF ELECTROLYTES ON THE OCCURRENCE OF COACERVATION IN THE SYSTEM: GELATIN–WATER–SODIUM SULPHATE. Minimum sodium sulphate concentrations (% w/w) required to produce coacervation at various electrolyte concentrations

Salt concentration (mmol/100 g gelatin solution)	$AlCl_3$	$CaCl_2$	KCl	KF	KBr	KI	K_2SO_4	$K_3[Fe(CN)_6]$	$K_4[Fe(CN)_6]$
0	7·81 (Blank)								
0·25	7·78	7·81	7·81	7·83	7·82	7·82	7·81	7·81	7·80
0·5	7·62	7·83	7·80	7·86	7·82	7·84	7·80	7·82	7·79
1·0	7·55	7·80	7·83	7·88	7·82	7·83	7·80	7·80	7·78
2·5	7·31	7·81	7·80	7·91	7·84	7·85	7·78	7·78	7·76
5·0	7·21	7·84	7·82	7·93	7·84	7·86	7·80	7·77	7·78
7·5	—	—	7·81	7·90	7·86	7·86	7·80	7·79	7·76
10·0	—	—	7·81	7·91	7·85	7·88	—	—	—

Temperature: 40° ± 0·1°, pH = 4·9 (pI).
Initial gelatin concentration: 10% w/w (5 g used).

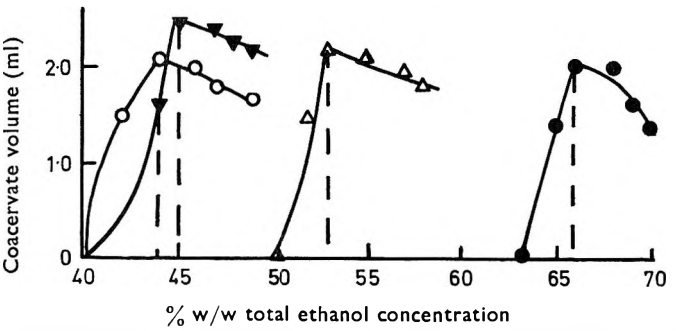

FIG. 3. Effect of electrolytes on changes in coacervate volume at various ethanol concentrations. Total gelatin concentration 4% w/w. Electrolyte concentration 2 mmole/100 g gelatin solution. Temp. 40° ± 0·05°, pH 4·9 (pI). ○——○, blank. ▼——▼, $CaCl_2$. △——△, $K_4[Fe(CN_6]$. ●——●, $AlCl_3$.

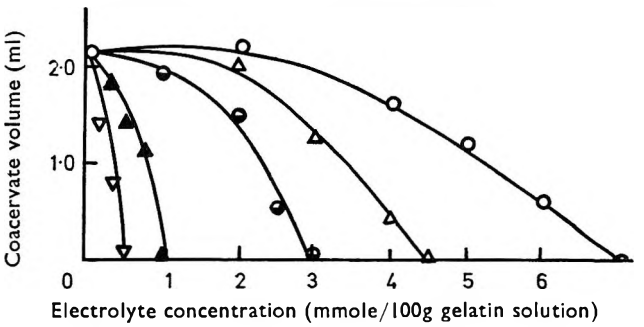

FIG. 4. Effect of electrolytes on changes in coacervate volume at a constant composition. Gelatin 4% w/w ethanol 44% w/w, water 52% w/w. Temp. 40° ± 0·05°. pH 4·9 (pI). ▽——▽, $AlCl_3$. ▲——▲, $K_4[Fe(CN)_6]$. ◓——◓, $CaCl_2$. △——△, K_2SO_4. ○——○, KI.

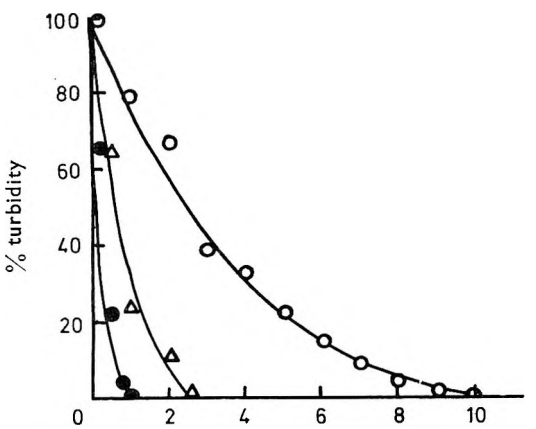

FIG. 5. Percentage turbidity plotted against electrolyte concentration (the turbidity in the absence of electrolytes was taken as 100%). Temp. 40° ± 0·05°. pH 4·9 (pI). ●——●, $AlCl_3$. △——△, $CaCl_2$. ○——○, KCl.

TABLE 2. EFFECT OF CALCIUM CHLORIDE ON THE COMPOSITION OF THE COACERVATES AND EQUILIBRIUM LIQUIDS IN THE SYSTEM GELATIN–WATER–ETHANOL

Calcium chloride concentration (mmole/100 g) gelatin solution)	Percentage w/w compositions					
	Coacervate			Equilibrium liquid		
	Gelatin	Ethanol	Water	Gelatin	Ethanol	Water
0	19·7	36·9	43·4	0·6	47·4	52·0
0·6	17·0	38·2	44·8	0·9	47·4	51·7
1·2	14·2	39·5	46·3	1·2	46·9	51·9
1·8	11·4	41·6	47·0	1·4	47·1	51·5
2·4	8·6	43·1	48·3	1·8	46·7	50·5
3·0	One phase					

Temperature: 40° ± 0·1°, pH = 4·9 (pI).
Percentage w/w compositions of the total mixtures: 4 (gelatin) 46, (ethanol) and 50 (water).

In the sodium sulphate system all electrolytes added, with the exception of potassium fluoride and aluminium chloride, had a negligible effect on the minimum sulphate concentrations necessary for coacervation (Table 1).

Coacervate volume plots, Fig. 3, show also the suppressive effect of the electrolytes in the ethanol system, where higher ethanol concentrations were required to produce the same coacervate volume. At a fixed ethanol concentration in the system the presence of electrolytes produced a gradual decrease in the coacervate volume. The effect again followed the valency rule (Fig. 4). Turbidity determinations, Fig. 5, show complementary results with the coacervate volume measurements.

The effect of calcium chloride on the composition of both the coacervate phase and equilibrium liquid is shown in Tables 2 and 3. The results for the ethanol system are plotted in Fig. 6. At constant compositions of gelatin, water and ethanol an increase in the calcium chloride concentration resulted in the composition of both phases approaching one another. At a sufficiently high calcium chloride concentration (point P, Fig. 6) coacervation was completely suppressed and the system remained in the one-phase region.

TABLE 3. EFFECT OF CALCIUM CHLORIDE ON THE COMPOSITION OF THE COACERVATES AND EQUILIBRIUM LIQUIDS IN THE SYSTEM: GELATIN–WATER–SODIUM SULPHATE

Calcium chloride concentrations (mmole/100 g) gelatin solution)	Percentages w/w compositions					
	Coacervate			Equilibrium liquid		
	Gelatin	Sod. sulph.	Water	Gelatin	Sod. sulph.	Water
0	14·4	7·0	78·6	0·6	11·5	87·9
0·6	14·1	7·1	78·8	0·6	11·8	87·6
1·2	14·2	6·9	78·9	0·6	11·4	88·0
1·8	14·0	6·9	79·1	0·6	11·0	88·4
2·4	14·3	6·9	78·8	0·8	11·0	88·2
3·0	14·4	7·2	78·4	0·8	10·9	88·3

Temperature: 40° ± 0·1°, pH = 4·9 (pI).
Percentage w/w compositions of the total mixtures: 4 (gelatin), 10·2 (sodium sulphate) and 85·8 (water).

In the sodium sulphate system the presence of calcium chloride produced no noticeable effect on the composition of both phases, Table 3. This may be attributed to the formation of the slightly soluble salt, calcium sulphate, coupled with insufficient Cl^- concentration to produce any peptizing effect in the presence of SO_4^{2-} ions.

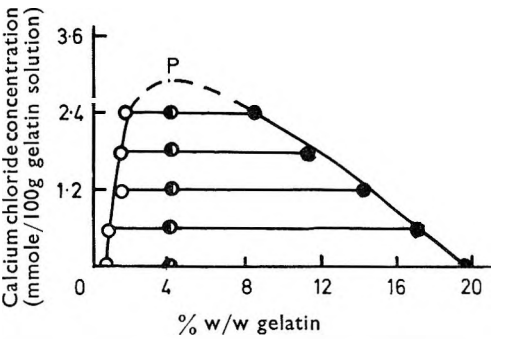

FIG. 6. Effect of calcium chloride on changes in the gelatin content of the coacervates, ●, and equilibrium liquids, ○. Composition of the total mixtures, ◐; gelatin 4% w/w, ethanol 46% w/w, water 50% w/w. Temp. 40° ± 0·1°. pH 4·9 (pI).

Discussion

The addition of low concentrations of electrolytes to isoionic gelatin results in a decrease of the mutual attractive forces due to the screening of the functional groups on gelatin. This produces the unfolding of the coiled structure and an increase in viscosity occurs (Overbeek & Bungenberg de Jong, 1949). The effect of electrolytes on coacervation may be explained on the same basis. The shielding of the functional groups by the ions of the added electrolyte decreases the inter- and intra-molecular attractive forces. The peptizing property of the ions may exert a secondary influence. Salts of the type 1–1 (e.g., KCl) would suppress the interaction by both the positive and negative groupings to the same extent. With salts of the type 1–4, 1–3 or 1–2 the polyvalent anions would, in the given order, screen off the positively charged basic groups to an increasingly greater extent than the effect of the monovalent cations on the negatively charged carboxyl groups of the aspartic and glutamic residues. This results in an imbalance of the original charge distribution, characteristic for the isoionic state and the mutual attractive forces diminish according to the electrolyte-sequence previously mentioned (Fig. 1).

At pH values away from the isoionic point, charge opposition occurs between the added polyvalent ions and the ionized groups on gelatin. Any repulsive force resulting from a pH variation would be overcome by the selective fixation of the polyvalent ions at the oppositely charged sites on the gelatin. Under such conditions coacervation occurs. The coacervates produced at pH 2·4 in the presence of ferrocyanide or ferricyanide ions showed a unique property. Whilst a clear single liquid phase was not restored on dilution with water, as occurred with other simple coacervating systems, a change in pH or the addition of an excess

of the above electrolyte solutions restored complete clarity. In this respect these coacervates are analogous to the known complex coacervates of the type: gelatin$^{(+)}$–water–acacia$^{(-)}$ where the ferrocyanide or ferricyanide ions replace the arabinate anions.

In the present work both the coacervate volume and turbidity measurements gave complementary results which showed visually the suppressive effect of electrolytes in the ethanol system (Figs 4 and 5). Analyses showed the changes produced in the phase composition by the presence of calcium chloride (Tables 2 and 3). In the ethanol system the coacervate phase showed more pronounced changes than its corresponding equilibrium liquid. The calcium chloride concentration was paralleled by a decrease in the gelatin and an increase in the liquid (ethanol + water) contents of the coacervate phase. This was due to the larger space occupied by the coiled structure as a result of unfolding under the influence of calcium chloride. At a sufficiently high concentration of calcium chloride the system remained clear in the one-phase region where the composition throughout would be the same.

The results reported here, together with the data presented in a previous paper on the effect of pH on gelatin coacervation (Khalil & others, 1968) clearly indicate the role played by the ionogenic groups on gelatin in the coacervation phenomenon. The results also showed that gelatin coacervation by non-electrolytes, e.g., ethanol was of different type from coacervation by electrolytes, e.g., sodium sulphate. The former system was extremely sensitive to pH changes and the addition of electrolytes whilst the latter resisted these factors due to the protection offered by the electrolyte used in coacervation. It is suggested that the coacervation of systems such as isoionic gelatin–water–non-electrolyte is comparable with complex coacervating systems such as gelatin$^{(+)}$–water–acacia$^{(-)}$. In both cases phase separation results from charge opposition. In the so-called complex coacervation this charge opposition is between the two colloids whilst with the simple coacervation it is between oppositely charged sites on the isoionic gelatin. The presence of ethanol, as a coacervating agent, was necessary to enhance this latter effect sufficiently to produce phase separation.

This suggestion is supported by the fact that both systems are affected, in a qualitatively similar fashion, by changes in pH and electrolytic impurities. It thus appears that the classification of coacervation phenomenon into "simple" and "complex" types is no longer valid.

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The action of ampicillin on *Aerobacter (Klebsiella) aerogenes*

A. C. R. DEAN AND R. W. SMITH

The action of ampicillin (α-aminobenzylpenicillin) on *Aerobacter* (*Klebsiella*) *aerogenes* (NCTC 418, *Bact. aerogenes* no. 240) has been investigated. Enzymatic destruction of the drug, although a necessary prelude to the growth of the sensitive strain, does not precede growth of resistant strains at concentrations to which they have been "conditioned". Instead a gradual inactivation occurs throughout the growth cycle. It is concluded that other factors are also involved in resistance. Cloxacillin, which was not destroyed by the cells, had little action on the ampicillin-sensitive strain and did not induce any ampicillin-destroying activity. The results are discussed in terms of the modes of action of penicillins and the origin of the resistance is examined.

AMPICILLIN (α-aminobenzylpenicillin) inhibits some Gram-negative organisms not markedly affected by benzylpenicillin. Resistance can, however, develop in these organisms (see for example Sutherland, 1964; Rolinson, 1955), and Hamilton-Miller (1965), who used twelve clinically-isolated *Klebsiella* strains, claimed that factors other than intracellular ampicillin-destroying enzymes (penicillinases) were involved. It has often been reported (see for refs. Rolinson & Stevens, 1961; Ayliffe, 1965) that the minimum inhibitory concentration of a penicillin is much higher for a large than for a small inoculum. This has been ascribed to an enzymatic destruction of the drug and indeed both Sutherland (1964) and Hamilton-Miller (1965) have used it to assess the part played by penicillinases in ampicillin resistance. In this paper the action of ampicillin on another strain of *Aerobacter* (*Klebsiella*) *aerogenes* is reported.

Experimental

The strain of *Aerobacter aerogenes* (NCTC 418, *Bact. aerogenes* no. 240) used was fully conditioned to a salts-glucose medium by thirty daily subcultures. This medium and the general techniques are described elsewhere (Dean & Moss, 1967). Ampicillin (Penbritin injection—Beecham Research Laboratories) was dissolved in sterile phosphate buffer pH 7·1. Experiments were made at 40° and the cultures were stirred and aerated by a stream of sterile air. Solid media were prepared by adding 1·5% agar to the salts-glucose medium. Penicillins were assayed by the method of Batchelor, Chain & others (1961) and disruption of the cells by ultrasonication was as described by Grant & Hinshelwood (1964). Cell mass was determined using a Hilger photoelectric absorptiometer calibrated in terms of the number in millions/ml of cells of a standard size. 10^{10} of these cells have a dry weight of 12·2 mg (Dean, 1967).

Results

EFFECT ON LAG AND MEAN GENERATION TIME

On the first exposure of *A. aerogenes* to ampicillin in liquid medium, the growth curve is not of the standard form. Instead an initial increase

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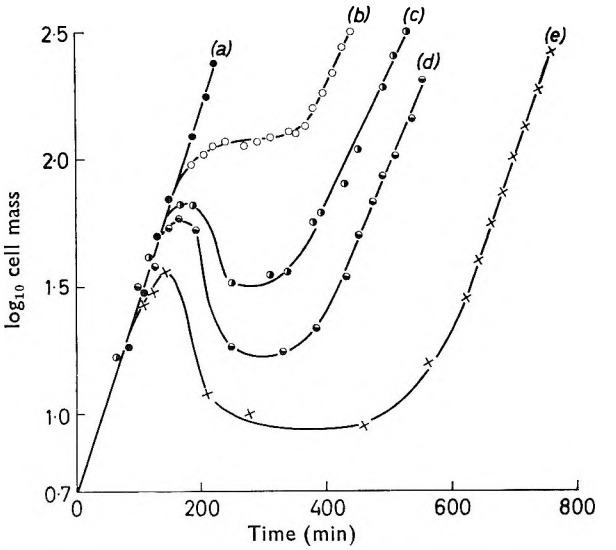

FIG. 1. Growth curves of the "untrained" strain in ampicillin medium. *a* no drug, *b* 10, *c* 50, *d* 100 and *e* 200 mg/litre.

in cell mass occurs without lag at a rate which is independent of the drug concentration (Fig. 1). Its extent is inversely related to the concentration. A lag then ensues before exponential growth sets in again and although at low drug levels the turbidity of the culture does not drop during this period, at higher levels it falls for a time before increasing again as the secondary phase of growth gets under way. The drop in turbidity is also greater the higher the concentration of drug. The mean generation time in the secondary phase is also largely independent of the concentration.

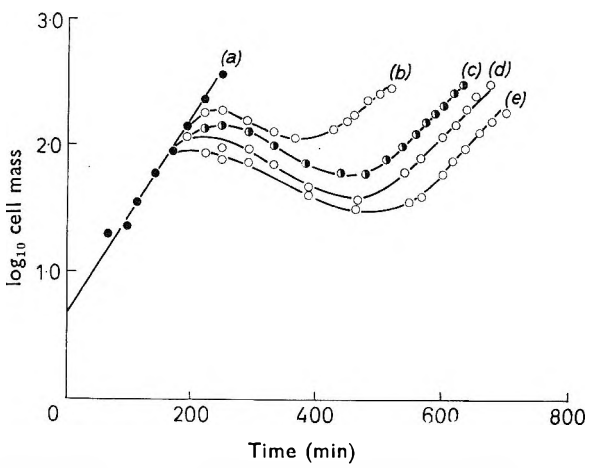

FIG. 2. Growth curves in ampicillin medium of a strain fully "trained" to 100 mg/litre. *a* 100, *b* 500, *c* 1000, *d* 1500 and *e* 2000 mg/litre.

For example in a series of twenty-four experiments carried out at concentrations between 25 and 1,000 mg/litre the mean generation time varied randomly in the range 41–53 min, mean 46 min. In drug-free medium it was 30–33 min. Since, as will be shown later, the inhibitory action of ampicillin is dependent on the inoculum size, an inoculum of 5×10^6 standard cells/ml was always used.

EFFECT OF REPEATED SUBCULTURE IN DRUG-MEDIUM

After many subcultures in a given concentration of ampicillin the growth curve is of the standard form. For example curve *a* of Fig. 2 was obtained with a strain which had received 31 daily subcultures in 100 mg/litre of drug. Fig. 2 also shows that "training" to 100 mg/litre resulted in the initial period of growth being greater, the ensuing drop in turbidity less and the lag before the secondary phase of growth much reduced. For example at the 1,000 mg/litre level the lag (obtained by extrapolating the linear portion of the secondary phase of the growth cycle to the original inoculum size) was only 280 min compared to about

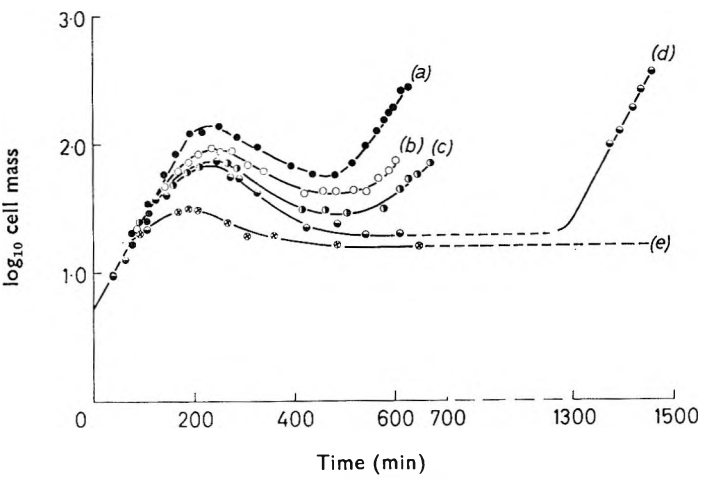

FIG. 3. Behaviour in tests at 1000 mg/litre during "training" to 100 mg/litre of ampicillin. Curves reading from the bottom upwards refer in turn to the growth curves obtained after 3, 4, 5, 6 and 50 serial subcultures in the presence of 100 mg/litre of drug. The lag of the untrained strain is about 7000 min.

7,000 min for the "untrained" strain. Moreover growth now occurred reasonably well at concentrations (i.e., 1,500 and 2,000 mg/litre) which were completely inhibitory before "training". The mean generation time during the secondary phase of growth, however, was little affected by "training", the values obtained falling within the range reported earlier for the "untrained" organism. Essentially similar results were obtained with strains "trained" to 10 and 50 mg/litre respectively. Nevertheless in other respects "training" is a gradual process which is illustrated in Fig. 3. Before "training" and after one subculture in the presence of

100 mg/litre of drug there was no detectable initial growth phase and hence these growth curves are not included in Fig. 3.

The resistance to 1,000 mg/litre of drug brought about by subculture in 100 mg/litre is not lost as easily as it is attained. For example, after four subcultures in 100 mg/litre the lag in 1,000 mg/litre was 1,260 min. Six subsequent subcultures in drug-free medium did not cause any increase in this lag but after 10 subcultures it had increased to 2,280 min and after 26 subcultures it was 7,000 min indicating that the resistance had been lost entirely.

CHANGES IN MASS, NUMBER AND VIABLE COUNT

The shape of the growth curve in the presence of 50 mg/litre of ampicillin has already been given (Fig. 1*c*) and the corresponding changes in mass, number and viable count are recorded in Table 1. Cell mass is given in terms of the equivalent number per ml of cells of a standard size. This datum is convenient since the ratio of the equivalent number (determined turbidimetrically) to the actual number obtained by microscopic counting gives a measure of the mean cell size (σ). The results in Table 1 show that, during the initial phase of growth, division is inhibited since cell mass increases without a concomitant increase in number (which actually drops) and σ therefore increases and indeed reaches its highest level. This has been confirmed by the determination of the distribution of cell sizes at various stages of the growth cycle. The viability also falls markedly. In the next phase, in which the mass (turbidity) of the culture drops, (see Fig. 1*c*) the viable count falls still further and reaches its lowest level but the total number of cells does not change. Thereafter logarithmic growth sets in and mass, number and viable count all increase.

TABLE 1. CHANGES IN MASS, NUMBER AND VIABLE COUNT DURING GROWTH IN AMPICILLIN MEDIUM (50 MG/LITRE)

Time from inoculation (min)	Mass/ml ($\times 10^{-6}$)	Number/ml ($\times 10^{-6}$)	Viable count/ml ($\times 10^{-6}$)	σ
0	5	26 (26)	22	0·19
220	39	13 (10)	1·7	3·00
285	15	19 (4)	0·2	0·79
435	26	30 (17)	1·9	0·87
555	170	290 (282)	300	0·59

The figures in brackets give the number of cells/ml after subtraction of those subsequently classified as ghosts.

σ, the ratio mass/number is a measure of the mean cell size.

At the end of the initial period of growth a large proportion of the cells were in the form of "incipient spheroplasts". Many of these subsequently lysed and the normal rod-shaped cells eventually outgrew the rest. The values for the number of cells/ml in Table 1 include those subsequently classified as empty envelopes or "ghosts". This can be accounted for and the corrected values given in brackets show that the cell number and mass both reach a minimum at the same time. Many of these cells, however, are non-viable. Isolated spheroplasts were rare

and were only observed to the extent of 2–3% at times 285 and 435 min (compare Gebicki & James, 1960). Increasing the osmotic pressure of the medium by 10 atmospheres by the addition of extra glucose did not increase their incidence. Nor did the further addition of magnesium sulphate as suggested by Weibull (1956b) for the stabilization of spheroplasts have any effect. Nevertheless the proportion of "incipient spheroplasts" was higher in the supplemented medium and the drop in the turbidity of the culture much less. The viability also remained higher, the minimum observed in the supplemented medium being 3% compared to 0·016% in the control. The corresponding lags in the supplemented and in the unsupplemented media were 1,140 and 1,800 min.

EFFECT OF SIZE OF THE INOCULUM

Decreasing the size of the inoculum increased the lag of the "untrained" organism in ampicillin medium. A strain "trained" to 100 mg/litre did not show the increase at that concentration but at higher levels there was an increased lag again (Table 2). The reported lags are the difference

TABLE 2. EFFECT OF INOCULUM SIZE ON THE LAG

Ampicillin (mg/litre)	Lag (min) at the given inoculum size per ml					
	5×10^6	5×10^5	5×10^4	5×10^3	5×10^2	5×10
	(a) "Untrained" strain					
50	400	870	2,400	∞	—	—
100	570	1,560	∞	—	—	—
200	1,100	∞	—	—	—	—
	(b) "Strain" trained to 100 mg/litre					
100	0	0	—	10	—	0
500	270	390	520	700	1,000	1,200
1000	340	540	600	1,000	∞	—

between the behaviour in the presence and in the absence of drug at the given inoculum sizes. A comparison of the death rate of the "untrained" organism at inoculum sizes of 5×10^5 and 5×10^6 cells per ml in drug medium (50 mg/litre) showed that in the initial phase of growth this rate was independent of the inoculum size. Thereafter the inoculum size had a marked effect (Fig. 4).

DESTRUCTION OF AMPICILLIN BY THE CELLS

At the end of the lag phase of "untrained" (sensitive) cells in medium containing initially 1,000 mg/litre of drug, its level had dropped to about 50 mg/litre. During the growth (without lag) in ampicillin medium (1,000 mg/litre) of a strain which had previously received 110 subcultures at that concentration, destruction also occurred. For example, when the cell mass had increased from the initial level of 5×10^6/ml to 38×10^6/ml the concentration of the drug was 350 mg/litre and when the mass had reached 119×10^6/ml it was 80 mg/litre. No drug could be detected 24 hr after inoculation.

Further experiments were made with resting cells from 24 hr cultures. The cells were separated by centrifugation and re-suspended in phosphate buffer (pH 7·1) containing 1,000 mg/litre of drug. Table 3 shows that intact cells of the sensitive strain destroyed the drug at a slow rate which was increased if the cell wall was first disrupted by ultrasonication; intact cells of a resistant strain (121 subcultures at 1,000 mg/litre) were more

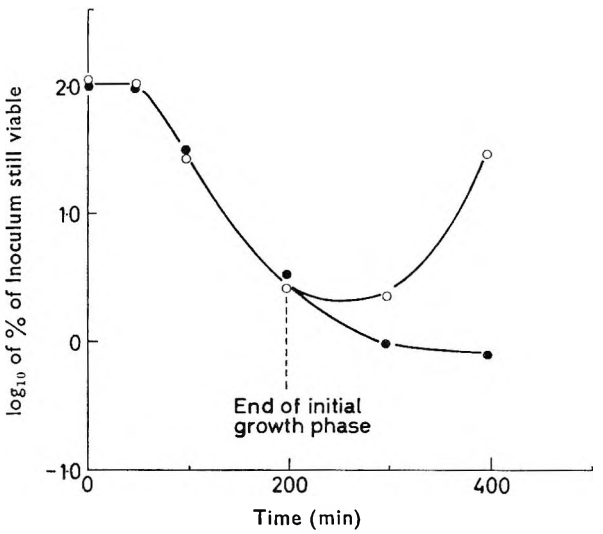

FIG. 4. Effect of inoculum size on the death rate during the first growth cycle in the presence of 50 mg/litre of drug. Inoculum ○, 5×10^6/ml; ●, 5×10^5/ml.

active and disrupted resistant cells very active. Heating the preparation of disrupted cells nullified the ampicillin-destroying activity. During the tests the viable count of the intact sensitive and resistant cells fell and at 24 hr was 29 and 30% respectively of the initial value. The medium in which the resistant cells were grown also possessed a heat-labile activity similar in magnitude to that found with intact resistant cells. This is probably a result of cell breakage either at the end of the growth cycle or during processing. Cells resistant to 1,000 mg/litre of ampicillin grew

TABLE 3. DECOMPOSITION OF AMPICILLIN BY RESTING CELLS

Strain	Ampicillin concentration (mg/litre) at the given time (hr)			
	0	1	7	24
Sensitive				
(*a*) Intact cells	1,020	930	890	665
(*b*) Broken cells	1,020	910	710	470
Resistant				
(*a*) Intact cells	1,020	835	765	90
(*b*) Broken cells	1,020	50	0	—
(*c*) Broken cells heated at 60°/10 min	1,020	900	—	—
Control on medium	1,020	995	990	790

The initial cell mass was identical throughout and corresponded to 20×10^8 standard cells/ml.

readily in the presence of 1,000 mg/litre of cloxacillin without destruction of the drug. 1,000 mg/litre of cloxacillin, however, had little action even on the ampicillin-sensitive strain. The lag was negligible and the mean generation time 37 min compared to 32 min in the control. The total population supported by the medium was slightly reduced and there was some clumping of the cells at 24 hr after inoculation. Moreover, one subculture in cloxacillin medium (1,000 mg/litre) did not enhance the ampicillin-destroyed activity of sensitive cells. Nor did cloxacillin (500 mg/litre) inhibit the destruction of ampicillin (500 mg/litre) by ampicillin-resistant cells.

Discussion

The pattern of behaviour during the initial period of growth of the "untrained" strain is consistent with the claim that, initially at least, penicillins inhibit division and synthesis of wall material to a greater extent than the other metabolic processes of the cell (Weibull, 1956a; McQuillen, 1956). The cells become larger and assume the "incipient spheroplast" form (see Reynolds, 1966, and references therein). This phase is followed by a period of lysis resulting from the rupture of the weakened cell wall. A lag then ensues before exponential growth sets in once more and when this growth begins most of the drug has already been destroyed, presumably by penicillin-β-lactamase (Cole & Sutherland, 1966). The inoculum-size effect observed in both the duration of the lag (Table 2) and the extent of death (Fig. 4) if interpreted as proposed by Sutherland (1964) and Hamilton-Miller (1965) confirms the production of penicillinase by the sensitive cells. It follows therefore that if the destruction of the drug entering the cells during the initial period of growth is great enough, little subsequent lysis would occur. This has been observed at low concentrations (10 mg/litre), but at higher levels sufficient inactivation does not take place and instead a dramatic drop in viability accompanied by the lysis of many of the cells results. This lysis facilitates the destruction of ampicillin since in this strain of *A. aerogenes* the penicillinase is largely intracellular (compare Citri & Pollock, 1966). That the degree of lysis and the length of the ensuing lag become greater and the duration of the initial growth phase becomes shorter as the concentration of drug increases, is consistent with the explanation just given.

Inactivation of the drug does not precede the growth (without lag) of "trained" strains when tested at the "training concentration" and the resistance here would appear to be due to another mechanism, possibly similar to the "intrinsic resistance" described by Ayliffe (1965). At higher concentrations, however, the behaviour characteristic of the "untrained" strain (Fig. 1) is also found with the "trained" strains although to a lesser extent (Fig. 2) and here the increased ability of the "trained" strains to destroy ampicillin is important although inactivation need only proceed until the level has been reduced to about the "training" concentration. The entire pattern of behaviour of the "untrained" and the "trained"

strains is compatible with adaptive theories of drug resistance (see Dean & Hinshelwood, 1966) and in this example it would be envisaged that during the lag a competition between death of the cells and the development of resistance occurs in a continually decreasing concentration of drug. On the other hand the selection of resistant mutants either from the original population or after they arise during the ensuing initial phase of growth must also be considered. The gradual nature of the "training" process as illustrated in Fig. 3 and reinforced by the relatively long time before training is complete (over 80 generations) argues against any simple selection of mutants and necessitates the assumption of a whole range of types. "Training" would then consist of the gradual increase of the better fitted of these until they comprised the entire population. It might be questioned how resistance could arise other than by the selection of mutants in a system in which initially uninterrupted growth only commences when the level of drug has dropped virtually to zero. During the lag, "training" followed by some "detraining" as the drug level falls could occur and since it has been shown that the latter is a slower process than the former, the cells would begin the second growth cycle at a slight advantage and so on. Eventually the lag disappears and on repeated subculture at a given concentration decomposition of the drug rather than being a necessary prelude to growth now accompanies it. This, together with the absence of an inoculum size effect when the "trained" strains were tested at the "training" concentration, reinforces the conclusion that factors other than an increased production of penicillinase are involved in the development of resistance. Alternatively, it could be argued that the resistance is explicable on the assumption that every molecule of ampicillin is decomposed immediately it enters the cell. A special priority for the adsorption of ampicillin molecules on to the enzyme would also be required and there is evidence that the adsorption of penicillins is general (Rogers, 1967). Moreover, such an explanation could not apply to the results obtained with cloxacillin since the relative immunity of the ampicillin-sensitive and the marked immunity of the ampicillin-resistant strains was not a consequence of its rapid inactivation. Indeed, little destruction of cloxacillin occurred during growth in its presence and this growth did not induce any ampicillin-destroying activity.

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The depletion and recovery of noradrenaline in the brain and some sympathetically innervated mammalian tissues after tetrabenazine

JAN HÄGGENDAL

The depleting effect of tetrabenazine on the monoamine levels appears generally to be less in peripheral tissues than in the central nervous system. The noradrenaline levels in brain and some sympathetically innervated tissues such as heart, submandibular glands, and skeletal muscle were examined in the rat after administration of the drug. The levels of 5-hydroxytryptamine, dopamine and noradrenaline were also estimated in the rabbit brain after tetrabenazine and compared with the levels in the rabbit heart. In both the brain and peripheral tissues the monoamine levels were strongly reduced 4 hr after tetrabenazine and increased thereafter, reaching normal levels after about 36 to 48 hr. The site of action of tetrabenazine is briefly discussed and compared to the site of action of reserpine.

CERTAIN benzoquinolizine derivatives like tetrabenazine cause sedation and reduce the levels of noradrenaline and 5-hydroxytryptamine (5-HT) in brain (Pletscher, Besendorf & Bächthold, 1958; Pletscher, Besendorf & Gey, 1959; Pletscher, Brossi & Gey, 1962; Quinn, Shore & Brodie, 1959). Tetrabenazine thus has several features in common with reserpine (Carlsson, 1965). When tetrabenazine is given before reserpine it partially affords protection against the long-lasting reserpine effects on the monoamine levels in brain, and in addition it antagonizes the effect of reserpine upon the gross behaviour. This indicates that the two drugs compete for the same site of action (Quinn & others, 1959; Carlsson & Lindqvist, 1966).

The effect of tetrabenazine on monoamine levels has been reported to be less pronounced in peripheral tissues than in the brain (Pletscher & others, 1962; Carlsson, 1965). Relatively high doses of tetrabenazine given to rabbits or guinea-pigs do not cause much reduction in the levels of 5-HT in blood platelets or small intestines (Quinn & others, 1959; Schwartz, Pletscher & others, 1960). On the heart, however, the results are inconsistent. In rabbits given 50 mg/kg intravenously, noradrenaline levels were found to be unchanged 4 and 24 hr later (Quinn & others, 1959). But in the guinea-pig 20 mg/kg intraperitoneally decreased the noradrenaline levels in heart and brain to about the same extent so that the levels between 1 and 4 hr after tetrabenazine administration were about half the normal (Schwartz & others, 1960).

With the histochemical fluorescence technique of Hillarp & others (see Corrodi & Jonsson, 1967) tetrabenazine has been shown to have a depleting effect in the peripheral tissues of the rat and mouse. The fluorescence of noradrenaline accumulated proximal to a ligation of the rat sciatic nerve disappears 4 to 8 hr after tetrabenazine, 100 mg/kg (Dahlström, 1966, 1967). It has also been observed that after tetrabenazine the noradrenaline fluorescence in the iris and vas deferens

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of the rat disappears (Malmfors, and also Norbert & Hamberger, personal communication).

The purpose of the present investigation has been to compare quantitatively the effect of tetrabenazine on the noradrenaline levels in brain and some sympathetically innervated tissues such as heart, submandibular glands, and skeletal muscle in rat. The levels of 5-HT, noradrenaline and dopamine in rabbit brain were also compared with the noradrenaline levels in the heart after administration of tetrabenazine.

Experimental

MATERIAL AND METHODS

Experiments were made on male albino rats of the Sprague-Dawley strain, weighing about 250 g, and on albino rabbits of either sex, weighing about 2 kg. Tetrabenazine was given intraperitoneally (100 mg/kg) to the rats and intravenously (50 mg/kg in single or repeated injections) to the rabbits. The animals were killed at various intervals after the injections. The experiments were done at room temperature (21–23°). Noradrenaline was estimated fluorimetrically (Bertler, Carlsson & Rosengren, 1958; Häggendal, 1963). Dopamine was estimated according to Carlsson & Waldeck (1958) with the modifications of Carlsson & Lindqvist (1962). The 5-HT estimation (Bertler, 1961) was modified in that the perchloric acid residue was re-extracted once with 0·4N perchloric acid.

Results

The results of the noradrenaline estimation on different tissues from rat are shown in Fig. 1. The values are given as per cent of normal values. Four hr after the administration of the drug, values were very low; the

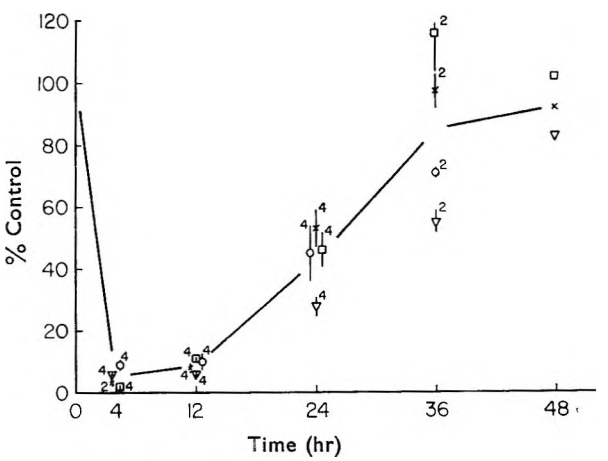

FIG. 1. The noradrenaline levels in per cent of normal values in rat brain (×), heart (▽), submandibular gland (□), and skeletal muscle (○) after tetrabenazine 100 mg/kg body weight, i.p. Means ± s.e. Small figures indicate number of observations.

mean being less than 10% of the normal levels in all the tissues. The levels increased similarly for the different tissues and were about normal 36 to 48 hr after the administration.

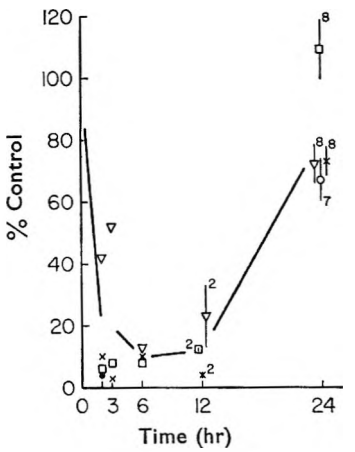

FIG. 2. The brain levels of noradrenaline (×), dopamine (□), and 5-hydroxytryptamine (○), and the heart levels of noradrenaline (▽) in per cent of normal values in rabbits after tetrabenazine 50 mg/kg body weight, i.v. in a single injection. 3 hr values: 50 + 50 mg/kg were given at an interval of 1½ hr and the animal was killed 3 hr after the first injection. 12 hr values: 50 mg/kg were given 4 times at an interval of 3 hr and the animals were killed 12 hr after the first injection. At 2 hr also the noradrenaline in skeletal muscle (●) was estimated. The points are single observations except at 12 and 24 hr where the values are means ± s.e. Small figures indicate number of observations.

The results from the estimation on rabbits are shown in Fig. 2. A similar pattern was found for the changes of the noradrenaline levels in the rabbits as in the rats. Dopamine and 5-HT in rabbit brain were changed in about the same way as noradrenaline. However, the dopamine level after 24 hr was higher than the noradrenaline level.

Discussion

The present results showed only small differences between the noradrenaline changes in brain and sympathetically innervated tissues after tetrabenazine. The doses used were high, being the same as those used by Quinn & others (1959) when they reported little or no noradrenaline depletion in peripheral tissues of rabbits. Differences in strain may be of importance for the explanation of the different results.

These results do not support the suggestion that tetrabenazine should have a relatively specific action on the central nervous system. That tetrabenazine has only a weak effect on the blood pressure compared with the effect of reserpine cannot be explained by suggesting that tetrabenazine has mainly a central nervous effect while reserpine acts centrally and peripherally.

Tetrabenazine, like reserpine, causes *in vitro* release of platelet 5-HT (Paasonen & Pletscher, 1959) and blockade of the specific storage mechanism of adrenal medullary granules (Carlsson, Hillarp & Waldeck, 1963). The two drugs probably have the same site of action (Carlsson & Lindqvist, 1966). After reserpine the noradrenaline stores in different tissues recover to normal values only after several weeks. The recovery after tetrabenazine, however, takes place within about 48 hr. Reserpine's action is thought to be irreversible so that normal levels depend upon the transport to the nerve terminals of fresh granules synthesized in the cell body of the neuron (Dahlström & Häggendal, 1966). The effect of tetrabenazine on amine storage granules appears, however, to be short-lasting and pronounced in both central and peripheral tissues.

Acknowledgements. I would like to express my gratitude to Prof. A. Carlsson and Miss M. Lindqvist for permission to include their results of monoamine estimations on rabbits after tetrabenazine treatment, and to Dr. A. Dahlström for help and discussions. For technical assistance I wish to thank Miss A. Wilén. For generous supply of tetrabenazine I am indebted to Roche-Produkter AB, Stockholm. The study was supported by the Swedish State Medical Research Council (grants B67-14X-166-03 and B68-14X-166-04).

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Relaxation of the guinea-pig tracheal chain preparation by N^6, 2′-*O*-dibutyryl 3′,5′-cyclic adenosine monophosphate

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N^6,2′-*O*-Dibutyryl 3′,5′-cyclic adenosine monophosphate (dibutyryl 3′,5′-AMP), isoprenaline and theophylline relax the guinea-pig tracheal chain preparation; whereas 3′,5′-cyclic adenosine monophosphate (3′,5′-AMP) does not. The relaxant effect of isoprenaline, but not that of dibutyryl 3′,5′-AMP, was blocked by propranolol. 3′,5′-AMP is hydrolyzed rapidly by beef heart phosphodiesterase whereas dibutyryl 3′,5′-AMP is not. The presence of equimolar concentrations of dibutyryl 3′,5′-AMP does not alter the rate of phosphodiesterase mediated hydrolysis of 3′,5′-AMP. These data are consistent with the theories that relaxation of the guinea-pig trachea may be mediated by 3′,5′-AMP and that dibutyryl 3′,5′-AMP acts by mimicking 3′,5′-AMP at its site of action.

CATECHOLAMINES and other hormones may stimulate the enzyme adenyl cyclase to convert adenosine triphosphate (ATP) to 3′,5′-cyclic adenosine monophosphate (3′,5′-AMP), which is inactivated by hydrolysis mediated by a specific 3′,5′-AMP phosphodiesterase to 5′-adenosine monophosphate (AMP) (Sutherland & Rall, 1960). The production of 3′,5′-AMP has been associated with the responses of various tissues to hormones, but it has not always been possible to show a direct cause-effect relation between administration of exogenous 3′,5′-AMP and these same physiological effects. It was generally accepted that the reason for this lack of effect was that nucleotides, such as 3′,5′-AMP, penetrated the cell wall little, if at all (Roll, Weinfeld & others, 1956; Leibman & Heidelberger, 1955), and in direct support of this contention, Robison, Butcher & others (1965) demonstrated that 3′,5′-AMP, when added to the media perfusing the isolated rat heart, did not gain access to the intracellular fluid in concentrations comparable to those seen after the administration of adrenaline.

Posternak, Sutherland & Henion (1962) synthesized a number of derivatives of 3′,5′-AMP to ". . . obtain substances that might have a better action on the intact animal, on isolated organs, or on tissue slices, than the original nucleotide; and obtain substances that might act as antagonists to the original nucleotide". They found that the N^6,2′-*O*-dibutyryl derivative (dibutyryl 3′,5′-AMP) was, among others, more active than the parent 3′,5′-AMP in producing hyperglycaemia in intact dogs. Subsequently, Butcher, Ho & others (1965) showed dibutyryl 3′,5′-AMP to be a more potent stimulant of lipolysis in isolated fat cells than 3′,5′-AMP. Bdolah & Schramm (1965) reported that dibutyryl 3′,5′-AMP (1×10^{-3}M) stimulated amylase secretion in rat parotid cells whereas 3′,5′-AMP (9×10^{-3}M) was inactive. Pasten (1966) observed that dibutyryl 3′,5′-AMP (50–375 μg/ml), like TSH, stimulated both the oxidation of [1-^{14}C] glucose to $^{14}CO_2$ and the incorporation of ^{32}P into phospholipid whereas 3′,5′-AMP (250 μg/ml) was inactive, and Imura, Matsukura & others (1965) indicated

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that dibutyryl 3′,5′-AMP was more effective than 3′,5′-AMP in stimulating adrenal corticosterone secretion in rats *in vivo*. These authors concluded as did Posternak & others (1962) that the greater effectiveness of the derivative is related to its resistance to hydrolysis by phosphodiesterase and perhaps to increased entry into cells. However, no evidence has been presented to deny the possibilities that the dibutyryl 3′,5′-AMP might act by stimulating adenyl cyclase or inhibiting phosphodiesterase.

Bueding, Butcher & others (1966) have shown that a positive correlation exists between the relaxant effect produced by physiological concentrations of adrenaline upon muscle from the taenia coli of guinea-pigs and an increased concentration of 3′,5′-AMP in this tissue. A logical extension of this evidence is that the smooth muscle relaxant effects of catecholamines are mediated by 3′,5′-AMP. The following experiments were made to determine if this mechanism might exist in the guinea-pig trachea and to ascertain the mechanism of action of dibutyryl 3′,5′-AMP.

Experimental

METHODS

Male Reed Willett strain guinea-pigs, 300–400 g, were used for the paired tracheal chain preparation (Foster, 1960). The tissues were suspended under 1 g tension in 10 ml of Krebs-Henseleit solution, 37°, constantly perfused with oxygen 95% and carbon dioxide 5%. Contractions and relaxations were recorded on a smoked drum via a light wooden lever at fourteenfold magnification.

Dibutyryl 3′,5′-AMP was prepared essentially as outlined by Posternak & others (1962). To a suspension of 150 mg of 3′,5′-AMP in 4·5 ml of dry pyridine was added 2·25 ml of n-butyric anhydride, and the mixture heated to reflux until a solution was obtained (6 min). The reaction mixture was cooled to room temperature and allowed to stand for 10 days. The reaction mixture was hydrolysed with 6 ml of water followed by concentration *in vacuo* to a solid residue. The crude product was chromatographed on Whatman No. 1 paper in an ethanol–ammonium acetate (0·5M) (5 : 2) system. The product spot was eluted with methanol and the solvent removed *in vacuo*. An ether precipitation from methanol provided the pure product, 120 mg, λ_{max}^{MeOH} 270 (ϵ = 14,634).

Beef heart phosphodiesterase was prepared as outlined by Butcher & Sutherland (1962). The enzyme was partially purified as described using ammonium sulphate fractionations, dialysis and freezing but was not fractionated on DEAE-cellulose. Its protein content, determined by the method of Warburg & Christian (1941), was 6·4 mg/ml. Its K_M value was 10^{-4}M. Either or both substrates, 4×10^{-4}M were incubated at 30° with 0·02 ml enzyme in a total volume of 2 ml containing 0·2 μmole EDTA, 4·0 μmole $MgSO_4$ and 80 μmole Tris-HCl buffer, pH 7·5. The reaction was stopped by boiling. One mg of lyophilized *Crotolus atrox* venom dissolved in 1 ml of Tris-HCl buffer was then added to the incubation. This mixture was incubated for 30 min at 30° and the reaction stopped by addition of 1 ml 20% trichloroacetic acid. The tubes were centrifuged

and the supernatant was applied to a Dowex 1-X8 (Cl form, 100–200 mesh) column. After washing, the inorganic phosphate was eluted with 1 ml of saturated potassium chloride solution. Phosphate was determined colorimetrically by the method of Fiske & Subbarow (1925).

Results and discussion

As previously shown by Foster (1966), the guinea-pig tracheal chain preparation was relaxed by isoprenaline (0·001–0·030 μg/ml) or the phosphodiesterase inhibitor, theophylline (0·4–25·0 μg/ml), intimating the existence of a relaxant mechanism similar to that in the taenia coli muscle. Furthermore, dibutyryl 3′,5′-AMP (500 μg/ml) relaxed the preparation (Fig. 1); however, equimolar amounts of 3′,5′-AMP were inactive. The

FIG. 1. Relaxation of the guinea-pig tracheal chain preparation by isoprenaline and dibutyryl 3′,5′-AMP. 1. Isoprenaline 0·03 μg/ml. 2, 3 and 4 Dibutyryl 3′,5′-AMP, 4, 160 and 500 μg/ml respectively. Time scale = 5 min.

onset and duration of response to dibutyryl 3′,5′-AMP were prolonged compared to those of isoprenaline. The response to 3′,5′-AMP in combination with submaximal concentrations of theophylline, was the same as that to theophylline alone, probably because 3′,5′-AMP did not enter the cell or did so at such an insignificant rate that partially inhibited phosphodiesterase was still capable of hydrolyzing it before it reached its active site.

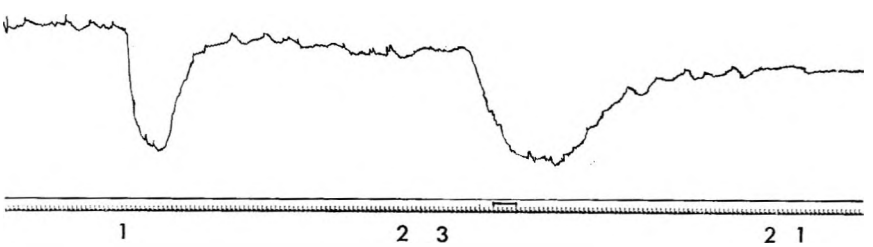

FIG. 2. The effects of the β-adrenergic blocking agent, propranolol, upon the responses of the guinea-pig tracheal chain preparation to isoprenaline and dibutyryl 3′,5′-AMP. 1. Isoprenaline, 0·03 μg/ml. 2. propranolol, 0·3 μg/ml. 3. Dibutyryl 3′,5′-AMP, 500 μg/ml. Time scale = 5 min.

Propranolol, in concentrations which blocked supramaximal concentrations of isoprenaline, did not alter the response to dibutyryl 3′,5′-AMP (Fig. 2).

The rate of hydrolysis of dibutyryl 3′,5′-AMP by phosphodiesterase was insignificant compared to that of 3′,5′-AMP (Fig. 3) and the presence of equimolar amounts of dibutyryl 3′,5′-AMP did not alter the rate of hydrolysis of 3′,5′-AMP by phosphodiesterase.

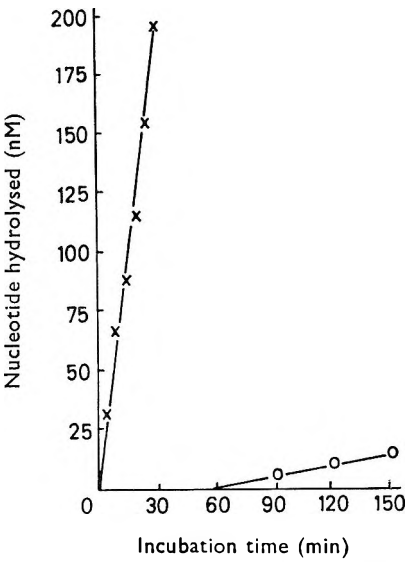

FIG. 3. Rates of hydrolysis of 3′,5′-AMP (×) and its dibutyryl derivative (○) in the presence of phosphodiesterase. Substrate concentrations, 4×10^{-4} M.

The data supports the contention that there is a relaxant mechanism in guinea-pig tracheal muscle similar to or identical with the mechanism in the taenia coli muscle wherein catecholamines induce the formation of 3′,5′-AMP accompanied by relaxation and the return to initial tone is governed by phosphodiesterase mediated hydrolysis of this 3′,5′-AMP.

Since the action of dibutyryl 3′,5′-AMP was not blocked by propranolol, it is improbable that it acts by releasing catecholamines which subsequently stimulate adenyl cyclase to form 3′,5′-AMP. It is also improbable that it unites with phosphodiesterase as a false substrate enhancing the activity of endogenously produced 3′,5′-AMP as do the methylxanthines, since it did not alter the rate of hydrolysis of 3′,5′-AMP by phosphodiesterase. The most logical explanation remaining for the activity of dibutyryl 3′,5′-AMP is that it mimics 3′,5′-AMP at its site of action. The longer onset of action observed with dibutyryl 3′,5′-AMP is probably the result of a slow entrance into the cell and the prolonged relaxation the result of its freedom from inactivation by phosphodiesterase and slow exit from the site of action.

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Blocking action of tetrahydrocannabinol upon transmission in the trigeminal system of the cat

A. J. LAPA, C. A. M. SAMPAIO, C. TIMO-IARIA AND J. R. VALLE

The influence of tetrahydrocannabinol upon polysynaptic reflexes involving the trigeminal system was investigated in cats by recording potentials from either the superior sensory nucleus of the trigeminal nerve or the nerve itself where it enters the pons. Stimulation of the lower eye-lid just sufficient to evoke a mild contraction of the orbicularis muscle was employed. Amplitude of the postsynaptic potentials began to decrease 5 to 10 min after injection of 0·8 or 1·0 mg/kg in most experiments. The presynaptic potential also underwent a striking depression whereas the tibialis nerve potentials, used as control, were practically unaffected. It seems that impairment of the impulse conduction along the presynaptic fibres instead of a central synaptic blockade was the dominant effect of THC. However, a blockade of synaptic transmission is likely to occur in other centres accounting for other neural effects of THC.

THE assumption that the trigeminal system is involved in the central action of cannabis goes back to the original observation by Gayer (1928) that the drug abolishes the rabbit blink reflex (Valle, Souza & Hyppolito, 1966). Sampaio, Lapa & Valle (1967) have demonstrated that cannabis, tetrahydrocannabinol and pyrahexyl induce the disappearance in dogs of the mandibular jerk following electrical stimulation of the central end of the lingual nerve. Thus, the influence of cannabis upon the polysynaptic reflexes which encompass trigeminal nuclei, needs a detailed analysis of the site of action of tetrahydrocannabinol (THC), one of the active principles of *Cannabis sativa* L.

Experimental

MATERIAL AND METHODS

Nine adult cats weighing 2·5–3·5 kg were anaesthetized with sodium pentobarbitone intraperitoneally. Anaesthesia was kept light throughout the experiments.

Stimulation of the trigeminal afferents was through hook electrodes implanted in the conjunctiva of the lower eye-lid. Rectangular pulses of 0·01 msec duration, and intensity just sufficient to evoke a mild contraction of the orbicularis muscle, were delivered to the conjunctiva every 2 sec through an isolation unit. The potentials were recorded from either the superior sensory nucleus of the trigeminal nerve or the nerve itself where it enters the pons, by means of a stainless electrode thoroughly isolated except for the tip. The stereotaxic technique for angle implantation was used to place the active electrode; the reference electrode was attached to the skull. Recording was made through a conventional RC coupled amplifier and the potentials were photographed directly from the oscilloscope display for further analysis.

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When the results of the first experiments appeared to be consistent, the dorsal root potential evoked by single pulse stimulation of the tibialis posterior nerve was also recorded from two animals, so that conduction along the afferent trigeminal nerve could be compared with conduction in a peripheral pathway afferent to the spinal cord.

An ethanolic solution of THC (10 mg/ml) was diluted with polysorbate 80 and saline and the fine suspension slowly injected intravenously in doses of 0·4, 0·8 or 1·0 mg/kg. Higher doses of 1·6 mg/kg were sometimes given, and in control experiments the vehicle alone was given.

At the end of the experiment the animal was killed by an overdose of the anaesthetic and the head perfused with 10% formaldehyde. The electrode position was then checked by histology.

Results

The records from the sensory nucleus of the trigeminal nerve showed a postsynaptic focal potential after a latency ranging from 1·2 to 4 msec. The presynaptic potentials, as recorded from the fibres afferent to the nucleus, appeared 0·8 to 1 msec after stimulation. In some experiments both potentials could be simultaneously recorded (Fig. 1A) from within the nucleus.

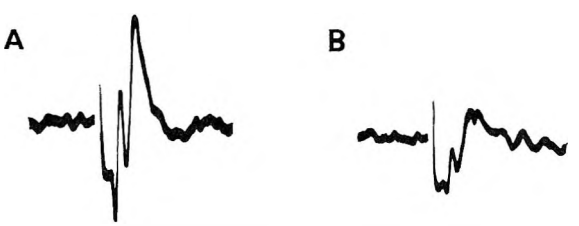

FIG. 1. Potentials recorded from the sensory nucleus of the trigeminal nerve. (A) Control, (B) Depression of the presynaptic and postsynaptic potentials 5 min after 0·8 mg/kg of THC being injected.

The amplitude of the postsynaptic potentials began to decrease 5 to 10 min after the injections of 0·8 or 1·0 mg/kg of THC in most experiments. Their latency did not change even under the influence of 1·6 mg/kg of the drug.

In preparations in which a second dose of THC greater than the first was administered, the depression of the potential provoked by the first dose was only slightly enhanced.

Fig. 1B shows the effect of 0·8 mg/kg of the THC on the amplitude of the pre- and postsynaptic potentials 5 min after the injection. The amplitude was strongly reduced whereas the latency remained unchanged. In Fig. 2 the changes of both pre- and postsynaptic potentials from another experiment are plotted as a function of time. Once again the time course of the depression of both potentials was identical, but the tibialis nerve action potentials were only slightly affected by the drug although the trigeminal potentials were reduced to a mere 5% 30 min after THC was administered.

In two experiments in which the potentials were recorded directly from the trigeminal nerve their reduction was comparable to the one observed in presynaptic intranuclear fibres.

Discussion

Although studies using microelectrodes should be done to establish if synaptic transmission is also impaired, our results indicate that failure to activate the trigeminal sensory nucleus, after administration of THC, may be attributed especially, if not exclusively, to a blocking action of the drug upon the impulse conduction along the presynaptic fibres. Such a depression was evident in all experiments in which afferent potentials were picked up. It might be thought that both nerve conduction and synaptic transmission were blocked. However, records showed increased latency of the postsynaptic focal potential, an effect which would certainly occur were a synaptic block also involved. Studying the effects of phenytoin on the spinal trigeminal nucleus, Fromm & Landgren (1963) found that this drug prevents transmission without interfering with the presynaptic conduction. A constant finding in their experiments was an increase in latency of discharge of the trigeminal neurons, as revealed by microelectrode recording.

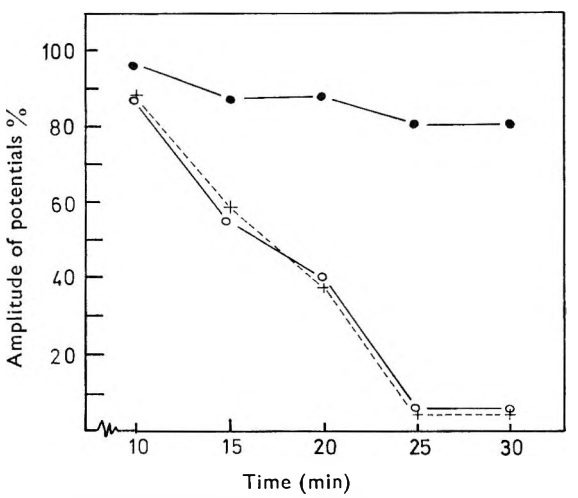

FIG. 2. Changes of the trigeminal nucleus potentials provoked by 1·0 mg/kg of THC. +, presynaptic potential. ○, postsynaptic potential.
The uppermost curve (●) represents the changes undergone by the dorsal root potential. Amplitude of the control potentials was considered as 100%.

In all cases THC provoked strong effects on the conduction by the trigeminal afferent fibres of the nerve impulse; whereas the tibialis nerve potentials were only mildly affected in the two preparations tested. This fact points to a selective action of THC. Such specificity seems to be usually overlooked when considering an explanation for the effects of psychotropic

drugs. Yet, a given drug may block conduction in one neural system and impair synaptic transmission in another. This might be so for certain central effects of THC itself. Xavier & Timo-Iaria (1963) observed that chlorpromazine transforms the polyphasic effects of stimulation of the midbrain reticular formation upon monosynaptic spinal reflexes into pure facilitation; whereas it depresses the reflexes when injected intravenously.

According with the findings of Boyd & Meritt (1965) with a THC derivative, several levels of the nervous system can be influenced by the drug. This point of view may explain other neural effects of the THC such as analgesia and respiratory depression (Valle, 1966).

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Potentiation by cocaine and 3,3-di(*p*-aminophenyl)-propylamine (TK 174) of the effect of isoprenaline and noradrenaline on isolated strips of cat spleen

G. P. LESZKOVSZKY AND L. TARDOS

Contractions of cat isolated spleen strips by noradrenaline and isoprenaline are brought about by interaction with α-adrenergic receptors. The effect of isoprenaline cannot be diminished by *in vivo* reserpine pretreatment. The effects of both noradrenaline and isoprenaline are potentiated by cocaine and by a new diphenylpropylamine derivative TK 174 [3,3-di(*p*-aminophenyl)propylamine]. Noradrenaline is potentiated more by TK 174 than by cocaine while the reverse is seen with isoprenaline. These results suggest that beside the inhibition of noradrenaline uptake another factor may be involved in the mechanism of action of cocaine, and to a lesser extent of TK 174.

PREVIOUSLY Leszkovsky, Tardos & others (1966, 1967a) reported that the peripheral effects of noradrenaline are much increased by the diphenylpropylamine derivative 3,3-di(*p*-aminophenyl)propylamine (TK 174). They have also suggested (Leszkovszky, Tardos & others, 1967b) that the mechanism of action of this substance is similar to that of cocaine.

Further experiments on isolated organs have been made to test this hypothesis. Isoprenaline was included because its inactivation in the organism differs from that of noradrenaline (Hertting, 1964). The isolated spleen strip was chosen because it is contracted by isoprenaline as well as by noradrenaline.

Experimental

METHODS

Cats weighing 2·6 to 3·7 kg were splenectomized under chloralose-urethane anesthesia. Some experiments were made on spleens from cats pretreated with reserpine (2 mg/kg of crystalline reserpine dissolved in 20% ascorbic acid) intraperitoneally, 24 hr before surgery.

The excised spleens were either used immediately or stored in Locke solution at 4° for 24 hr. Like Bickerton, Rockhold & Micalizzi (1962) and de Geus, Bernards & Verduyn (1956), we found the sensitivity to sympathomimetic amines of spleens so stored not to be diminished.

Strips—1 cm wide and 2 cm long—were cut from the spleens and attached under 1·5 g tension to an isotonic lever (magnification of 1 : 20). The 30 ml organ bath was vigorously gassed with pure oxygen and maintained at 38·5°. The bathing fluid had the following composition (%): NaCl 0·75, KCl 0·042, $CaCl_2$ 0·024, $NaHCO_3$ 0·024, NaH_2PO_4 0·014, glucose 0·1. The experiment was begun after an equilibration period of about 40 min. Noradrenaline and isoprenaline were used in constant concentrations of $1{\cdot}48 \times 10^{-6}$M and $2{\cdot}018 \times 10^{-4}$M, respectively. Cocaine and TK 174 were added to the organ bath 5 min before the amine. As

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the contraction of splenic smooth muscle is rather slow, a contraction was recorded until it reached a maximum, then the preparation was washed. The vertical heights of contractions measured on the kymographs were evaluated; mean values with their standard errors are listed in the results.

The drugs used were: (−)-noradrenaline bitartrate, (±)-isoprenaline hydrochloride, Dibenamine hydrochloride, propranolol hydrochloride, cocaine hydrochloride, reserpine and TK 174 (molecular weight: 241·32).

Results

Noradrenaline, $1{\cdot}48 \times 10^{-6}$M, induced contractions of 20 to 30 mm which were halved by $3{\cdot}30$ to $6{\cdot}60 \times 10^{-7}$M Dibenamine.

Increasing equimolar concentrations of cocaine and TK 174 were tested. High concentrations ($1–2 \times 10^{-4}$M) of both produced contractions of spleen strips. This phenomenon, however, was not observed with the lower concentrations used to potentiate catecholamine effects. Between two concentrations, noradrenaline was added to the bath at least twice to detect accidental changes in the sensitivity of the organ. Contractions recorded in the presence of cocaine or TK 174 were compared with those immediately preceding them. Table 1 shows the heights of contractions recorded both before and after the addition of the test substance, as well as the difference between them in both absolute (mm) and relative (percentage) terms.

TABLE 1. POTENTIATION OF THE EFFECT OF NORADRENALINE ($1{\cdot}48 \times 10^{-6}$M) ON ISOLATED STRIPS OF CAT SPLEEN

Substance	Conc. M	No. of exps	Height of contractions* before the addition of the substance to be tested: before (mm)	Height of contractions* before the addition of the substance to be tested: after (mm)	Increase due to the substance tested in: absolute (mm)	Increase due to the substance tested in: relative (%)
					terms	
Cocaine hydrochloride	$8{\cdot}8 \times 10^{-7}$	5	22·8 ± 2·9	33·6 ± 5·6	10·4 ± 5·0	47
	$4{\cdot}4 \times 10^{-6}$	5	26·8 ± 4·4	47·8 ± 7·7	21·0 ± 6·6	78
	$2{\cdot}1 \times 10^{-5}$	5	35·8 ± 5·5	65·6 ± 13·2	29·8 ± 9·4	84
TK 174	$1{\cdot}7 \times 10^{-7}$	6	30·7 ± 4·5	39·2 ± 4·9	8·5 ± 2·9	28
	$8{\cdot}3 \times 10^{-7}$	6	26·4 ± 5·1	47·4 ± 6·6	20·1 ± 6·4	79
	$4{\cdot}2 \times 10^{-6}$	6	36·2 ± 11·8	65·8 ± 14·3	29·7 ± 4·8	82
	$2{\cdot}1 \times 10^{-5}$	5	36·4 ± 10·2	73·8 ± 15·0	37·4 ± 6·2	103

* Mean values ± s.e.

It can be seen from Table 1 that the effect of noradrenaline is enhanced by both cocaine and TK 174. The potency of TK 174 is approximately five times that of cocaine.

Isoprenaline, $2{\cdot}018 \times 10^{-4}$M also produced contractions of 20–30 mm. These were fully abolished by Dibenamine ($3{\cdot}30$ to $6{\cdot}60 \times 10^{-7}$M) but were left unchanged by propranolol ($0{\cdot}676$ to $1{\cdot}69 \times 10^{-5}$M). They were not inhibited by reserpine pretreatment; in 9 strips taken from spleens of 5 reserpinized cats, $2{\cdot}018 \times 10^{-4}$M isoprenaline produced contractions of 33·9 ± 9·2 mm. This mean value is higher than any of those observed on non-pretreated cat spleens (see Table 2, column 4) but it does not differ significantly from the larger of these values.

The potentiating effect on isoprenaline contractions of cocaine and TK 174 was examined as in the experiments with noradrenaline. Detailed results are in Table 2.

TABLE 2. POTENTIATION OF THE EFFECT OF ISOPRENALINE ($2{\cdot}018 \times 10^{-4}$M) ON ISOLATED STRIPS OF CAT SPLEEN

Substance	Conc. M	No. of exps	Height of contractions* before the addition of the substance to be tested: before	Height of contractions* before the addition of the substance to be tested: after	Increase due to the substance tested in: absolute (mm)	Increase due to the substance tested in: relative (%)
			mm	mm	terms	
Cocaine hydrochloride	$1{\cdot}8 \times 10^{-7}$	8	$30{\cdot}3 \pm 4{\cdot}3$	$37{\cdot}0 \pm 5{\cdot}1$	$6{\cdot}7 \pm 3{\cdot}3$	22
	$8{\cdot}8 \times 10^{-7}$	9	$26{\cdot}7 \pm 4{\cdot}2$	$31{\cdot}7 \pm 3{\cdot}3$	$5{\cdot}0 \pm 1{\cdot}6$	18
	$4{\cdot}4 \times 10^{-6}$	8	$24{\cdot}8 \pm 3{\cdot}1$	$35{\cdot}0 \pm 3{\cdot}1$	$10{\cdot}1 \pm 1{\cdot}3$	41
	$2{\cdot}1 \times 10^{-5}$	13	$32{\cdot}9 \pm 5{\cdot}1$	$52{\cdot}0 \pm 4{\cdot}6$	$19{\cdot}1 \pm 5{\cdot}8$	58
TK 174	$8{\cdot}3 \times 10^{-7}$	9	$16{\cdot}7 \pm 4{\cdot}4$	$19{\cdot}8 \pm 3{\cdot}8$	$3{\cdot}1 \pm 1{\cdot}5$	18
	$4{\cdot}2 \times 10^{-6}$	14	$30{\cdot}7 \pm 6{\cdot}5$	$29{\cdot}9 \pm 5{\cdot}7$	$-0{\cdot}8 \pm 6{\cdot}7$	−3
	$2{\cdot}1 \times 10^{-5}$	12	$29{\cdot}1 \pm 4{\cdot}1$	$39{\cdot}3 \pm 5{\cdot}4$	$10{\cdot}2 \pm 2{\cdot}9$	45

* Mean values ± s.e.

It is clear from these data that the effect of isoprenaline on the cat isolated spleen strip is potentiated by both cocaine and TK 174. The latter is effective only at the higher ($2{\cdot}1 \times 10^{-5}$M) concentration while the former is active at the concentration 5 times lower.

Discussion

Isolated spleen strips are contracted by both noradrenaline and isoprenaline. This effect is due to interaction with α-adrenergic receptors; it can be blocked by low concentrations of Dibenamine. The effect of isoprenaline could not be blocked by propanolol. We found isoprenaline to have about one hundredth of the potency of noradrenaline, as did Bickerton (1963) and Kizaki & Abiko (1966) in experiments made on the spleens of various species.

The effect of isoprenaline is due to its direct action on the receptors, as it is not diminished by a reserpine pretreatment sufficient to deplete catecholamines from cat spleen (Thoenen, Tranzer & others, 1966). The same action has recently been shown for another α-adrenergic effect of isoprenaline by Gay, Rand & Wilson (1967).

Cocaine increased the effect of noradrenaline in accordance with the literature on its potentiation of various sympathetic effects. The new diphenylpropylamine derivative TK 174 also increased this noradrenaline effect. TK 174 has been found to be more potent than cocaine in potentiating the effect of noradrenaline on the guinea-pig isolated vas deferens (Leszkovszky & others, 1967b). Now it has been shown to be more potent on the isolated spleen. Thus, it may be supposed that TK 174 inhibits uptake of noradrenaline into nerve terminals more actively than cocaine. Our results suggest, however, that another factor may play a role in the effect of cocaine. We found that the effect of isoprenaline is also potentiated by cocaine. According to most investigators, the effects of isoprenaline are not increased by cocaine (Smith, 1963; Stafford, 1963;

Andén, Corrodi & others, 1964; Hardman, Meyer & Clark, 1965; Trendelenburg, 1966; Bhagat, Bovell & Robinson, 1967). Thus, our observation seems to be contrary to most of the published data. A result similar to ours has recently been published by Gay & others (1967) who found in experiments on isolated perfused ear arteries of the rabbit that the vasoconstrictor action of isoprenaline was much increased by cocaine. It seems worthwhile to note that in those reports which failed to demonstrate any potentiation by cocaine of isoprenaline effects, various β-adrenergic actions of isoprenaline were being examined. Like us, Gay & others (1967) found it was an α-adrenergic effect, resulting in smooth muscle contraction, that was potentiated by cocaine.

Although the effect of isoprenaline was potentiated less by TK 174 than by cocaine, for noradrenaline their potency ratio was reversed. This shows that besides the block of noradrenaline uptake there must be some other factor in the mechanism of action which is not equally strong for both substances.

The possibility of a direct muscular action of cocaine is suggested by the recent work of Bevan & Verity (1967) who stated that cocaine increased the maximum response of both normal and nerve-free (denervated) strips of rabbit aorta to noradrenaline.

This other action of TK 174 (possibly direct muscular) is weaker than that of cocaine. Considering this fact together with the lack of any central nervous excitatory activity of TK 174 (Leszkovszky & others, 1967b), we suggest that TK 174 can advantageously be used as a pharmacological tool for studying supersensitivity to sympathomimetic amines and related questions.

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A note on the effects of some cholinergic and anticholinergic drugs on the aggressive behaviour and spontaneous electrical activity of the central nervous system in the ant, *Formica rufa*

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The influence of various cholinergic and anticholinergic drugs on the aggressive behaviour and electroencephalogram was investigated in the ant, *Formica rufa*. Both atropine and scopolamine decreased aggressiveness and electrical activity of the brain whilst tubocurarine caused an opposite effect. The possible role of nicotinic and muscarinic receptors in the central nervous system of the ant is discussed.

THE role of acetylcholine in the central nervous system of insects is not established. Effects obtained with various cholinergic drugs might be taken to indicate the existence of cholinergic transmission mechanisms (Schallek & Wiersma, 1948, 1949; Schallek, Wiersma & Alles, 1948). On the other hand, eserine does not increase the action of acetylcholine in preparations of insect terminal abdominal ganglia (Prosser, 1940; Turner, Hagins & Moore, 1950).

Acetylcholine in the insect nervous system has been identified by various techniques (Chang & Kearns, 1955; Lewis & Smallman, 1956; Colhoun, 1958). Very high concentrations of acetylcholine (143 μg/g tissue wet weight) and acetylcholinesterase (137 μg acetylcholine hydrolysed g/tissue/hr) were obtained in the cockroach (*Periplaneta americana*) brain (Colhoun, 1958, 1959).

We have assessed the influence of some cholinergic and anticholinergic drugs on the aggressive behaviour and spontaneous electrical activity of the central nervous system in the ant, *Formica rufa*. This is an extension of previous work from our laboratory, concerned with the action of some neurohormones and psychotropic drugs on the brain of this species (Kostowski, Beck & Mészáros, 1965, 1966; Kostowski, 1966).

EXPERIMENTAL

Aggressiveness of ants. The modified test of ants attacking a beetle was used (Kostowski, 1966). 15 ants, *F. rufa*, were placed in a Petri dish (10 cm in diameter), surrounded with water. After 15 min a beetle, *Geotrupes* sp., was placed in the dish and the number of attacking ants was counted after 1, 2 and 3 min. The three counts of attacking ants were averaged and multiplied by ten to give an index of aggressiveness (Kostowski, 1966). Nicotine and (+)-tubocurarine dissolved in 0·6% saline were injected into the abdominal cavity by microsyringe in amounts of 0·5–1·0 μlitre. All other drugs were administered orally in honey.

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Spontaneous electrical activity of ant brain. The electroencephalogram (EEG) was recorded from the optic lobes of the ant by the method of Kostowski & others (1966). Tungsten wire electrodes 40–50 μ in diameter, insulated by epoxide varnish were used. The EEG records from the optic lobe surface were made with a Biofizpribor (USSR) EEG apparatus. All drugs were injected in 0·6% saline into the abdominal cavity in 0·5 μlitre amounts with a microsyringe.

Drugs. The drugs used were: pilocarpine hydrochloride (Polfa), eserine salicylate (Vis), atropine sulphate (Polfa), scopolamine hydrobromide (Polfa), nicotine sulphate (Tescat Lab), (+)-tubocurarine chloride (Burroughs Wellcome).

RESULTS

Aggressiveness of ants. The results are in Fig. 1. In control experiments the index of aggressiveness (IA) was 67·8. Atropine or scopolamine (0·3–0·5 μg/mg body weight) decreased aggressiveness after 2–4 hr (IA = 44·8 and 47·5 respectively). Tubocurarine (0·1–0·2 μg/mg) increased the aggressiveness after 0·5–1·0 hr (IA = 76·5). A slight but not

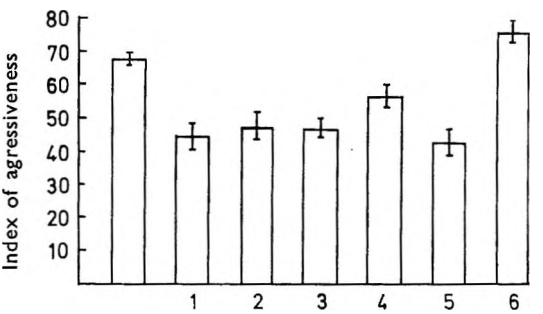

FIG. 1. The effect of drugs on aggressiveness of ants. Unlabelled column, control (40 experiments). 1, Atropine (15 exp.), 2, scopolamine (13 exp.). 3, Pilocarpine (18 exp.). 4, Eserine (25 exp.). 5, Nicotine (10 exp.). 6, Tubocurarine (10 exp.). Vertical bars represent the standard error. Statistically significant differences were calculated according to Student's *t*-test. They are: $P < 0{\cdot}02$ for 1, 2 and 3. $P < 0{\cdot}05$ for 4 and 6. Differences were significant between 5 and 6 ($P < 0{\cdot}002$), 3 and 4 ($P < 0{\cdot}005$), 4 and 5 ($P < 0{\cdot}05$), and 1 and 4 ($P < 0{\cdot}05$).

significant fall of aggressiveness was observed 2–4 hr after eserine (0·1–0·15 μg/mg) whilst both pilocarpine (0·5 μg/mg) and nicotine (0·05 μg/mg) markedly decreased aggressiveness (IA = 47·2 and 43·7 respectively) after 1–2 hr. Toxic effects such as rigor, ataxia and death were observed after larger doses of nicotine (1–2·0 μg/mg). Tremor and clonic movements of the extremities were sporadically observed after the usual doses of eserine. Aggressiveness between ants, disturbances of locomotor activity, inability to maintain an upright posture and slight ataxia occurred 0·5–1 hr after injection of tubocurarine.

Spontaneous electrical activity of ant brain. The characteristic EEG pattern of the optic lobe of the ant in control experiments consisted of

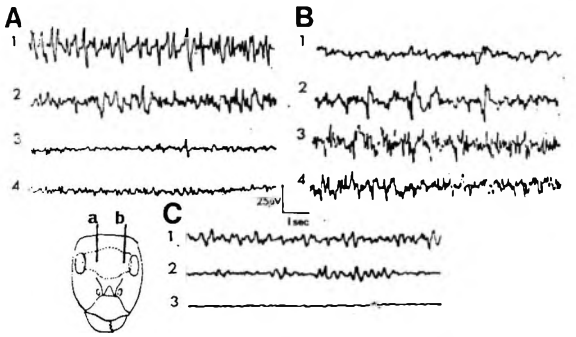

FIG. 2. EEG of ants. Points of leads marked on the diagram of the head. 1, Control. 2, 2 min; 3, 5 min; 4, 20 min after A, scopolamine or B, pilocarpine (0·1 μg/mg weight). C, 1, Control, 2, 5 min; 3, 15 min after atropine (0·2 μg/mg).

potential changes of 5–50 μV with a frequency of 3–6/sec. Of the drugs investigated, pilocarpine and tubocurarine (0·1 μg/mg) increased the amplitude and frequency of the potential changes. 5–15 min after injection of drugs, the frequency rose as high as 8–12/sec and the amplitude sporadically to 100 μV (Figs 2 and 3). This effect persisted 30 min or more. Both scopolamine and atropine (0·1–0·2 μg/mg) caused a decrease of amplitude 5–15 min after administration (Fig. 2). A similar effect

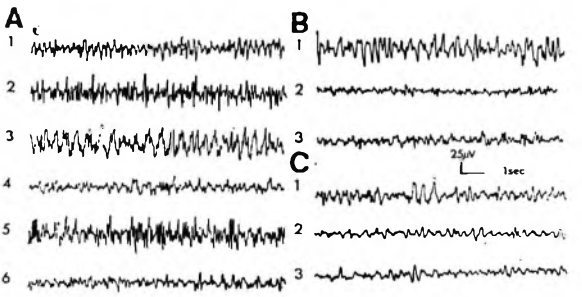

FIG. 3. EEG of ants. A 1, Control. 2, 5 min; 3, 15 min; 4, 35 min after tubocurarine (0·1 μg/mg). 5, 5 min; 6, 20 min after a further injection of tubocurarine (0·1 μg/mg). B. 1, Control; 2, 5 min; 3, 15 min after nicotine (0·05 μg/mg). C. 1, Control. 2, 5 min; 3, 15 min; after eserine (0·1 μg/mg).

was observed 5–15 min after nicotine (0·05 μg/mg). Eserine (0·1 μg/mg) did not cause a clear effect. 8–10 experiments were made with each drug. The results are summarized in Table 1.

DISCUSSION

On the basis of the results it may be possible to postulate the existence of cholinergic transmission in the central nervous system of the ant. Cholinergic blocking agents such as atropine or scopolamine decreased the aggressiveness and the amplitude of the EEG record. On the other

hand, the muscarinic stimulant pilocarpine caused an increase of amplitude and frequency of the EEG record but the effect on aggressiveness was similar to that obtained with atropine or scopolamine. The nicotinic and antinicotinic drugs caused opposite effects on aggressiveness as well as on spontaneous electrical activity of the ant brain. Decreased aggressiveness and increased amplitude of the EEG record were observed after nicotine. Tubocurarine increased aggressiveness and caused the appearance of high amplitude, fast waves in the EEG. It seems possible that

TABLE 1. THE EFFECT OF DRUGS ON AGGRESSIVENESS AND EEG PATTERN OF THE ANT, *F. rufa*.

Drug	Aggressiveness	Changes in EEG pattern	
		Amplitude	Frequency
Pilocarpine	Decreased	Increased	Increased
Eserine	Slightly decreased	Slight decrease or no change	No change
Nicotine	Decreased	Decreased	Decreased or no change
Atropine	Decreased	Decreased	No change
Scopolamine ..	Decreased	Decreased	No change
D-Tubocurarine ..	Increased	Increased	Increased

these effects might be related to the existence of both nicotinic and muscarinic receptors in the central nervous system of the ant. Since the antinicotinic drug, tubocurarine, produces excitation of the CNS of the ant we may suppose that nicotinic receptors are involved in inhibition in central pathways. Although some of the drug responses are contradictory it may be possible that muscarinic receptors in the central nervous system are involved in excitation or in excitation and inhibition but further investigation is needed.

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A note on the blockade of uptake of noradrenaline by 4-chloro-αα-dimethylphenethylaminopropan-2-one in rodents

W. LIPPMANN

4-Chloro-αα-dimethylphenethylaminopropan-2-one blocked uptake of [^{3}H]noradrenaline in the heart, had no effect on the endogenous levels of catecholamines or 5-hydroxytryptamine in the heart, brain or adrenals and decreased the [^{3}H]noradrenaline-depleting activity of metaraminol, but not reserpine, in rodents. The compound appears to act by interfering with the active transport of noradrenaline through the nerve cell membrane.

VARIOUS 4-chlorinated aralkylamines cause alterations in the tissue levels of the monoamines. 4-Chloro-*N*α-dimethylphenethylamine decreases the brain 5-hydroxytryptamine (5-HT) concentration without appreciable effect on the noradrenaline concentration in the rat (Pletscher, Bartholini & others, 1964; Lippmann & Wishnick, 1965a) or guinea-pig, whereas in the mouse and rabbit, there is but little effect on either concentration (Pletscher & others, 1964). In the brain of the cat, 4-chloro-*N*α-dimethylphenethylamine causes a large decline in the 5-HT concentration, but not in the noradrenaline concentration, in the hypothalamic area and in the diencephalo-thalamic area (Lippmann & Wishnick, 1965a). 4-Chloro-αα-dimethylphenethylamine causes no decrease in 5-HT or noradrenaline in the brain of the mouse or guinea-pig (Dubnick, Leeson & others, 1963). In the rat brain after administration of di(3,4-dichlorophenethyl)amine, there is a rapid transient decrease of 5-HT, noradrenaline and dopamine as well as a concomitant increase of 5-hydroxyindolylacetic acid (5-HIAA). After treatment with *N*α-dimethyl-4-nitrophenethylamine, a rapid transient decrease of 5-HT and 5-HIAA occurs in the rat brain (Pletscher, Da Prada & others, 1966). In the rat and guinea-pig brain the decrease in 5-HT after 4-chloro-*N*α-dimethylphenethylamine is slow and is accompanied by a slow decrease in 5-HIAA (Pletscher & others, 1964). Studies have been made on the structurally related 4-chloro-aralkylamine compound 4-chloro-αα-dimethylphenethyl aminopropan-2-one (AY-14,948) and this report describes the effects of AY-14,948 on the uptake and storage of the monoamines.

EXPERIMENTAL

For the determination of radioactive noradrenaline levels in tissues, male albino mice (Canadian Breeding Laboratories, 24–26 g) or male albino rats (Charles River, 60–80 g) were injected in the tail vein with 0·25 ml containing 5 μc (±)[7-^{3}H] noradrenaline* hydrochloride (New England Nuclear Corp.) and 0·85 μg (−)-noradrenaline hydrochloride in a solution of 0·75% sodium chloride and 0·01N hydrochloric acid. Drugs were injected intraperitoneally in 0·5 ml saline unless otherwise specified. Control animals received injections of the appropriate vehicle. The

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* 2-Amino-1-(3,4-dihydroxyphenyl)-[1-^{3}H]ethanol.

tissue samples were homogenized in ice-cold 0·4N perchloric acid and centrifuged. A portion of the supernatant fluid was transferred to a vial containing a mixture of 1 ml methanol, 3 ml ethanol and 10 ml toluene phosphor [0·4% of 2,5-diphenyloxazole and 0·005% of 1,4-di(5-phenyl-oxazol-2-yl)benzene], and the total radioactivity was measured by liquid scintillation counting. The counting efficiency was 22%. Brain catecholamine levels were determined in the rats as described by Lippmann & Wishnick (1965b). Brain 5-HT levels were estimated by the fluorimetric procedure of Bogdanski, Pletscher & others (1956) on aliquots of the final acid extract (Mead & Finger, 1961). The levels of heart noradrenaline in acetic acid eluates from aluminium oxide columns were determined by oxidation with ferricyanide (Whitby, Axelrod & Weil-Malherbe, 1961). Adrenal catecholamines were isolated and determined as previously described (Lippmann & Wishnick, 1965b). Monoamine oxidase activity was measured by the method of Kraml (1965) and catechol-*O*-methyl transferase according to Anderson & D'Iorio (1966).

Drugs used were: reserpine (Serpasil; Ciba Co. Ltd.) and metaraminol bitartrate (Aramine; Merck Sharpe and Dohme Ltd.). AY-14,948 was synthesized by Dr. A. Langis (Ayerst Laboratories) and was in the form of the hydrochloride salt. Student's *t*-test was used to evaluate the data.

TABLE 1. EFFECT OF AY-14,948 ON THE UPTAKE AND RELEASE OF [^{3}H]NORADRENALINE IN THE MOUSE AND RAT HEART

A. MOUSE

Drug	Time drug given before or after [^{3}H]noradrenaline	Radioactivity content	
		Counts/min/g ± s.e.	% of control
None	1 hr, before	4,394 ± 197	
AY-14,948	1 hr, before	2,516 ± 94	58
None	1 hr, after	3,551 ± 163	
AY-14,948	1 hr, after	3,285 ± 126	93

AY-14,948 was administered at 50 mg/kg, i.p. The animals were killed 4 hr after the drug. There were 27 animals in the control and 21 in the treated group.

B. RAT

None	1 hr, before	16,193 ± 1,461	
AY-14,948	1 hr, before	5,029 ± 453	31
Imipramine	1 hr, before	796 ± 69	5
None	1 hr, after	15,588 ± 956	
AY-14,948	1 hr, after	15,316 ± 785	96
Imipramine	1 hr, after	16,596 ± 843	106

AY-14,948 was administered at 50 mg/kg, i.p., and imipramine at 20 mg/kg, i.p. The animals were killed 4 hr after the drug. There were 12 animals in the control and 10 in the treated group.

RESULTS

The effects of AY-14,948 on the uptake and release of [^{3}H]noradrenaline ([^{3}H]-NA) in the mouse and rat heart are shown in Table 1. AY-14,948 was administered (50 mg/kg, i.p.) to the animals one hr before or after [^{3}H]-NA and the radioactivity contents of the hearts determined. When the AY-14,948 was given before the [^{3}H]-NA and the animals were killed 4 hr after administration of the aralkylamine, the radioactivity of the mouse and rat heart had decreased by 42 and 69% respectively.

No appreciable decrease in [^{3}H]-NA level was observed in either the mouse or rat heart when AY-14,948 was given after it. In the rat, imipramine (20 mg/kg, i.p.) caused a 95% drop in the [^{3}H]-NA when given before and no decline when given after it. Thus, AY-14,948, like imipramine, caused a decline in the uptake and had no effect on the release of [^{3}H]-NA in the heart.

TABLE 2. EFFECT OF AY-14,948 ON CATECHOLAM NE AND 5-HT CONTENTS OF VARIOUS TISSUES OF THE RAT

Drug	Time animals killed after AY-14,948, hr	Heart catecholamines, μg/g ± s.e.	P	Adrenal catecholamines, μg/pair ± s.e.	P
None		0·52 ± 0·04 (13)		32·5 ± 1·5 (17)	
AY-14,948	7·5	0·61 ± 0·03 (7)	>0·1	29·1 ± 1·6 (8)	>0·1
AY-14,948	16·5	0·45 ± 0·06 (8)	>0·1	32·3 ± 2·3 (7)	>0·5
AY-14,948	24·0	0·52 ± 0·03 (8)	>0·5	25·6 ± 3·1 (6)	>0·05
		Brain catecholamines, μg/g ± s.e.		Brain 5-HT μg/g ± s.e.	
None		0·58 ± 0·02 (7)		0·61 ± 0·05 (7)	
AY-14,948	7·5	0·59 ± 0·03 (8)	>0·5	0·56 ± 0·06 (8)	>0·1
AY-14,948	16·5	0·53 ± 0·02 (7)	>0·1	0·64 ± 0·08 (8)	>0·5
		Heart catecholamines, μg/g ± s.e.		Adrenal catecholamine, μg/pair ± s.e.	
None		0·65 ± 0·07 (13)		21·9 ± 0·6 (15)	
AY-14,948	3	0·58 ± 0·04 (8)	>0·1	20·6 ± 0·7 (8)	>0·1
AY-14,948	24	0·56 ± 0·05 (7)	>0·1	22·0 ± 1·7 (7)	>0·5
		Brain catecholamines, μg/g ± s.e.		Brain 5-HT μg/g ± s.e.	
None		0·42 ± 0·02 (15)		0·56 ± 0·02 (15)	
AY-14,948	3	0·36 ± 0·03 (8)	>0·1	0·57 ± 0·01 (8)	>0·5
AY-14,948	24	0·42 ± 0·02 (7)	>0·5	0·53 ± 0·03 (7)	>0·1

The animals were injected with AY-14,948 at 50 mg/kg, i.p. The tissues were removed at the times indicated. The number of animals is in parentheses.

Table 2 shows the effects of AY-14,948 on catecholamine and 5-HT contents of various tissues of the rat. There were no changes in the catecholamine contents of the heart, brain, or adrenals at 3, 7·5, 16·5 or 24·0 hr after administration of AY-14,948 (50 mg/kg, i.p.). There were also no changes in the 5-HT content of the brain under these conditions.

At a concentration of 1×10^{-4}M, AY-14,948 caused no inhibition of monoamine oxidase or catechol-*O*-methyl-transferase activities *in vitro*. One hr after the treatment of rats with AY-14,948 (50 mg/kg, i.p.) there was no inhibition of the monoamine oxidase activity in the brain or liver and no inhibition of the catechol-*O*-methyltransferase activity in the liver.

The effects of AY-14,948 on the activity of noradrenaline-releasing agents in the mouse heart were determined and are shown in Fig. 1. Mice received [^{3}H]-NA and 15 min later were given AY-14,948 (100 mg/kg, i.p.). Five min after the latter treatment the animals were injected with the noradrenaline releasers metaraminol (0·3 mg/mg, i.v.) or reserpine (0·5 mg/kg, i.v.). The animals were killed 1 hr after the initial treatment and the levels of noradrenaline in the hearts were determined. While AY-14,948 caused no appreciable change in the [^{3}H]-NA content, metaraminol and reserpine caused declines of 45 and 39%, respectively, in the

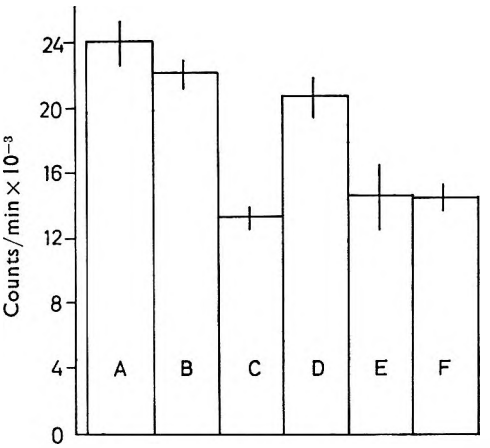

FIG. 1. Effects of AY-14,948 on the activity of noradrenaline-releasing agents in the mouse heart. A. Control. B. AY-14,948. C. Metaraminol. D. AY-14,948 + metaraminol. E. Reserpine. F. AY-14,948 + reserpine.

[^{3}H]-NA content in the heart. AY-14,948 significantly blocked the release obtained after metaraminol but not after reserpine.

DISCUSSION

AY-14,948 acts to block the uptake of [^{3}H]-NA into the storage sites and does not cause a release of the stored [^{3}H]-NA. In addition, it causes no changes in the endogenous levels of catecholamines in the heart, brain or adrenals and 5-HT in the brain. These activities of AY-14,948 are similar to those found with drugs such as imipramine and chlorpromazine. These latter drugs which cause a block in the uptake of [^{3}H]-NA (Axelrod, Hertting & Potter, 1962) also do not alter the endogenous catecholamine levels (Gey & Pletscher, 1961; Sulser & Brodie, 1961).

In comparison with other 4-chlorinated aralkylamines, AY-14,948 is similar to 4-chloro-$\alpha\alpha$-dimethylphenethylamine in its effect on noradrenaline in the rat heart, as both AY-14,948 (Table 2) and the latter compound (Dubnick & others, 1963) cause no changes in the endogenous level. In contrast, AY-14,948 does not decrease the brain 5-HT (Table 2), whereas 4-chloro-*N*α-dimethylphenethylamine does (Pletscher & others, 1964; Lippmann & Wishnick, 1965a). In the 4-chloro-α-methylphenethylamine series the methyl group on the carbon atom adjacent to the amino-function is necessary for the 5-HT-decreasing activity observed at 16 hr in rat brain. Compounds lacking this branching could be rapidly metabolized by monoamine oxidase (Pletscher & others, 1964, 1966; Fuller, Hines & Mills, 1965). Within this series the compound with the primary amino-group is the most effective in causing the decline in 5-HT. The most effective chloro-α-methylphenethylamine isomer is one in which there is a single chlorine atom substituted in the 4-position. The unchlorinated α-methylphenethylamines do not lower 5-HT levels. Noradrenaline

levels are not appreciably affected after administration of the chloro-α-methylphenethylamines under these conditions (Fuller & others, 1965). Other aralkylamines related to AY-14,948 have different activities in depleting brain noradrenaline and 5-HT, and are effective for varying durations. Di(3,4-dichlorophenethyl)amine, 4-chloro-*N*-(3,4-dimethylcyclohex-3-enylmethyl)phenethylamine and di-(-chlorophenethyl)amine decrease both the 5-HT and noradrenaline and only for a short time. *N*α-Dimethyl-4-nitrophenethylamine, 2-methyl-4-nitrophenethylamine and 3-chloro-*N*α-dimethylphenethylamine cause a drop in 5-HT of a short duration, and no fall in noradrenaline. 4-Chloro-*N*α-dimethylphenethylamine and di(4-chloro-α-methylphenethyl)amine decrease only the 5-HT, and for a long period, with no fall in noradrenaline (Pletscher & others, 1966). AY-14,948 thus also differs from these drugs in that it caused no decline in 5-HT or noradrenaline at any of the various time intervals.

AY-14,948 produces no inhibition *in vitro* or *in vivo* of the two major enzymes involved in the inactivation of the catecholamines, i.e. the monoamine oxidase and catechol-*O*-methyl transferase. In contrast, 4-chloro-*N*α-dimethylphenethylamine (Pletscher & others, 1966) causes inhibition of the monoamine oxidase *in vitro*.

The metaraminol-induced decrease in [^{3}H]-NA is blocked by a pretreatment with AY-14,948, but that caused by reserpine is not. Imipramine, desipramine and chlorpromazine block the uptake of noradrenaline and act by interfering with the active transport through the nerve cell membrane (Axelrod & others, 1962). Desipramine blocks the release of [^{3}H]-NA induced by metaraminol, but not that by reserpine (Stone, Porter & others, 1964; Carlsson & Waldeck, 1965). Thus, AY-14,948 is similar to desipramine in its actions and appears to act by interfering with the active transport through the nerve cell membrane.

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Rate of accumulation and plateau plasma concentration of drugs after chronic medication

SIR,—The plasma concentration of a drug reaches a steady state level after long-term administration of a fixed dose at fixed time intervals (Boxer, Jelinek & others, 1948; Augsberger, 1954; Krüger-Thiemer, 1960; Krüger-Thiemer & Bunger, 1961).

Assuming the operation of first order kinetics for such a dosage regimen (Teorell, 1939; Dost, 1953) the plasma concentration of a drug A during the intervals when it maintains a plateau blood level can be expressed by the following equation (Dost, 1953; Rossum & Tomey, 1968):

$$C_{A(pl)} = C_{Am}[e^{-t/\tau_2}/(1 - e^{-\Delta t/\tau_2}) - e^{-t/\tau_1}/(1 - e^{-\Delta t/\tau_1})] \quad .. \quad (1)$$

Here $C_{A(pl)}$ is the plasma concentration, τ_1 the time constant for absorption, τ_2 the time constant for elimination, Δt the fixed dosage interval, t the time from the commencement to the end of each interval (from time 0 to Δt) and C_{Am} a constant depending on the dose Q_A^0, the apparent volume of distribution V and the time constants [$C_{Am} = Q_A^0.\tau_2/(\tau_2 - \tau_1)V$].

The plateau concentration therefore oscillates between a minimum value (when $t = 0$) and a maximum value (Dost, 1953; Krüger-Thiemer & Bunger, 1961; Wiegand, Buddenhagen & Endicott, 1963).

The average plateau plasma concentration of the drug over a time interval Δt may be obtained by integration of $C_{A(pl)}$ over the interval Δt and subsequent division by Δt, as shown in the following equation:

$$\bar{C}_{A(pl)} = \frac{1}{\Delta t}\cdot\int_0^{\Delta t} C_{A(pl)}dt = \frac{C_{Am}}{\Delta t}(\tau_2 - \tau_1) = \frac{Q_A^0}{V}\cdot\frac{\tau_2}{\Delta t} \quad .. \quad (2)$$

This equation is identical to that of Wagner, Northam & others (1965) and discussed by Krüger-Thiemer (1966).

Since the biological half-life of the drug corresponds to τ_2 as $t_{\frac{1}{2}} = 0{\cdot}693\ \tau_2$, the average plateau plasma concentration may also be written as:

$$\bar{C}_{A(pl)} = 1{\cdot}44\ .\ \frac{Q_A^0}{V}\cdot\frac{t_{\frac{1}{2}}}{\Delta t} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

The value Q_A^0/V may be calculated from a plasma concentration curve after administration of a single dose. In addition, such a curve also provides information about the degree of absorption (Krüger-Thiemer, 1960, 1966). The plateau concentration is directly proportional to the maintenance dose and half-time for elimination and is inversely proportional to the dosage interval and the volume of distribution.

The time, $tc_{\frac{1}{2}}$, which the average plasma concentration takes to reach half the average plateau concentration provides a measure of the rate of accumulation of the drug. A calculation of $tc_{\frac{1}{2}}$ is possible from an equation representing the average plasma concentration of each dosage interval; for example that for the j^{th} interval is given by the following equation (8):

$$\bar{C}_{A(j)} = \frac{C_{Am}}{\Delta t}[\tau_2(1 - e^{-j\cdot\Delta t/\tau_2}) - \tau_1(1 - e^{-j\cdot\Delta t/\tau_1})] \quad .. \quad (4)$$

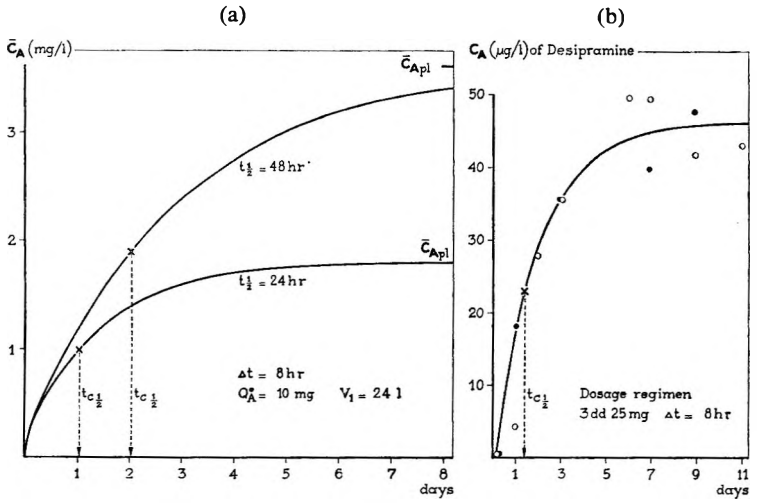

FIG. 1. Drug accumulation curves. (a) The average plasma concentration of two drugs with biological half-lives of 24 and 48 hr after a fixed multiple dosage regimen as calculated from equation (4). The plateau level is proportional to the biological half-life while also the rate of accumulation is practically proportional to $t_{\frac{1}{2}}$. (b) Plasma concentration of desipramine in a patient receiving a dose of 25 mg three times a day (Δt = 8 hr). The accumulation curve has been obtained by combining data of Hammer & others (1967) determined in two different accumulation experiments, made several weeks apart.

TABLE 1. THE PLATEAU LEVEL AND RATE OF DRUG ACCUMULATION. The average plateau plasma concentration obtained ($\bar{C}_{A(pl)}$), the half-time for accumulation ($tc_{\frac{1}{2}}$) and the time for accumulation of the drug to 90% of the plateau level ($tc_{90\%}$) have been calculated from equation (5). The calculations are based on the administration of a fixed dose of drugs (Q^o_A = 10 mg) in a multiple dosage regimen, a volume of distribution (V = 36 litres i.e. sum of extra- and intracellular fluid in a normal man), the time constant for absorption is kept constant for all drugs (τ_1 = 0·72 hr) while the biological half-life ($t_{\frac{1}{2}}$) and the dosage interval (Δt) is varied.

$t_{\frac{1}{2}}$ (hr)	Δt (hr)	$\bar{C}_{A(pl)}$ (mg/l)	$tc_{\frac{1}{2}}$ (hr)	$tc_{90\%}$ (hr)
3	1	1·20	3·79	10·8
6	2	1·20	6·76	20·7
6	4	0·60	6·76	20·7
12	4	1·20	12·74	40·6
12	8	0·60	12·74	40·6
24	4	2·41	24·73	80·5
24	8	1·20	24·73	80·5
24	12	0·80	24·73	80·5
48	8	2·41	48·73	160·2
48	12	1·60	48·73	160·2
96	12	3·21	96·74	319·7
96	24	1·60	96·74	319·7

The average plasma concentrations of two drugs with $t_{\frac{1}{2}} = 24$ and 48 hr calculated from this equation are shown in Fig. 1a. An experimental accumulation curve of desipramine administered to a patient 3 times per day ($\Delta t = 8$hr) for 11 days, and based on the data of Hammer, Ideström & Sjöqvist (1967), is presented in Fig. 1b. The $tc_{\frac{1}{2}}$ calculated from this experiment in this patient is 33 hr.

Since $tc_{\frac{1}{2}} = j.\Delta t$ when $\bar{C}_{A(j)} = 0{\cdot}5\ \bar{C}_{A(pl)}$ the accumulation half-time of a drug may be calculated by combining equations (2) and (4). The following equation is obtained if in addition $j\,.\,\Delta t \gg \tau_1$:

$$tc_{\frac{1}{2}} = t_{\frac{1}{2}}\,[1 + 3{\cdot}30 \log \tau_2/(\tau_2 - \tau_1)] \quad .. \quad .. \quad (5)$$

For most drugs especially those with a biological half-life of more than 12 hr $\tau_2 \gg \tau_1$, this equation can generally be reduced to $tc_{\frac{1}{2}} = t_{\frac{1}{2}}$. This implies that the half-time for accumulation of a drug is approximately equal to its half-time for elimination and is independent of the dosage interval. The drug accumulation is anticipated to have reached its plateau after a time-interval equal to $3\frac{1}{2}$ times the biological half-life (see Table 1). So far a drug with an elimination half-time of 2 days given by a multiple fixed dosage regimen, accumulation occurs at such a rate that the plateau plasma concentration is reached after one week.

The computed plateau plasma concentration half-times for accumulation, and the times at which the plateaux are practically reached are presented for a number of drugs in Table 1.

It may be concluded that the biological half life provides a good approximation of the degree of accumulation. This conclusion has special importance for patients receiving long term medication.

A full discussion of consequences and a detailed mathematical analysis of the equations are in the press (Rossum & Tomey, 1968).

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The effect of staphylococcus α-toxin on the release of acetylcholine in the coaxially stimulated isolated ileum preparation of the guinea-pig

SIR,—Feldberg & Kellaway (1938) were the first to describe the action of staphylococcal toxin on the guinea-pig isolated ileum preparation. Since then its action has been extensively studied (e.g., Brown, Pritchard & Quilliam, 1959; Rašková & Vaněček, 1964; Brown & Quilliam, 1965). The most obvious features of the effect of the toxin on smooth muscles *in vitro* are a slowly developing contracture with subsequent diminution or absence of responses to stimulant drugs and failure to respond to a further dose of the toxin. However, the mechanism of action remains far from clear. The slow contracture it produces may be a direct effect on the smooth muscle cells or due to liberation of a contracting substance from the ileum. Botulinus toxin depresses transmitter release from myenteric plexuses (Harry, 1962) and there remains the possibility that staphylococcus toxin behaves similarly. We have examined this question.

The guinea-pig isolated ileum was stimulated coaxially according to Paton (1955). Pieces of ileum (4–5 cm), taken at least 10 cm proximal to the ileo-caecal junction, were placed in a 5 ml bath containing modified Krebs solution (Eccles, 1952) at 37° and gassed with a mixture of oxygen 95% and carbon dioxide 5%. The distal end of the ileum was tied to a short polyethylene tube allowing the gut lumen to be washed. One platinum electrode (anode) was placed in the lumen of the preparation and the other was placed in contact with the bath fluid opposite the anode and outside the lumen. The ileum was stimulated transmurally with supramaximal rectangular pulses of 400 μsec duration at a rate of 6/min. Stimulation at a frequency of 30/sec was usually required for 3 to 10 sec to produce tetanic responses. The sensitivity to acetylcholine and histamine was also tested. Contractions were recorded by an isotonic lever writing on smoked paper. Acetylcholine output was measured in the presence of eserine salicylate 5×10^{-6} g/ml in the bath. The sequence and duration of acetylcholine collections during the periods without and with the electrical stimulation is shown in Table 1. Acetylcholine activity of samples was assayed immediately on another piece of ileum in the presence of eserine salicylate 12·5 μg/litre, and morphine hydrochloride, 6 mg/litre. This ileum preparation was allowed to equilibrate with the eserinized solutions for 45 min to completely inactivate cholinesterases (Paton, 1957). That the activity was due to acetylcholine was checked. It was stable in acid but destroyed by boiling in alkaline solution, all the biological effects were antagonized by atropine (10^{-8} g/ml).

A filtrate of *Staphylococcus pyogenes* strain Wood–46 was used. Preparation of the toxin was as described by Johanovský (1956). The haemolytic potency of the toxin, expressed in haemolytic units (H.U.) assessed according to Brown & others (1959), was 5·1 H.U./mg of toxin and the toxin had an intravenous LD50 for albino mice of 4·9 H.U. (3·7–6·4 H.U.)/20 g body weight. The toxicity was lost either after heating to 90° or by gassing with oxygen 95% and carbon dioxide 5% for 10 min. After each of those two procedures, inactivated toxin or the culture media were not spasmogenic on the coaxially stimulated guinea-pig ileum.

In our experiments 13 H.U./ml or more produced a slowly developing contracture, the extent of which was dose-dependent. The contracture was followed by a gradual diminution and finally abolition of the electrical and drug-induced responses. The contracture response had a latency of from 30 to 60 sec and washing out of toxin after a contact time of 10 sec did not impair the contracture. Fig. 1A shows a contracture after 20 H.U./ml of α-toxin.

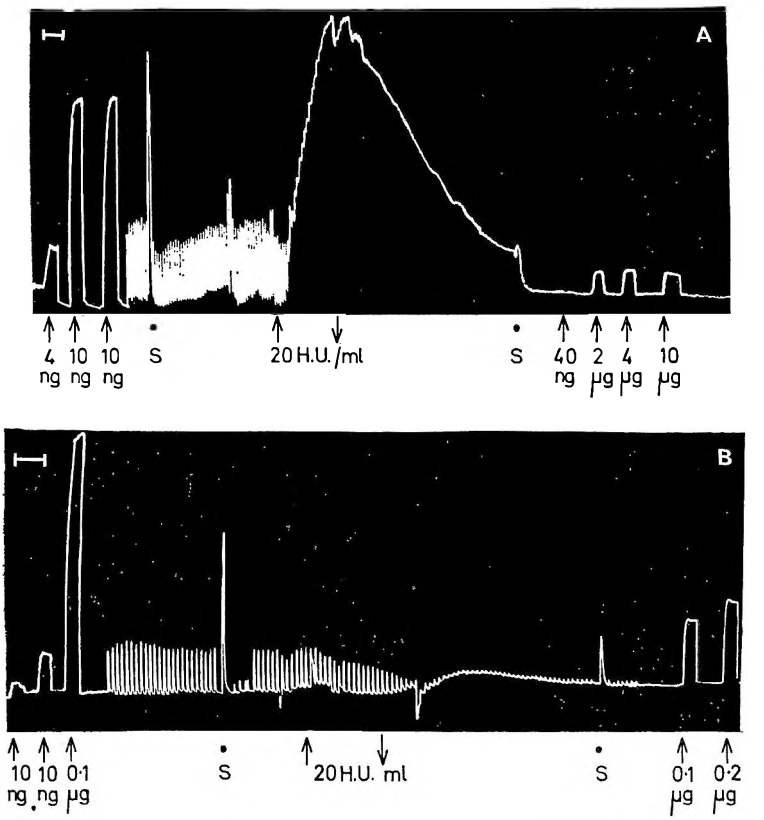

FIG. 1. Coaxially stimulated isolated guinea-pig ileum preparation. Stimulation by rectangular pulses 400 μsec duration and intensity of current to elicit maximal response. A. Experiment in which the toxin is followed by a contracture. B. An experiment without contracture. ↓↑ period of contact with 20 HU/ml of staphylcoccal α-toxin. Figures are doses of acetylcholine. S. 3 sec stimulation with frequency 30 shocks/sec.

TABLE 1. ACETYLCHOLINE OUTPUT FROM THE GUINEA-PIG ISOLATED ILEUM PREPARATIONS EXPRESSED IN NG/MIN/ML OF BATH FLUID AND STATED AS ACETYLCHOLINE HYDROCHLORIDE

Expt No.	Acetylcholine output before and after the 3 min contact with 20 H.U. of α-staphylotoxin								
	Before			After					
	Spont.	Stim.	Spont.	Spont.	Stim.	Spont.	Stim.	Spont.	
1	1·3	1·7	2·6	—	1·7	1·4	1·8	1·5	During stim. periods supramaximal pulses frequency of 1/10 sec were used. Duration of collection periods 20 min.
2	1·2	1·5	1·4	—	1·1	0·5	1·2	—	
3	0·9	2·5	1·1	—	2·2	0·9	2·8	1·0	
4	1·7	2·6	2·5	1·9	2·1	1·1	1·6	1·5	
5	0·5	0·6	0·4	0·3	0·4	0·4	—	—	
6	0·3	0·3	0·2	0·2	0·3	0·3	—	—	
7	0·6	1·5	1·0	0·65	1·0	0·6	1·2	0·7	During stim. periods supramaximal pulses frequency of 2/sec were used. Duration of collection periods 10 min.
8	1·75	2·4	1·6	—	1·7	1·0	1·5	1·2	
9	2·0	4·1	3·6	2·4	3·6	3·4	4·6	2·6	
10	0·4	1·6	—	—	1·1	0·4	1·2	—	
11	0·7	1·9	0·8	—	1·7	0·8	3·2	—	

This response was found in two thirds of our experiments. In the other third, however, desensitization occurred without appearance of the contracture. In all experiments the responses to acetylcholine and electrical stimulation were eventually abolished.

The output of acetylcholine was measured during rest and during stimulation at frequencies of 1/10 sec and 2/sec. Results are in Table 1. There was no change in the output of acetylcholine before or after the addition of the toxin. Even when the frequency of stimulation was raised to 10/sec there was no fall in the acetylcholine output after the toxin at 20 H.U./ml.

Thus, no evidence for changed nervous activity was found and our results accord with those of Brown & others (1959, 1965), Brown & Quilliam (1965) and Thal & Egner (1961) that the toxin has a direct action on smooth muscle and its effect is probably not mediated through nervous plexuses. We have found that toxin does not alter the acetylcholine output.

Since the nerve plexuses are the main source of the acetylcholine output (Paton, 1957, 1963; Harry, 1962; Johnson, 1963), the action of the toxin appears to be entirely postsynaptic and on the smooth muscle itself. The irreversible action of the toxin after a short period of contact argues either for a rapid enzyme-like action with immediate damage of the smooth muscle membrane, or for a fixation of the toxin with the development of gradual changes. A direct action of the toxin upon the contractile substance does not accord with the findings of Gulda & Seč (1966) that the toxin had no effect on actomyosin.

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Effects of guanoxan on intestinal smooth muscles

SIR,—Guanoxan has adrenergic neuron blocking properties (Augstein & Green, 1964) and combines the chemical structure of both guanethidine and benzodioxan in one molecule. Introduced as an antihypertensive agent, one of its reported side-effects is an urgency to defaecation, some minutes after its administration (Peart & MacMahon, 1964), or diarrhoea (Frohlich, Dustan & Page, 1966).

We have previously noticed that the administration of guanoxan to our anaesthetized animals often induced defaecation (Bueno, de Castro & Sollero, 1967).

In 20 dogs anaesthetized with pentobarbitone sodium (30 mg/kg), the intestinal contractions were examined *in situ* by means of an extensible balloon filled with saline which was intraluminally inserted in the small intestine and connected to a water manometer. After intravenous administration of guanoxan, and paralleling the fall in blood pressure, an immediate and short-lasting contraction was seen.

With the isolated ileum of the guinea-pig suspended in Tyrode solution at 37·5°, guanoxan evoked contractions in doses beginning with 2 μg/ml. Repeated administration did not induce tachyphylaxis and a direct dose-effect relation was also seen (Fig. 1). The contractions were not blocked by an antihistamine drug (promethazine 0·5 μg/ml), a 5-hydroxytryptamine-blocking agent (BOL 8 μg/ml) or a ganglion blocking drug (hexamethonium 8 μg/ml).

On the rabbit isolated duodenum suspended in Tyrode solution at 37·5°, guanoxan also evoked contractions in doses starting with 1 μg/ml. These contractions are not blocked by atropine sulphate (2 μg/ml) or hexamethonium (8 μg/ml).

It seems that there is a direct action of guanoxan on the smooth muscle fibres and since one of the most common side-effects during antihypertensive therapy is diarrhoea this mechanism should also be re-evaluated for those drugs having a guanidine nucleus in their molecules.

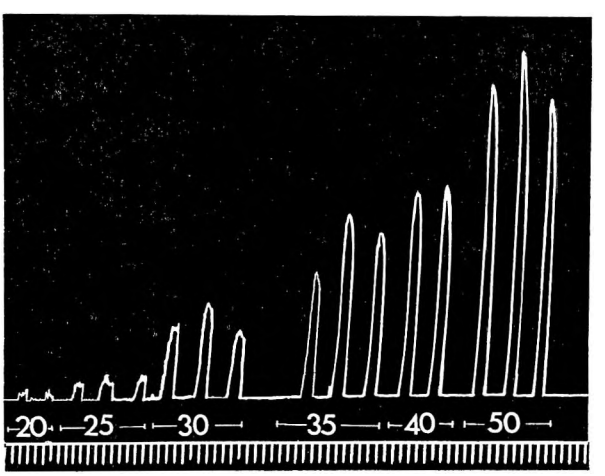

FIG. 1. Guinea-pig isolated ileum. Bath volume: 20 ml. Temperature: 37·5°. Contractions evoked by guanoxan (20, 25, 30, 35, 40 and 50 μg—total doses) added to the bath. The interval between each contraction was 60 sec. Note the dose-effect relation and the absence of tachyphylaxis.

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The effect of the chronic administration of sodium barbitone on the exploratory behaviour of rats

SIR,—Contrary to previous experience with rats (Leonard, 1967) it now appears that the chronic administration of sodium barbitone affects the reward-motivated rather than fear-motivated behaviour, and therefore it was of interest to investigate the action of the barbiturate on unlearned behaviour, for example, exploratory activity. The Y-box test of Steinberg, Rushton & Tinson (1961) appeared to provide a simple, quantifiable method for the determination of exploratory activity.

Female rats (initially 45–55 g) originally of the Wistar strain were housed singly throughout the experiment. Sodium barbitone was added to the drinking water in increasing concentrations over a period of 5 weeks and then withdrawn. The initial dose of barbitone was 100 mg/kg/day and this was increased by increments of 100 mg/kg/day every week. Sodium saccharin (20 mg/100 ml) was added to the drinking water to disguise the bitter taste of the barbiturate and also to the drinking water of the untreated animals. To ensure maximal activity all animals were kept on reversed (12 hr) lighting. The experimental and untreated rats were individually put into the Y-box during the period of barbiturate administration and after its withdrawal as shown in Fig. 1. The total number of entries into the arms of the Y-box in 3 min was recorded. The exploratory activity was measured on the second day after withdrawal of the barbiturate from the experimental rats, as it has been shown previously that this coincided with the period of maximal withdrawal hyperexcitability (Leonard, 1967). All rats were allowed free access to food and water apart from the time during which they were in the Y-box. They were disturbed as little as possible and the experiment was conducted in the room in which they were housed. A diffuse red light enabled the animals to be observed during the time in which they were in the Y-box.

The results (Fig. 1) show that sodium barbitone does not affect the exploratory behaviour and that familiarity with the Y-box does not lead to a noticeable reduction in exploratory activity since the mean response of the untreated animals did not change appreciably during the course of the experiment. When the barbiturate was withdrawn the mean number of entries was reduced by about 70% and was still significantly reduced 4 weeks later. This post-withdrawal depressant effect is surprising because apart from the hyperexcitability and occasional spontaneous convulsions that occurred during the first few

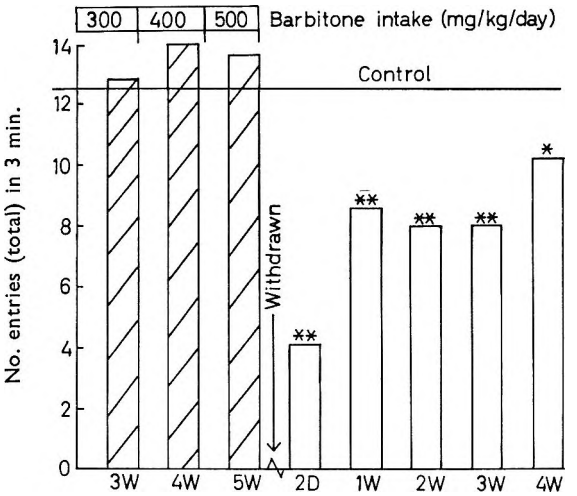

FIG. 1. Effect of the long term administration of sodium barbitone on exploratory behaviour. The ordinate shows the mean number of entries into the arms of the Y-box and the abscissa shows the time, in weeks (W) or days (D), at which the exploratory behaviour was determined. The results are given as the mean values for 6 experimental and 6 untreated rats. The significance of the results, using Students *t*-test, shown as $**P<0{\cdot}001$ and $*P<0{\cdot}05>0{\cdot}02$. Hatched columns before and open columns after sodium barbitone had been withdrawn from the drinking water.

days after withdrawal, there appeared to be no superficial difference in behaviour between the treated and untreated groups of rats for example, when handled, or in response to an auditory stimulus such as a click. Furthermore, no difference was observed between the experimental and untreated groups of rats in the excitability of the central nervous system as assessed by electroshock or chemical convulsants, 10 days after withdrawal (Leonard, 1968).

These results suggest that the effect of a barbiturate on exploratory behaviour after chronic administration is different from the acute effects. Rushton & Steinberg (1963) found that amylobarbitone increased the exploratory behaviour in low doses but that doses greater than 30 mg/kg produced a depression of the exploratory behaviour.

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Iodination of angiotensin II

SIR,—Recent reports concerning radioimmuneassay methods for the measurement of angiotensin II, using angiotensin iodinated with radioactive iodine, have shown that both synthetic angiotensin II, and the naturally occurring hormone may be estimated at very low concentrations (Catt & Coghlan, 1967; Boyd, Landon & Peart, 1967; Catt, Cain & Coghlan, 1967). It has also been suggested that immunologically active but biologically inactive fragments of angiotensin II in the circulation may interfere with the estimation since the antigenic portion of the molecule is close to the C-terminal end and this could result in differing values for angiotensin II obtained by bioassay and radioimmuneassay (Catt & Coghlan, 1967). A further possible source of error arises from the iodination procedure used, as a result of which there is contamination of the iodinated angiotensin with either unchanged angiotensin or with angiotensin which has been modified in ways other than the introduction of iodine atoms. Of the contaminating reactions the most likely is oxidation, since a potent oxidizing agent is used in all published methods of iodination.

I have investigated the efficiency of the iodination method of Hunter & Greenwood (1962) and found that using 5 μg of angiotensin II and 1 mc of $Na^{131}I$ in the reaction mixture, only about 1% of the angiotensin molecules were iodinated. Neither paper nor column chromatography using several systems proved particularly effective in separating the iodinated hormone from unchanged angiotensin and from other products of the reaction. However, after a prior precipitation of most of the inorganic salts present with methanol, paper electrophoresis using acetic acid: sodium acetate buffer, pH 3·2 and an applied voltage of 7 V/cm resulted in clear separation of iodinated angiotensin from angiotensin and the other products of the reaction. The iodinated angiotensin migrated much more slowly towards the cathode (0·2 cm/hr compared with 1·5 cm/hr) than angiotensin. The iodinated angiotensin could be readily eluted from the paper with methanol. The use of iodinated angiotensin prepared in this way should improve both the specificity and sensitivity of radioimmuneassay procedures using this substance.

Because of previous reports concerning the loss of the biological activity of iodinated angiotensin II (Cruz-Coke, 1946; Wolf, Mendlowitz & others, 1961, 1962a, b, c; Barbour & Bartter, 1963) the effect of modifying the method of Hunter & Greenwood for iodinating synthetic angiotensin II has also been investigated. Excess iodine has been used in place of excess chloramine T, sufficient iodine being liberated from the NaI to ensure complete iodination. The iodinated derivative was eluted after electrophoresis. Amino-acid analysis by Dr. T. Bellair of the Russell Grimwade School of Biochemistry, University of Melbourne, according to Spackman, Stein & Moore (1958) showed that the iodinated material obtained contained the amino-acids present in angiotensin II in approximately the expected proportions but it had no biological activity when tested by two methods (Regoli & Vane, 1964; Osborn, Louis & Doyle, 1966). Subsequent experiments also showed that it differed from angiotensin II in the ability of chymotrypsin to destroy it, as judged by a comparison of the rate of formation of iodo-asparaginyl-arginyl-valyltyrosine and the loss of biological activity of angiotensin II under comparable conditions.

The present results and of those of Catt & Coghlan (1967) suggest that structural modifications of angiotensin involving loss of biological activity do not necessarily affect radioimmuneassay procedures.

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Evaluation and control of variability in hormone-stimulated lipolysis in rat adipose tissue

SIR,—We use adrenaline-stimulated lipolysis in isolated rat epididymal fat pads to assay the antilipolytic property of various prostaglandins (Pike, Kupiecki & Weeks, 1967). For accurate assays and screening tests, small pieces of tissue have proved to be impractical because of the variation in the lipolytic response. This variation has been minimized (Schusterová, Krčíková & others, 1964; Carlson, 1965; Finger, Page & Feller, 1966) by distributing tissue from several rats among replicate vessels or by mincing the fat with scissors and using samples from the minced pool. We have extended and evaluated a method using finely chopped tissue pools to control variability between replicate samples whereby up to 30 replicates with 200 mg of tissue each may be used.

The distal portion of fat pads from 12 to 16 rats (male, 260 to 300 g) are removed under ether anaesthesia and incubated for 2 hr in about 125 ml of Krebs-Ringer bicarbonate solution (without glucose, with 3·2% albumin, always aerated with air 95% and carbon dioxide 5%. This pre-incubation allows basal lipolysis to become minimal (Vaughan, 1967). Each fat pad is cut into "cubes" with a McIlwain chopper (H. Mickle, Gomshall, Surrey) (McIlwain & Buddle, 1953), set for 0·73 mm thickness and 80 to 85 strokes/min. The chopped tissue is pooled in 75 ml of Krebs solution and mixed continuously using a magnetic stirrer. Any tissue not completely chopped is removed and the mixture is then centrifuged for 3 min at 1000 rev/min to separate shreds of filter paper from the chopper pad. The floating cake of chopped tissue is then divided among two or three 50 ml beakers and resuspended with stirring in 25 ml of Krebs solution at room temperature. Tissue may be kept for at least 2 hr without loss of lipolytic activity while stirred and aerated at room temperature, but no more than 30 min in a cake. Just before tissue samples are weighed, they are centrifuged again, and samples of 200 to 205 mg are

weighed and added to 10 ml of Krebs solution with 0·1 μg/ml adrenaline. The tissue is dispersed by brief mixing using a Vortex, Jr, and incubated for 60 min at 37° in a shaker set at 100–110 strokes/min. The fluid is aerated using a small polythene tube passed through a loose-fitting plastic cap with the tip placed just above the fluid. A specially designed incubation flask

TABLE 1. VARIANCE COMPONENTS IN ADRENALINE-STIMULATED LIPOLYSIS IN RAT ADIPOSE TISSUE

		Lipolysis rate μmole glycerol/g/hr							
		Tissue snips				Chopped tissue			
Expt*	No. rats	Means	Variation between	Component**	CV	Means	Variation between	Component	CV
		Control of within-rat variability by chopping tissue							
I	10	Right 4·75	Rats	2·30	33·4	1 9·66	Rats	10·29	34·0
		Left 4·35	Sides within-rats	0·59	16·9	2 9·22	Samples within-rats	0·38	6·6
		Mean 4·55	Snips	1·40	25·9	Mean 9·44	Glycerol†	0·30	5·8
		Control of between-rat variability by pooling chopped tissue							
II	10					Right 11·79	Rats	11·91	26·9
						Left 13·90	Sides within-rats	5·62	18·5
						Mean 12·84	Glycerol	0·31	4·4
						Pool 10·98	Samples	1·11	9·7
							Glycerol	0·37	5·6
		Comparison of variability between tissue snips and pooled chopped tissue							
III	9	Right 7·03	Rats	0·77	12·4	Pool 17·76	Samples	3·45	10·5
		Left 7·17	Sides within-rats	0·00	—		Glycerol	0·50	4·0
		Mean 7·10							

* See text for description. ** Variance component (s^2) associated with each source of variation (Bliss 1967). CV Coefficient of variation ($s/\bar{X} \times 100$). † Variance component between replicate glycero determinations.

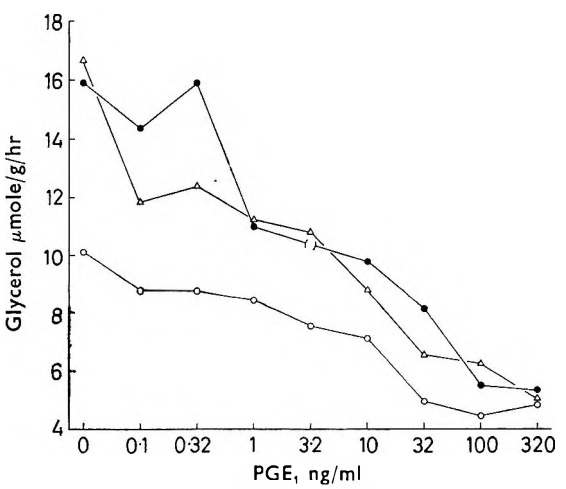

FIG. 1. Inhibition of adrenaline-induced lipolysis in chopped rat adipose tissue by prostaglandin E_1. Three replicate determinations, samples weighed consecutively from one tissue pool, the second and third determinations started 22 and 42 min after the first. Each point represents a single flask. Basal lipolytic rate without adrenaline was 0·87 μmole/g/hr. Entry ( ) is 18·0, discarded as due to experimental error. Replicate 1, ●; 2, △; 3, ○.

prevents tissue fragments from adhering to the sides.* Lipolysis is evaluated by glycerol release. We used the enzymic fluorometric method of Laurell & Tibbling (1966), slightly modified. Conventional tissue snips were 50 to 60 mg pieces cut from the distal tip and treated similarly using 1 ml of Krebs solution.

The three experiments in Table 1 demonstrate the variability in the lipolytic response and the degree to which it can be controlled. In the first experiment (I), two snips were cut from each pad and the remainder of both pads were chopped and divided between two flasks. Comparing the two techniques, the coefficient of variation between rats was the same, however that for snips within rats was 25·9 and that for chopped samples within rats was only 6·6. In the second experiment (II), each pad was chopped and the individual lipolysis rates and that of a tissue pool of all pads determined (7 flasks). The coefficient of variation between rats was 26·9, but was reduced to 9·7 between samples from the tissue pool. The third experiment (III) was to demonstrate, in tissue from the same animals, that both within-rat and between-rat variability was controlled. Snips were cut as in the first experiment, then chopped tissue from all rats was pooled (10 flasks). The within-rat coefficient of variation for snips was the same as in experiment I. Likewise, that between samples from the tissue pool was similar to experiment II. The variability from the glycerol assay was relatively small and there was no consistent difference between right and left pads.

It should be noted that in all experiments the lipolytic rate of chopped tissue was over twice that of tissue snips. Presumably, the greater surface to mass ratio permitted more efficient exchange of metabolites.

Fig. 1 illustrates three replicate concentration-effect curves for prostaglandin E_1 inhibition of lipolysis. Tissue samples for the replicate curves were weighed consecutively, without subdividing the pooled tissue. The first two curves are virtually identical, but the third showed less lipolytic activity. Control vessels from subsequent assays for antilipolytic activity demonstrated that subdivision and resuspension of the tissue prevented this loss of activity. Seven experiments were conducted within a two-week period. In each experiment the tissue was subdivided into two pools and two samples were tested from each pool. There was no difference in lipolytic rate of the two tissue pools, although the second was used 25 min after the first. The coefficient of variation for replicate samples was 7·7.

We are indebted to Dr. F. P. Kupiecki for helpful advice during this study.

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* Details of the flask and of the laboratory procedure may be obtained *direct* from the authors.

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Uptake of debrisoquin and guanethidine by human blood platelets

SIR,—Although there has been much work on the uptake of 5-hydroxytryptamine (5-HT) by platelets, the possibility that these cells might also accumulate other substances has only recently been investigated. Solomon & Zieve (1967) have described the uptake of histamine, procaine, quinidine and reserpine by platelets, energy-dependent mechanisms being involved in the uptake of quinidine.

Since it is known that guanethidine is selectively taken up into sympathetic nerve endings (Schanker & Morrison, 1965; Chang, Costa & Brodie, 1965; Boullin, 1966, 1968), and recent work indicates that the uptake mechanism that concentrates 5-HT in platelets resembles the uptake mechanism of the sympathetic neuron (Pletscher, 1968), we have investigated the uptake of the anti-hypertensive drugs debrisoquin and guanethidine into human blood platelets suspended in normal plasma at 37°, using the technique described by Stacey (1961) for 5-HT. Tritiated guanethidine (Boullin, 1968) and carbon-14 labelled debrisoquin (specific activity 0·9 μc/mg) were used.

Both compounds were taken up by platelets suspended in plasma containing concentrations between 10^{-6} and 10^{-4}M. The ratio of the concentration of drug/ml of packed platelets (C_1) to the concentration/ml of plasma (C_0) varied between 6:1 and 25:1 at the end of 90 min incubation (Table 1). The accumulation process appeared to involve energy-dependent mechanisms, since the uptake of guanethidine and debrisoquin was reduced by 98% (s.e. $\pm$ 2%, 4 experiments with each compound) during incubation at 3° for 90 min. Efflux experiments with guanethidine showed that little drug was lost from platelets after uptake had occurred. In 4 experiments where platelets were re-suspended in drug-free plasma after an initial incubation for 90 min in medium containing 10^{-5}M guanethidine, 9·3% (s.e. $\pm$ 1·3%) was lost during the first 30 min re-incubation, but efflux then diminished.

Our finding that blood platelets can take up antihypertensive drugs until the concentration in the cells is 25 times greater than in the plasma, raises the possibility that this accumulation may be of some significance. Hardisty & Stacey (1955) reported that whole blood contains 4·2 μl platelets/ml, and our results agree with this. On this basis it may be calculated that, if the initial plasma level of drug is 1 μg/ml, 4 μl platelets will take up 0·1 μg if the C_1/C_0 ratio is 25:1. In other words the final distribution of drug in blood will be 90% in platelets and 10% in plasma. As guanethidine is neither lost from, nor metabolized by platelets (unpublished observations), the platelet-bound fraction will represent an increasing proportion of the total blood content as plasma levels decline due to metabolism. For example, if the plasma level declines by 90% over 40 hr, the platelet-bound portion will then represent 50% of the total. Clearly the actual proportion which the platelet-bound fraction represents will depend on the degree of uptake and the rate of decline

TABLE 1. UPTAKE OF DEBRISOQUIN AND GUANETHIDINE BY BLOOD PLATELETS

Drug	Concentration ratio C_1/C_0		
	Initial plasma concentration (M)		
	10^{-6}	10^{-5}	10^{-4}
Debrisoquin	—	14·5 ± 2·6	6·2 ± 0·3
Guanethidine	25·1 ± 1·6	20·1 ± 0·7	11·0 ± 1·0

C_1/C_0 is the ratio of the concentration of drug/ml packed platelets: concentration/ml plasma at the end of incubation.
Results are the mean ± s.e. obtained in 4–5 experiments.

of plasma levels, the above calculations being based on available data for debrisoquin plasma levels in man (Roche Products, Ltd., 1967).

Since the experiments described were carried out under conditions which may be considered to approximate to those occurring *in vivo* in man, there seems to be a *prima facie* case for considering that uptake of the above compounds may occur in patients undergoing anti-hypertensive therapy, and that any circumstance that interferes with the uptake of debrisoquin or guanethidine into platelets, or causes the discharge of these drugs from the platelets, may alter the magnitude and duration of their clinical effects.

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Absence of inhibitory effects of catecholamines on lower vertebrate arterial strip preparations

SIR,—Many pharmacological studies on spiral strips of large arteries from mammals have been reported and both α- and β-adrenotrophic receptors have been demonstrated (Furchgott, 1952, 1954; Furchgott & Bhadrakom, 1953; Bevan, 1960; Maxwell, 1965; Paterson, 1965). In the present work, the pharmacological responses of spiral strips of arteries from lower vertebrates have been examined as part of an investigation of the evolution of the autonomic innervation of the vasculature.

Spiral strips were cut from both right and left systemic arteries of the sleepy lizard (*Tiliqua rugosa*), the toad (*Bufo marinus*) and from the ventral aorta of the trout (*Salmo trutta*) and the eel (*Anguilla occidentalis australis*). The arterial strips from the sleepy lizard and toad were suspended in McKenzie solution as used by Campbell, Burnstock & Wood (1964). The teleost arterial strips were suspended in a modified Krebs solution as used by Bülbring (1953). Recordings were made at 25° either with an isotonic frontal writing lever or isometrically with a tension transducer.

In the lizard, toad, trout and eel, adrenaline tartrate and noradrenaline bitartrate monohydrate caused contraction of the arterial strips. The threshold concentration (salt) for contraction of the systemic artery of the sleepy lizard

was 10^{-9} g/ml and 10^{-8} to 10^{-7} g/ml for the toad. In teleosts the threshold concentration was higher and more variable (10^{-4} to 10^{-6} g/ml).

Isoprenaline hydrochloride contracted the lizard systemic artery preparation, although it was the least potent of the three catecholamines; the minimal sensitivity was 10^{-5} g/ml. However, isoprenaline hydrochloride (10^{-9} to 10^{-4} g/ml) had no effect on either the toad systemic artery or the teleost ventral aorta. Application of isoprenaline never produced a relaxation in any of the preparations, even when the strips had been brought initially to a state of moderate contraction by another agent, for example, acetylcholine 10^{-7} g/ml or noradrenaline 10^{-7} g/ml (Table 1). In contrast, experiments on mammalian aortic muscle have shown that the β-effects of isoprenaline can be revealed in this way (Furchgott, 1952; Furchgott & Bhadrakom, 1953).

The contractile effects of the catecholamines in the lizard and toad preparations were blocked by the α-blockers, phentolamine methanesulphonate 10^{-7} to 10^{-6} g/ml, phenoxybenzamine hydrochloride 5×10^{-7} to 10^{-6} g/ml and Dibenamine 5×10^{-7} to 10^{-6} g/ml. Reversal of the contractile response of the catecholamines to relaxation was never observed, whereas reversal of the action of adrenaline on the rabbit aorta after α-blockade by Dibenamine has been reported (Furchgott, 1952; Furchgott & Bhadrakom, 1953). The β-blocker, pronethalol, 5×10^{-7} to 10^{-6} g/ml blocked the contractile actions of catecholamines on the lizard and toad preparations. The effects of α- and β-blockers on the response of the teleost ventral aorta to adrenaline and noradrenaline appeared to be similar to their effects on the toad, but were difficult to assess because of the variability in the control response.

In conclusion, it appears that there are no catecholamine receptors which mediate inhibition in the systemic artery muscle of the lizard or toad, or in the ventral aorta of the eel and trout. However, in the lizard, the potency ratio for noradrenaline, adrenaline and isoprenaline for contractile effects is consistent with that observed for α-stimulation in mammals. Thus, the catecholamine receptors in this preparation appear to be of the α-type, and the blockade of the responses to catecholamines by the β-blocker, pronethalol, must be unspecific. Arterial strips from lower vertebrates could prove to be useful vascular smooth muscle preparations for studies of the excitatory action of sympathomimetic agents uncomplicated by their effects on receptors which mediate relaxation.

TABLE 1. COMPARISON OF THE RESPONSES OF SPIRAL STRIPS OF LARGE ARTERIES TO CATECHOLAMINES (CONCENTRATIONS IN G/ML) IN DIFFERENT VERTEBRATE CLASSES, AND THE ACTIONS OF α- AND β-BLOCKERS ON THESE RESPONSES

	Mammalia*	Reptilia	Amphibia	Teleosteii
Noradrenaline	+ (10^{-10}–2×10^{-6})	+ (10^{-9})	+ (10^{-8}–10^{-7})	+ (10^{-6}–10^{-4})
Adrenaline	+ (10^{-10}–2×10^{-6})	+ (10^{-9})	+ (10^{-8}–10^{-7})	+ (10^{-6}–10^{-4})
Isoprenaline	+ (10^{-6}) − (10^{-9}–10^{-8})	+ (10^{-6})	no effect	no effect
α-Blockers	Block +	Block +	Block +	Block +†
β-Blockers	Block −	Block +	Block +	Block +†

+ = contraction. − = relaxation. Block + = blockade of contractile response. Block − = blockade of inhibitory response.
*Figures from Furchgott & Bhadrakom (1953).
† See text.

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The anticholinesterase activity of physostigmine

SIR,—When physostigmine in solution undergoes decomposition, its urethane grouping is first lost and a colourless compound termed eseroline is formed; subsequent oxidation leads to rubreserine, a red quinone, which is later converted to eserine blue or eserine brown (Ellis, 1943). We have now investigated the anticholinesterase activities of these degradation products.

Samples of eseroline, rubreserine, eserine blue and eserine brown were kindly supplied by Mr. G. Smith, Department of Pharmacy, Heriot-Watt University. They were dissolved in freshly distilled water and stored at 4° until required. The anticholinesterase activities of the different solutions were compared with that of physostigmine.

In the first experiments, comparisons were made using horse serum as the source of pseudocholinesterase and acetylcholine as the substrate. Both the Warburg manometric technique and the biological method which involves measuring the residual acetylcholine on a piece of isolated tissue (rat colon, rat uterus, guinea-pig ileum) were used. All the degradation products of physostigmine were less active than the parent compound; eserine blue, the most potent, was 100–500 times less active whilst eseroline and rubreserine were 10 times less active than eserine blue. When the comparisons were made using both serum and red blood cells of rabbit, horse and man as the sources of enzyme and acetylcholine as the substrate, all of the degradation products were more active against the pseudocholinesterases than against the true enzymes; on the other hand, physostigmine at a very much lower concentration was equally active against both enzymes.

Finally, tests using the chromodacryorrhoea response in rats (Burgen, 1949) showed that eserine blue, rubreserine and eseroline were about 1,000 times less active than physostigmine in potentiating the *in vivo* action of acetylcholine.

The results are of relevance in that ophthalmic solutions of the British Pharmaceutical Codex are now required to be sterile, and physostigmine eye-drops B.P.C. (Supplement, 1966) may be sterilized by heat. Hydrolysis may occur during the heating process, resulting in the formation of an inactive colourless compound, eseroline, before the appearance of the pink oxidation product, rubreserine. Thus solutions of physostigmine may be colourless but relatively inactive.

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Book Review

PRINCIPLES OF PHYSICAL CHEMISTRY FOR BIOLOGY AND PHARMACY. By L. Saunders. Pp. ix + 438 (including Index). Oxford University Press, London, 1966. 63s. U.K. only.

As this book is aimed at students of biology and pharmacy, it might be as well to emphasize at the start that it is an undergraduate textbook of physical chemistry, not of physical pharmacy or physical biochemistry. To achieve orientation and brevity, special criteria have been applied in selecting material and the result is an unusual but interesting balance between depths and shallows. Some topics have been skimmed over, others extensively treated and developed mathematically step-by-step to give a basis in the physics of chemical processes, particularly those of chemical kinetics, thermodynamics, electrochemistry, chromatography and physical methods of determining molecular size and shape, "topics . . . which are of greater interest in pharmacy and biology." The treatments are relatively simple and for students having a foundation in logarithms, series, differential and integral calculus, most of the steps should be recognizable. However, there are some situations in wave mechanics, entropy, distribution of energies, multiple partition and diffusion, in which more than the usual thin knowledge of mathematics is required and many will grope through these proofs and skip parts of them. Two appendices, on the mechanics of rotors and the distribution of molecular energies, are of more value to the good than the bad mathematician and will not help the laggards and gropers.

Thermodynamics is given as a general background throughout the text rather than as a separate entity and this is a praiseworthy feature. Usually the foundation is built upon energy interconversion and the first law, but here these topics are in the first chapter, in company with rather bald definitions of units and constants, and a 50 page interval deprives the section on work of expansion of gases of its appropriate antepast; some recapitulation would be valuable to condition the student to the thermodynamic approach and need not disrupt the otherwise excellent unity between kinetic theory and internal energy of gases.

A major problem is the assimilation of new and difficult concepts, such as entropy, free energy and chemical potential. They are better described here than in most textbooks, and a real attempt is made to give them meaning.

The conciseness of the treatment is often pleasurable but sometimes leaves a dry air, which could be relieved by inserting experimental results and worked examples at strategic points.

Comprehension would be improved by numbering and cross-referencing intermediate steps in proofs more generously (e.g., on pp. 55, 59, 113) and defining important conditions or limitations fully in words, as symbols may not be understood unless redefined in context or cross-referenced (e.g., $\Delta E = 0$ on p. 56). Nevertheless, arguments are clearly presented, diagrams very helpful (as in the explanation of maximum work on p. 57) and the text is on the whole a model of brevity and simplicity.

Included in some 400 pages are chapters on Atoms, Molecules and Chemical Bonds, Gases, Liquids, The Solid State, Solutions, Two-phase Systems and Surface Chemistry, Chemical Kinetics, Thermochemistry and Chemical Equilibrium, Electrical Conductivity, Potentiometry, Radiation and Molecules, Chromatography and Ion Exchange and Physical Methods for Studying Molecules. The last two chapters go beyond the usual by presenting in some depth the theoretical principles of separation methods and methods for size and weight determination of macromolecules, as well as the more expected topics such as spectroscopic, magnetic and other methods of determining molecular structure. Though these chapters present a useful summary, the emphasis and balance are not fully acceptable, the structure topics being less well done than the macromolecular ones. In four places on pp. 367–8, statements about the effect of mutual proximity of double bonds, auxochromes or aromatic rings on the position of maxima are liable to be misinterpreted and though some data and examples are quoted, the text stops short of perspicacity by failing to connect with ideas of electron delocalization or electron affinity, though such ideas are partially used in the discussions on Dissociation Constants and Dipole Moments. In fact little is added to the earlier discussion on ultraviolet, visible and infrared absorption in Chapter 12, where incidentally the near infrared is given as up to $30\,\mu$ rather than $2{\cdot}5\,\mu$ as is more usual. Surprisingly, little is said about gel separation based upon molecular size differences, an important new weapon in macromolecular biochemistry, though in compensation, enzyme kinetics and inhibition receive an unusually detailed treatment. There are also other brief references to biological phenomena, such as membrane potentials, buffers, Donnan equilibrium and chelation.

To a pharmacist, the sections on solution, disperse systems, interfaces, surfactants, micelles, and viscosity, though lucid, are inadequate, and while mention is made of some pharmaceutical aspects such as biological half-life of drugs and the prediction of decomposition rate, the book is somewhat short on specific pharmaceutical examples and applications. The 70 problems with answers, at the back, are useful and are mostly of a kind which can be solved by substitution if the correct equations are found (though no. 68 involves some guesswork about symbols). There is an adequate index and a useful bibliography but 4 pages of thermochemical data seem somewhat extravagant. The book is well-produced, contains some 140 line figures and has relatively few misprints, only three of which are serious (pp. 116–7, 211, 238). It can be recommended for its emphasis on mathematical principles and its careful explanations of important processes, which make it complementary to books on experimental techniques, applications and systems required to cover the needs of pharmacy and biology students.

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Letters to the Editor

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