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Research Papers

Identifying the names and dosage of drugs

E. C. POULTON

Printing the different doses of a drug (morphine sulphate) larger or in different colours on labels was found to increase the rate at which they could be discriminated from each other by students. Rate of discrimination was measured by the mean time taken both to sort packs of labels, and to search among a matrix of labels. Printing the different doses of an antibiotic preparation (Pro-stabillin-A.S.) in large numerals of different colour made them as quick to discriminate from each other as were different coloured labels. It is believed that changes of this kind in printing could ease the job of finding the required medical preparation, and might reduce the number of instances in which the wrong preparation is given in error.

EVERY year instances are reported of patients receiving the wrong medical preparations. They have come to light either because the error was brought to the attention of the coroner, or because a claim was made for damages. The number which never come to light may be greater. The author believes that in many of these instances the error need not have occurred had the names and dosage of the drugs been clearer. For even if all those concerned had failed to read the label, a brief glance might then have suggested that all was not well, and a proper check instigated

Clearer inscriptions would also ease the job of finding the correct medical preparation, especially when the illumination is poor. The need is illustrated by the statement of a district supervisor that it was her habit to purchase each of the standard drugs used by her midwives from a different drug firm. Thus the different colour and design of label conventionally used by each company helped the midwives to distinguish between the drugs in poor illumination.

Very little experimental work appears to have been published which is directly relevant to the needs of rapid and correct identification. The number of colours which can be unambiguously identified ranges from about 17 for unpractised people (Eriksen & Hake, 1955), to about 50 for the highly practised (Hanes & Rhoades, 1959). The identification of drugs and dosage by colour is thus of value principally to people who handle a very limited number of preparations. Colour coding is being used successfully for gas cylinders, for different types of insulin, and for local anaesthetics used by dentists. But the diversity of medical interest means that colour by itself cannot be a sufficient method of identification for general use, because there are unlikely to be adequate variations available to avoid duplication and its concomitant dangers.

The experiments reported here were designed to investigate the effects of the size and colour of inscriptions upon the ease with which they could be distinguished. Two different experimental techniques for measuring discriminability were used: sorting and searching.

From the Medical Research Council, Applied Psychology Research Unit, Cambridge.

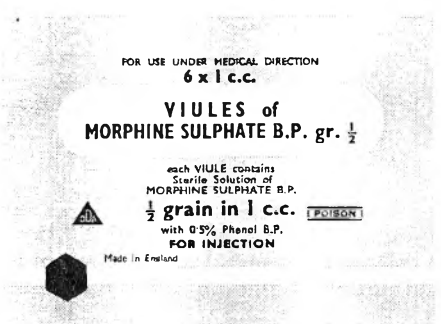
Method

EXPERIMENTAL SUBJECTS

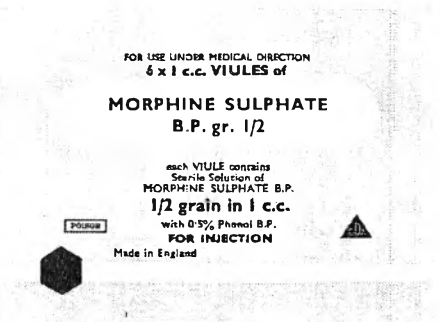
These were male undergraduates of Cambridge University. A separate group of 10 or 12 men was used for each experiment.

EXPERIMENTAL MATERIALS

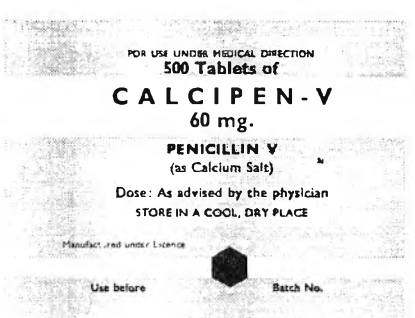
The labels were all 3 × 2 inches. They represented either the fronts of cartons containing six 'viules', or the labels stuck onto bottles. In *Experiments* 1–3, 5 series of labels were examined as listed in Table 1.



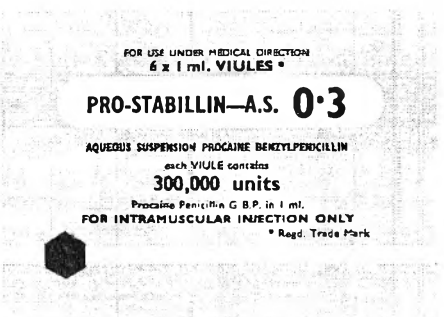
All black



Larger black



Medium sized, different colours



All Large black

FIG. 1. Some examples of labels used. All 2/3 original size. See text and Tables 1 and 2.

Each series contained 5 doses. The labels were all Cambridge blue except for a white area normally $2\frac{1}{2} \times \frac{1}{2}$ inches. Here "MORPHINE SULPHATE" was printed in black on one line in capital letters 2·3 mm high, and generally on the line below: "B.P. gr." followed by the dose in grains: 1, $\frac{1}{2}$, $\frac{1}{3}$, $\frac{1}{4}$, $\frac{1}{6}$. The colours in which the doses were printed, and the heights of the numerals used in the fractions, are listed in Table 1.

The numeral 1 of the "gr. 1" was always 2·4 mm high. For the fractions the numerator was normally printed directly above the denominator, separated from it by a horizontal line whose length varied from 1·2 to 2·0 mm; but for the "larger-black" series the numerator

and denominator were on the same line, separated by an oblique line 3·3 mm long, e.g. 1/2. In the "all different colours" series, gr. 1 was red, gr. $\frac{1}{2}$ light green, gr. $\frac{1}{3}$ middle blue, gr. $\frac{1}{4}$ light brown, and gr. $\frac{1}{6}$ was black. In the "some different colours" series, gr. 1 and gr. $\frac{1}{6}$ were both black, gr. $\frac{1}{3}$ and gr. $\frac{1}{4}$ were both red, and gr. $\frac{1}{2}$ was light green.

The "all black" series was actually in use, and the layout of these labels was different. The drug name and dose were always printed on a single line. For the gr. 1 label the white area was consequently reduced to a width of $\frac{3}{8}$ inch. For the remaining labels "VIULES OF" was printed on the white area above the drug and dose. For the gr. $\frac{1}{2}$ and $\frac{1}{3}$ all the capitals were taller, 2·9 instead of 2·3 mm high. On the blue area of the labels was always printed the remaining details required either by law or by the convention of the company.

For *Experiment* 4, six series were used, each having three doses. The colours and the heights of the numerals are listed in Table 2. The series of "small different colours" contained the light green gr. $\frac{1}{2}$, the middle blue gr. $\frac{1}{3}$, and the red gr. $\frac{1}{6}$ of the morphine sulphate labels used in the previous experiments.

In the three series of large numerals, black, red, and different colours, the numerals 0·3, 0·6 and 0·9, were 5·9 mm high. They were printed at the end of the same line as the name of the drug: "PRO-STABILLIN—A.S." (Aqueous suspension of procaine benzylpenicillin) which was in black capitals 3 mm high. The black numerals, which are the ones currently in use, were printed towards the end of a white area $2\frac{1}{2} \times \frac{3}{8}$ inches. The different colours series, which were the same as for the morphine sulphate labels, light green, red and middle blue, and the red series had a wider area of white $2\frac{1}{2} \times \frac{1}{2}$ inches.

The series of "medium-sized different colours" had a white area $3 \times \frac{3}{8}$ inches. On this was printed "CALCIPEN—V" (penicillin V as calcium salt) in capital letters 3 mm high, and on the line below the dose in numerals 2·9 mm high. For the 60 mg label the name and dose were both black; for the 125 mg label both were light green, and for the 250 mg label the name was in black and the dose in red.

Of the different coloured labels, "SULPHADIMIDINE SUSPENSION" was printed in black capitals 3 mm high on a white area $3 \times \frac{3}{8}$ inches extending across an otherwise Cambridge-blue label. "SULPHAGUANIDINE SUSPENSION" was printed similarly on a yellow label. "TRIPLE SULPHONAMIDES SUSPENSION" (sulphathiazole, sulphanilamide and sulphadimidine) was printed in middle-blue capital letters 2·4 mm high. "SUSPENSION" was on the white area below, and the rest of the label was pink.

For the *Sorting* Experiments 1 and 2, packs of 60 cards were prepared for each series, containing 12 specimens of each of the five doses. The cards were arranged in random order, but were all the same way up. French chalk was used to prevent the cards from sticking together. The cards had to be sorted into two tins, each $4\frac{1}{2} \times 4\frac{1}{2}$ and $1\frac{3}{4}$ inches deep, one for selected the other for discarded cards. The tins were placed side by side on a table in front of the student.

For the *Searching* Experiment 3, the packs of cards were arranged in a 6 × 10 matrix for each series on sheets of black cardboard 20 × 25 inches so that there was a black margin of about $\frac{1}{4}$ inch separating each card from the next. The arrangement was random apart from the following restrictions: (*a*) every row of six cards contained at least one card of each of the five kinds; (*b*) every column of 10 cards contained two cards of each kind; and (*c*) two cards of the same kind could not be adjacent in a row or column.

For the *Searching* Experiment 4 a pack of 36 cards was prepared for each of the six series, and contained 12 specimens of each of the three kinds of label in the series. The pack was arranged in a 6 × 6 matrix on a sheet of black cardboard 20 × 15 inches. The arrangement was random apart from the restriction that every row and column contained two cards of each kind. For both searching experiments (3 and 4) twelve brass-coloured curtain rings 1 inch in diameter had to be placed on the 12 selected cards of one kind.

Illumination during all the experiments was either by good daylight from an adjacent window, or by a 60-watt anglepoise lamp located about 2 ft 6 inches directly above the display.

EXPERIMENTAL DESIGN

In Experiments 1–3, the five series of labels and five doses were arranged in a graecolatin square (Fisher, 1935). This meant that each student discriminated each dose only once, and worked with each series only once. Five students were needed to examine every combination of dose and series. A second five students performed the same conditions in the reverse order to the first five students. A corresponding design was used in Experiment 4 for the six series of labels and three kinds of label within each series. This called for two subgroups each of six students.

PROCEDURE

In the *Sorting* Experiment 1 the student held a pack of cards with the faces upwards. At a signal from the experimenter he had to sort the 12 cards of one particular dose into the left hand tin, and the remainder into the other tin. He was told to work as quickly as he could without making any errors. The experimenter timed him with a stop watch. When he had finished he was told how long he had taken, and the cards were inspected for errors. He was then given the next pack to sort.

The procedure was identical for Experiment 2, except that (*a*) the pack of cards had to be held face downwards, so that the student could not look at the next card while disposing of the previous one; and (*b*) he was told that if he noticed that he had put a card in the wrong tin, he was to remove it and put it in the correct tin. As a result there were never any errors at the end of a trial.

In the *Searching* Experiments 3 and 4 the student held 12 curtain rings, and had to place one on each card of a particular kind. He was told to work as quickly as he could without missing any of the 12 specified cards. The experimenter again timed him, and told him at the end how long he

had taken. Errors never occurred. The experiment was preceded by a practice to teach him the search systematically, taking each row or column in turn. Unsystematic searching was found to give very variable times, since the student might have to search the whole display a second time in order to locate the last specified card. The practice was on a matrix of the same size as the experimental matrices, but carrying simple shapes instead of cards. The student practised placing rings first on one shape and then on another, until he had been convinced of the necessity of a systematic strategy. The average practice was 1·5 trials.

Results

The mean times taken are given in Tables 1 and 2. Analysis of variance showed that differences as great as those between the means of Experiment 2 would occur by chance less often than once in 1,000 experiments.

TABLE 1. MEAN TIME TAKEN TO IDENTIFY DOSES OF MORPHINE SULPHATE

Printing of doses Series	Numeral height in mm	Sorting (sec per card)		Searching (sec per ring) Exp. 3
		Face up Exp. 1	Face down Exp. 2	
All black	1·6	0·77	1·27	1·96
All red	1·9	0·74	1·22	1·78
Larger black	2·4	0·82	1·12	1·63
Some different colours	1·9	0·77	1·13	1·46
All different colours	1·9	0·67	1·14	1·40
0·5% point for the reliability of differences		0·16	0·10	0·28

TABLE 2. MEAN TIME TAKEN TO IDENTIFY LABELS

Printing of doses Series	Numeral height in mm	Searching (sec per ring) Exp. 4
All large red	5·9	1·06
All large black	5·9	0·97
Small different colours	1·9	0·90
Medium sized different colours	2·8	0·87
Large different colours	5·9	0·77
Different coloured labels	—	0·78
0·3% point for the reliability of differences	—	0·20

The same held for Experiments 3 and 4, but differences as large as those between the means of Experiment 1 would occur by chance more often than once in 20 experiments. This is indicated in the bottom line of the tables by an estimate of the size of the smallest difference between any two means in the corresponding column which can be accepted as reliable statistically. The probability level of 5% which is accepted by convention has been divided by 10 in Table 1 since there are 10 possible comparisons, and by 15 in Table 2.

The results of Experiments 2 and 3 in Table 1 show that discrimination took longest with the "all black" series at present in use, and reliably less time was taken both with larger black numerals and with numerals

printed in different colours. The slight advantage in Experiment 3 of different colours over size was not reliable statistically. There was virtually no difference between the series containing some different colours and the series containing all different colours.

The results of Experiment 4 in Table 2 show that large numerals printed in different colours were reliably quicker to discriminate from each other than were large numerals printed all in one colour, either black or red. They were in fact discriminated as quickly as were complete labels printed in different colours. The slight advantage shown by doses printed in small or medium-sized numerals of different colours, over doses printed large in a single colour, was not reliable statistically.

There were no errors in Experiments 2–4. In the *SEARCHING* Experiments 3 and 4 this was presumbly because the student could see where he had put his rings. In the *SORTING* Experiment 2 it was because he never made an error without spotting it at once, and had been instructed to correct any error he spotted. There were altogether six errors in the sorting Experiment 1. All involved failing to select one of the 12 cards which had to be picked out from the rest. Half the errors were for the "all red" numerals, but the overall number is too small to allow statistical treatment.

Discussion

The results show that inscriptions can be discriminated from each other more quickly either when they are printed in lettering larger than has generally been the practice (Table 1), or when the key differences are printed in contrasting colours (Tables 1 and 2). A combination of larger lettering in contrasting colours makes for the quickest discrimination (Table 2). No added advantage is to be gained by printing the complete labels in contrasting colours.

When only three different colours were used for the five doses of morphine sulphate (Table 1, Some different colours), the doses were discriminated from each other about as quickly as when five different colours were used (Table 1, All different colours). This suggests that the students cannot have been using colour alone as a basis for their discriminations, but rather a combination of colour and dose.

Sorting with the cards held face downwards in Experiment 2 was about equally sensitive to the differences in printing as was searching in Experiment 3. However, sorting with the cards held face upwards in Experiment 1 did not discriminate very adequately between the five series. This was presumably because with the cards held face upwards the student could be deciding where to place the next card while he was disposing of the last. Thus sorting time was determined almost entirely by the relatively long movement time, which was the same for all conditions, and hardly at all by the differences in the time needed to discriminate between the different inscriptions. An earlier pilot experiment, using a separate group of 17 students, also failed to discriminate between the series. Here the cards had to be sorted into five tins, each labelled with one of the cards, and ordered by dose from gr. 1 on the left to gr. $\frac{1}{6}$ on

the right. The overriding difficulty appeared to be to remember the correct location of the tins without having to check their labels each time, a difficulty which was shared by all five series.

Acknowledgements. I am most grateful to Dr. B. Garforth, Deputy Production Manager of the Fine Chemical Department of Boots Pure Drug Co., Ltd., for discussing these problems with me and for providing the labels used in the experiments. Financial support from the Medical Research Council is also gratefully acknowledged.

References

Eriksen, C. W. & Hake, H. W. (1955). *J. exp. Psychol.*, **50**, 153–160.
Fisher, R. A. (1935). *The Design of Experiments*, Edinburgh: Oliver & Boyd.
Hanes, R. M. & Rhoades, M. V. (1959). *J. opt. Soc. Amer.*, **49**, 1060–1064.

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Method of graphical analysis of 2 + 2 and 3 − 3 biological assays with graded responses

B. T. WARNER*

Three examples of 2+2 and 3+3 biological assays with graded responses are analysed graphically. The method described has the advantage that only two "general purpose" nomograms are necessary for the analysis of assays with a wide range of ratios of successive doses and numbers of observations; assays with block restrictions may also be analysed. The accuracy of the method is discussed. An appendix gives the theory of the method and of the construction of the nomograms.

THE biological assay of solutions of known composition but unknown concentration can be considered complete only when the relative potency and its fiducial (or confidence) limits have been determined. Preferably, in my opinion, the experimenters themselves should make these determinations; however, biologists often find the standard statistical computations tedious and liable to error. In this paper some alternative graphical methods are described; they are offered for use as a check on the standard methods and, in many cases, as a substitute for them.

A similar procedure was first devised by the author for the analysis of routine tests of synthetic compounds in an industrial pharmacological research laboratory. However, the statistical bases of many of the tests and of parallel line dilution assays (Finney, 1952) are equivalent. Hence two "general purpose" nomograms have been prepared for 2 + 2 and 3 + 3 assays with graded responses.

The method described here differs from previous graphical approaches in three main ways. First, a log-dose response diagram is plotted, and hence less arithmetic is required. Secondly, the present method differs from others (Healy, 1949; Gridgeman, 1951; Leech & Grundy, 1953) by using an approximation to the fiducial limits of the relative potency instead of the exact limits, although it resembles these and most other graphical procedures in estimating variability of the observations by the range. Thirdly, these nomograms may be used for a wide range of ratios of successive doses and numbers of observations, and for assays with block restrictions. However, as with other methods, the ratio of successive doses of both test and standard preparations must be the same within any one assay, and there must be equal numbers of observations on each dose of both preparations.

The nomograms and their use

The nomograms are illustrated in Figs 1 and 2. For use they are printed on transparent photographic film. Each nomogram is about 12 × 10 inches in size, the response sum scales on the left hand sides being in inches. The theoretical basis of constructing the nomograms is described in a statistical appendix.

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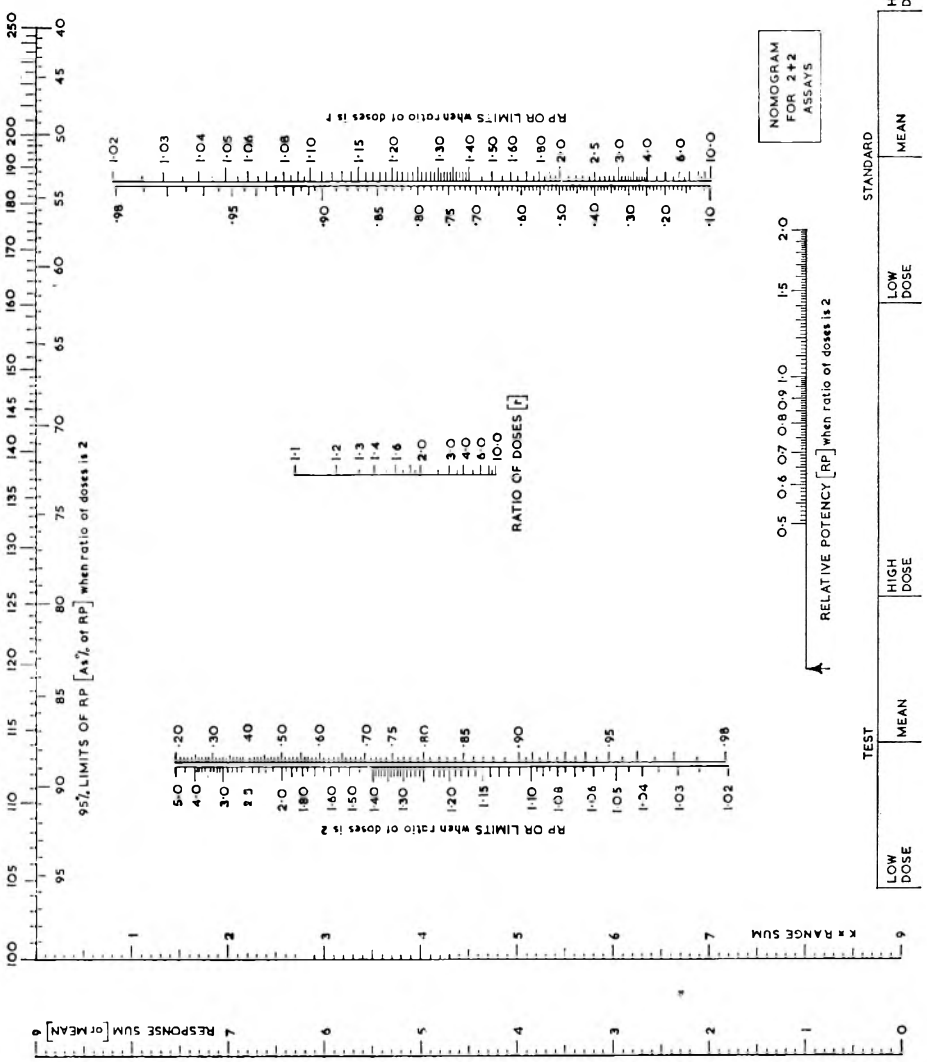

FIG. 1. Nomogram for 2 + 2 assays. Diagram is exactly half size.

Full-size copies of both the nomograms, Figs. 1 & 2, printed on acetate sheet, will be made available until December 1965 at the inclusive price of 12s. 6d. Cash with order to The Pharmaceutical Press, 17, Bloomsbury Square, London, W.C.1.

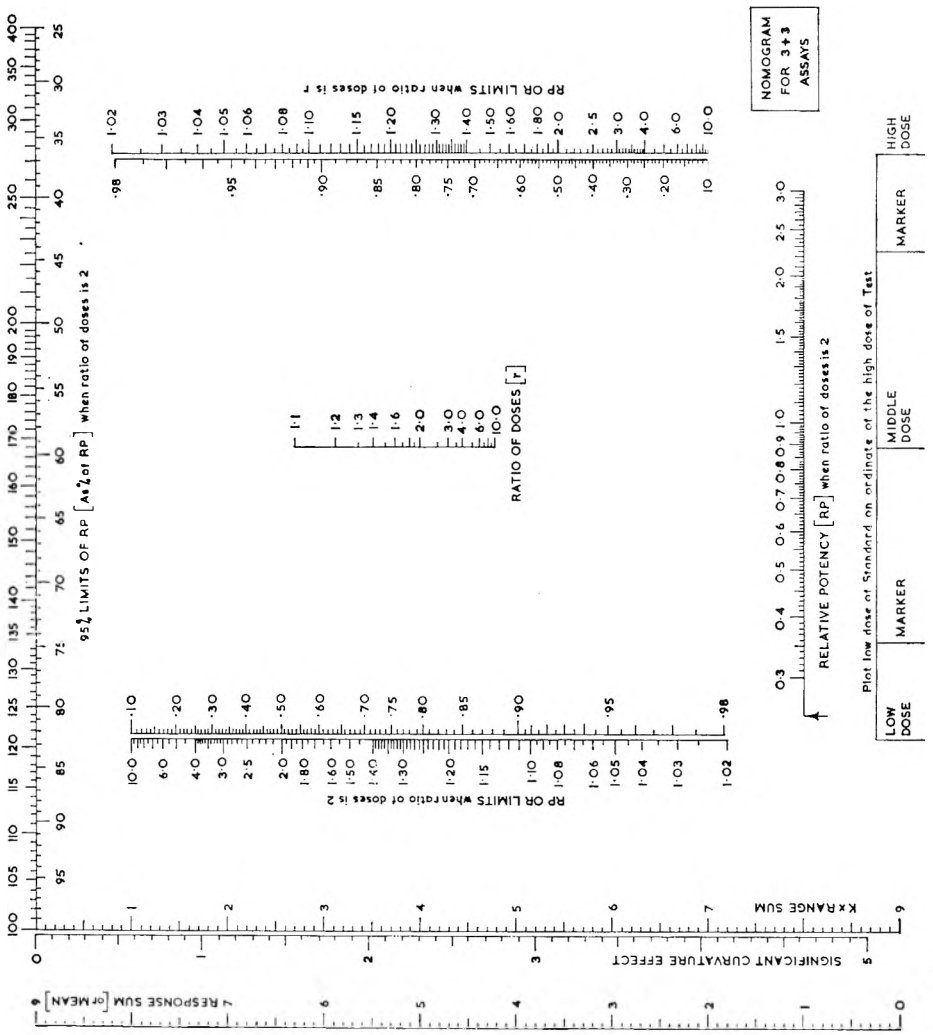

FIG. 2. Nomogram for 3 + 3 assays. Diagram is exactly half size.

The results given initially by the nomograms are those that would be obtained if the ratio of successive doses was 2. Each nomogram includes an alignment diagram to convert the results if the ratio is not 2.

The use of the nomograms is illustrated by 3 examples.

EXAMPLE 1: A 2 + 2 ASSAY WITHOUT BLOCK RESTRICTIONS

Table 1 shows the data and calculations and Fig. 3 the graphical construction for a 2 + 2 assay without block restrictions. For each dose the response sum and range (i.e. highest — lowest response) are determined. Paper graduated in inches and tenths (or twentieths) is used to plot the data. A suitable scale for plotting the response sums is chosen: in this example, 50 units per inch. If it is more convenient, the response means may be plotted.

TABLE 1. A 2 + 2 ASSAY OF PENICILLIN
(data from British Pharmacopoeia, 1958, p. 904)

	Test		Standard	
	0·6 ml (T_1)	1·2 ml (T_2)	0·6 ml (S_1)	1·2 ml (S_2)
Responses	156 155 163 158	190 194 190 190	172 171 157 177	202 199 200 200
Sum	632	764	677	801
Range	8	4	20	3

The response sums are plotted on a scale of 50 units per inch :
Relative potency, RP = 0·80
Range sum = 35, K = 2·08, K × range sum = 72·8
95% limits of RP = 90%, 111% of 0·80
= 0·72, 0·888
Parallelism effect, $L'_1 = 764 - 632 - (801 - 677) = 8$, which is not significant at the 5% level, since $2|L'_1| < K \times$ range sum.

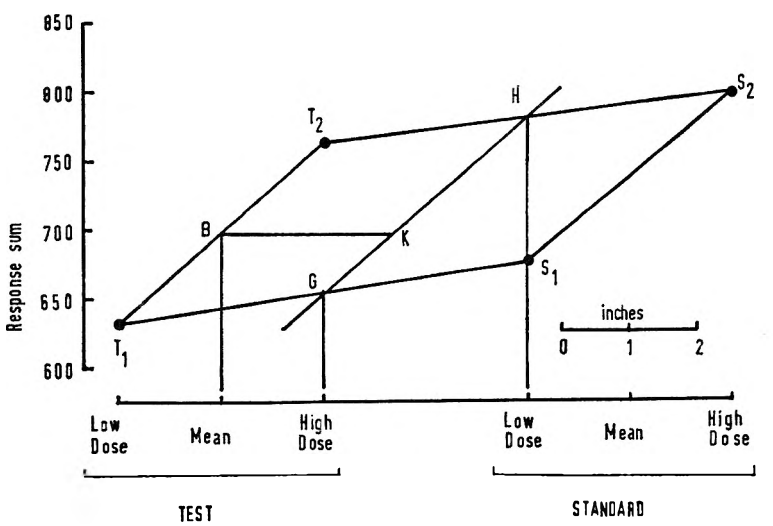

FIG 3. Graph for 2 + 2 assay (data from Table 1).

The dosage scale is shown at the bottom of the nomogram (Fig. 1); the low and high doses of the test preparation and the low and high doses of the standard preparation are placed successively at intervals of 3 inches. Denoting the plotted points by T_1, T_2, S_1, S_2 respectively, the quadrilateral $T_1T_2S_2S_1$ is drawn (Fig. 3). The line GH joining the points where T_1S_1 cuts the ordinate for the high dose of the test preparation and T_2S_2 cuts the ordinate for the low dose of the standard preparation has a slope equal to the mean slope of the log-dose response lines of the two preparations. The horizontal distance, BK, from the point where T_1T_2 cuts the "test mean" ordinate to the line GH is measured against the scale on the nomogram to determine the relative potency.

To find the 95% fiducial limits (abbreviated to 95% limits in the remainder of this paper) of the relative potency, the sum of the ranges is multiplied by the factor K found from Table 2. The nomogram is placed over the

TABLE 2. VALUES OF K IN 2 + 2 ASSAYS

Number of responses on each dose	Response sums plotted		Response means plotted	
	No block restrictions	With block restrictions	No block restrictions	With block restrictions
2	3·36	5·83	1·68	2·92
3	2·31	3·27	0·770	1·09
4	2·08	2·50	0·520	0·624
5	2·01	2·10	0·403	0·419
6	2·00	1·84	0·334	0·306
7	2·01	1·66	0·287	0·237
8	2·02	1·52	0·253	0·190
9	2·05	1·41	0·228	0·157
10	2·08	1·32	0·208	0·132

graph so that the line GH cuts the scale for "K × range sum" at the point corresponding to its calculated value measured on the same scale as the response sums (or means) have been plotted; it is essential that the "K × range sum" scale is vertical, i.e. parallel to the ordinates on the graph. In this example, the value of 72·8 at 50 units per inch is represented by a length of 1·456 inches on the scale. The point where the line GH cuts the scale for the 95% limits gives these limits as percentages of the relative potency.

Two validity tests are usually made in 2 + 2 assays: the slope should be significant, but the parallelism effect should not. If, when determining the 95% limits, the line GH cuts the scale for these limits, the slope is significant, at least at the 5% level. The parallelism effect, L_1', is found by substituting the response sums or means, whichever have been plotted, in the formula

$$L_1' = T_2 - T_1 - (S_2 - S_1)$$

In 2 + 2 assays, this effect is significant at the 5% level if

$$2|L_1'| > K \times \text{range sum}$$

In this example, the assay is valid and, since the ratio of high to low doses is 2, the relative potency and its limits do not have to be converted.

EXAMPLE 2: A 2 + 2 ASSAY WITH BLOCK RESTRICTIONS

Table 3 shows the data and calculations for a 2 + 2 assay in which the doses were given in random order, in blocks of 4; the standard analysis is a randomised block analysis. The ratio of the high to the low dose of each preparation is 1·5, and the relative potency and its limits have to be converted at the final stage of the graphical analysis.

TABLE 3. A 2 + 2 ASSAY OF VASOPRESSIN ON RAT BLOOD PRESSURE (from Walsh, J., personal communication)

Block	Responses to				Deviations from response mean				Range
	T_1	T_2	S_1	S_2	T_1	T_2	S_1	S_2	
1	31	40	29	38	− 8·75	− 11·25	− 8·25	− 9	3·00
2	40	56	40	46	0·25	4·75	2·75	− 1	5·75
3	46	55	40	53	6·25	3·75	2·75	6	3·50
4	42	54	40	51	2·25	2·75	2·75	4	1·75
Sum	159	205	149	188		0	0	0	14·00
Mean	39·75	51·25	37·25	47					

Ratio of doses, r = 1·5, K = 2·50, K × range sum = 2·50 × 14·00 = 35·0
Parallelism effect, L'_1 = 205 − 159 − (188 − 149) = 7, which is not significant at the 5% level, since $2|L'_1| < K \times$ range sum.
Assuming r = 2, relative potency, RP, = 1·25
and 95% limits of RP = 86%, 116% of 1·25
= 1·075, 1·45
Since r = 1·5, converted relative potency = 1·14
with 95% limits of 1·045, 1·24

Assuming the ratio of doses is 2, the relative potency is determined by the graphical procedure described for Example 1.

As shown in Table 3, the deviation of each observation from the mean of the responses to its particular dose is found; these deviations should add to zero, and this provides a check on their determination. The range of the deviations of the 4 observations in each block is determined. The sum of these ranges is used in the same way as the range sum in an assay without block restrictions to determine the 95% limits of the relative potency and to test the parallelism effect.

The three scales labelled "RP or limits when ratio of doses is 2", "RP or limits when ratio of doses is r", and "Ratio of doses" form the alignment diagram which is used to convert the relative potency and its 95% limits when the ratio of doses differs from 2. The procedure is the same for the relative potency and each of its limits. A straight-edge is placed to cut the appropriate scales at the value found assuming the ratio of doses is 2 and at the value of the actual ratio of doses. The straight-edge will then cut the third scale at the correct value with the actual ratio of doses.

Each "RP or limits" scale has separate calibrations for values greater and less than 1. The relative potency (and its limits) will remain greater or less than 1 when converted to any other value of the ratio of doses. For relative potencies or limits between 0·98 and 1·02, the following approximate formula can be used.

RP (or limit) when ratio of doses is r =

$$1 + \frac{\log_{10} r}{\log_{10} 2} \{[\text{RP (or limit) when ratio of doses is 2}] - 1\}$$

EXAMPLE 3. A 3 + 3 ASSAY WITH BLOCK RESTRICTIONS

Table 4 shows the data and calculations, and Fig. 4 the graphical construction for a 3 + 3 assay with block restrictions.

TABLE 4. A 3 + 3 ASSAY OF THE POTENCY OF KALLIDIN RELATIVE TO BRADYKININ ON GUINEA-PIG ILEUM

(from Shorley, P. G., personal communication)

	Responses to						Deviations from response mean						
	Kallidin			Bradykinin			Kallidin			Bradykinin			
Block	T_1	T_2	T_3	S_1	S_2	S_3	T_1	T_2	T_3	S_1	S_2	S_3	Range
1	16·0	36·5	53·5	27·5	46·5	54·5	4·3	−1·7	1·3	4·0	4·2	1·7	6·0
2	10·5	33·5	52·0	23·5	34·0	51·0	−1·2	−4·7	−0·2	0	−8·3	−1·8	8·3
3	8·5	44·5	51·0	19·5	46·5	53·0	−3·2	6·3	−1·2	−4·0	4·2	0·2	10·3
Sum	35·0	114·5	156·5	70·5	127·0	158·5	−0·1	−0·1	−0·1	0	0·1	0·1	24·6
Mean	11·7	38·2	52·2	23·5	42·3	52·8							

The response means are plotted on a scale of 10 units per inch
Ratio of doses, r = 1·4, K = 0·409, K × range sum = 0·409 × 24·6 = 10·06
Parallelism effect, L'_1, = 52·2 − 11·7 − (52·8 − 23·5) = 11·2, which is significant at the 5% level, since $|L'_1| > K \times$ range sum
Combined curvature effect, L_2, = 0·40 + 0·25 = 0·65, which is significant at the 5% level, as judged by the "significant curvature effect" scale.
Difference of curvature effects, L'_2, = 0·40 − 0·25 = 0·15, which is not significant at the 5% level, as judged by the "significant curvature effect" scale.
Assuming r = 2, relative potency, RP = 0·80
and 95% limits of RP = 85%, 118% of 0·80
= 0·68, 0·94
Since r = 1·4, converted relative potency = 0·90
with 95% limits of 0·83, 0·97

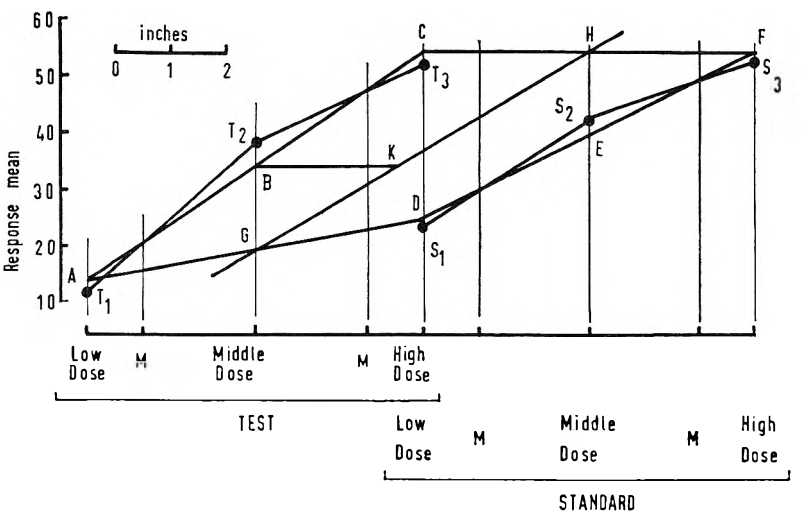

FIG. 4. Graph for a 3 + 3 assay (data from Table 4).

The dosage scale is shown at the bottom of the nomogram (Fig. 2); the low, middle and high doses of each preparation are plotted at intervals of 3 inches, with the high dose of the test preparation and the low dose of the standard preparation at the same point on the scale. Let the plotted

points, either response sums or response means, be T_1, T_2, T_3, S_1, S_2, S_3. For each preparation two marker ordinates, M, between each pair of doses and 1 inch from the low dose or high dose ordinate are used.

The regression line for the test preparation is constructed by drawing a line through the points where T_1T_2 and T_2T_3 cut the marker ordinates; suppose it cuts the ordinates of the three doses of the test preparation at A, B, C respectively. The regression line for the standard preparation is similarly constructed to cut the ordinates of the doses of the standard preparation at D, E, F respectively.

The line GH joining the points where AD and CF cut the ordinates for the middle doses of the test and standard preparations respectively has a slope equal to the mean slope of the two regression lines. The horizontal distance BK from B to the line GH is measured against the scale on the nomogram to determine the relative potency.

The range sum is determined as in 2 + 2 assays with block restrictions. In this example, the deviations for the doses do not add exactly to zero because the response means are corrected to one decimal place. The values of K for 3 + 3 assays are given in Table 5.

TABLE 5. VALUES OF K IN 3 + 3 ASSAYS

Number of responses on each dose	Response sums plotted		Response means plotted	
	No block restrictions	With block restrictions	No block restrictions	With block restrictions
2	1·00	1·96	0·501	0·981
3	0·73	1·23	0·245	0·409
4	0·67	0·96	0·169	0·241
5	0·66	0·82	0·132	0·163
6	0·65	0·72	0·109	0·120
7	0·66	0·65	0·094	0·093
8	0·66	0·60	0·083	0·075
9	0·68	0·59	0·075	0·062
10	0·68	0·53	0·068	0·053

Four validity tests may be made in 3 + 3 assays. If when determining the 95% limits, the line GH cuts the scale for the limits, the slope is significant, at least at the 5% level.

The parallelism effect, L_1', is found by substituting the response sums or means, whichever have been plotted, in the formula

$$L_1' = T_3 - T_1 - (S_3 - S_1)$$

In 3 + 3 assays, this effect is significant at the 5% level if

$$|L_1'| > K \times \text{range sum}$$

Note that this criterion for significance differs from that for 2 + 2 assays.

The combined curvature effect, L_2, and the difference of curvature effect, L_2', are found from the lengths of T_2B and S_2E. The length T_2B is given a positive or negative sign according to whether T_2 is above or below B; similarly, S_2E is positive or negative according to whether S_2 is above or below E. The effects are given by

$$L_2 = T_2B + S_2E$$
$$L_2' = T_2B - S_2E$$

Either of these effects is significant at the 5% level if the distance indicated by $|L_2|$ or $|L_2'|$ on the scale for the "significant curvature effect" is greater than the distance on the "K × range sum" scale used in determining the 95% limits. Thus, in this example, $|L_2 = 0{\cdot}65$, and the distance corresponding to 0·65 on the "significant curvature effect" scale is greater than 1·006 (= 10·06/10) on the "K × range sum" scale; hence L_2 is significant.

In this example, both L_1' and L_2 are significant and it would be conventional to regard the assay as invalid. However, to illustrate the method, the relative potency and its 95% limits have been determined and converted to allow for the ratio of successive doses being 1·4.

Discussion

The accuracy of the graphical method described may be assessed by comparing the results of the three worked examples with those obtained by standard computational methods using the root mean square estimate of the residual standard deviation and determining the exact fiducial limits using Fieller's theorem.

In all cases the tests of validity gave the same results by both methods. The relative potencies and their limits are compared in Table 6. It will be seen that the comparison is good in all cases.

TABLE 6. COMPARISON OF RELATIVE POTENCIES AND 95% LIMITS IN 3 EXAMPLES

	Standard method		Present graphical method	
	Relative potency (RP)	95% limits of RP	Relative potency (RP)	95% limits of RP
Example 1 ..	0·801	0·705–0·898	0·80	0·72 –0·888
Example 2 ..	1·137	1·040–1·245	1·14	1·045–1·24
Example 3 ..	0·898	0·824–0·975	0·90	0·83 –0·97

It should be emphasised that limits determined from the nomograms are "exact" only when the relative potency of corresponding doses of the two preparations is 1. Table 7 shows that as the relative potency deviates more from 1, the approximate limits differ more from the exact fiducial limits, and the fiducial probability of the approximate limits becomes less than 0·95.

In the author's experience, the approximation is accurate enough for most research purposes when the fiducial probability is 0·90 or more, i.e. (with ratios of doses of 2) when the relative potency lies between 0·6 and 1·67 in a 2 + 2 assay, or between 0·5 and 2·0 in a 3 + 3 assay. When accuracy is of critical importance it may be necessary to use the approximation only for relative potencies closer to 1, e.g., between 0·8 and 1·25. In a particular assay, the difference between the approximate and exact limits may be roughly estimated from Table 7 by interpolation; this would be a guide to whether the exact limits should be computed.

The nomograms described here have a general applicability to a wide range of assays. They may be used as the basis of simpler nomograms for

TABLE 7. EXACT PERCENTAGE FIDUCIAL LIMITS CORRESPONDING TO APPROXIMATE LIMITS DETERMINED FROM NOMOGRAMS, AND FIDUCIAL PROBABILITIES OF APPROXIMATE LIMITS

The relative potencies and limits given are for ratios of doses, r, equal to 2. The fiducial probabilities of the approximate limits are given in parenthesis; they were determined for a residual mean square with 24 degrees of freedom. The derivation of this table is described in the Appendix.

2 + 2 *Assays*

Approximate % limits from nomograms	Relative Potency								
	0·4	0·6	0·8	0·9	1·0	1·11	1·25	1·67	2·5
90–111	82–117 (0·73)	86–113 (0·89)	89–111 (0·94)	89–111 (0·95)	90–111 (0·95)	90–112 (0·95)	90–113 (0·94)	89–116 (0·89)	85–122 (0·78)
80–125	62–133 (0·79)	72–126 (0·90)	77–124 (0·94)	79–124 (0·95)	80–125 (0·95)	81–127 (0·95)	81–130 (0·94)	80–140 (0·90)	75–161 (0·79)
60–167	23–162 (0·84)	38–151 (0·91)	51–153 (0·94)	56–159 (0·95)	60–167 (0·95)	63–178 (0·95)	65–196 (0·94)	66–264 (0·91)	62–439 (0·84)
40–250	3–150 (0·83)	10–174 (0·92)	24–191 (0·94)	32–213 (0·95)	40–250 (0·95)	47–309 (0·95)	52–420 (0·94)	57–1030 (0·92)	67–3700 (0·88)

3 + 3 *Assays*

Approximate % limits from nomograms	Relative Potency								
	0·4	0·6	0·8	0·9	1·0	1·11	1·25	1·67	2·5
90–111	86–114 (0·83)	88–112 (0·93)	89–111 (0·95)	90–111 (0·95)	90–111 (0·95)	90–112 (0·95)	90–112 (0·95)	89–113 (0·93)	88–116 (0·88)
80–125	72–129 (0·83)	77–125 (0·93)	79–124 (0·95)	80–125 (0·95)	80–125 (0·95)	80–126 (0·95)	80–127 (0·95)	80–130 (0·93)	78–138 (0·88)
60–167	42–164 (0·83)	51–160 (0·93)	57–161 (0·95)	59–163 (0·95)	60–167 (0·95)	61–171 (0·95)	62–176 (0·95)	63–196 (0·93)	61–239 (0·89)
40–250	15–206 (0·93)	25–206 (0·93)	34–223 (0·95)	37–235 (0·95)	40–250 (0·95)	43–270 (0·95)	45–298 (0·95)	48–403 (0·93)	49–684 (0·90)

specialised use in routine assays or tests with a fixed number of observations and a fixed ratio of successive doses.

In this Department, a specialised nomogram has been prepared for the rat tail-pinch test for analgesics. The antagonism by nalorphine of the compound under test is measured by the dose ratio, which is mathematically equivalent to a relative potency. A test of significance of the antagonism is made by seeing whether the value of unity, corresponding to no antagonism, lies between the lower and upper limits of the dose ratio. Our experience with this nomogram has been favourable; it is reasonably accurate and has been welcomed by the experimenters.

Appendix: notes on the construction of the nomograms and on Table 7

Following Giet (1956), the term "modulus" is used to indicate the scale for plotting a variable: if a variable x has modulus μ, it is plotted at a distance μx along the scale. For illustration we shall consider the use of the nomogram for a 3 + 3 assay with n observations on each dose, a ratio of doses of 2 and no block restrictions in the assay design.

Graphical construction. The method of construction of the individual regression lines for 3 + 3 assays is a special case of the method of advancing centroids given by Askovitz (1957). The relative potency scales on the nomograms are logarithmic with lengths of $1\frac{1}{2}$, 3 and $4\frac{1}{2}$ inches corresponding to relative potencies of 0·5, 1·0 and 2·0 respectively. Suppose the response sums are plotted with modulus λ. The modulus of the logarithm to base 2 of the dose is the distance between successive doses on the dose scale; let this distance be d. Then the modulus of the slope of the regression line will be $n\lambda/d$.

Estimation of the residual standard deviation. The range sum is used to estimate the residual standard deviation following the methods described in the preface of Pearson & Hartley (1958). The mean range, $\bar{w}$, is distributed approximately as

$$\frac{c\chi\sigma}{\sqrt{\nu}}$$

where σ is the true residual standard deviation, χ is a chi-variate on ν degrees of freedom, and ν and the scale factor c are found from Table 30 of Pearson & Hartley (1958). In the illustrative 3 + 3 assay without block restrictions, σ is estimated by s, given by $s = \bar{w}/c = W/6c$, where W is the sum of the ranges.

Determination of fiducial limits. The geometry of the method of determining the approximate fiducial limits has previously been used, for example by De Beer (1945). In Fig. 5, GH is a line with slope b and OV

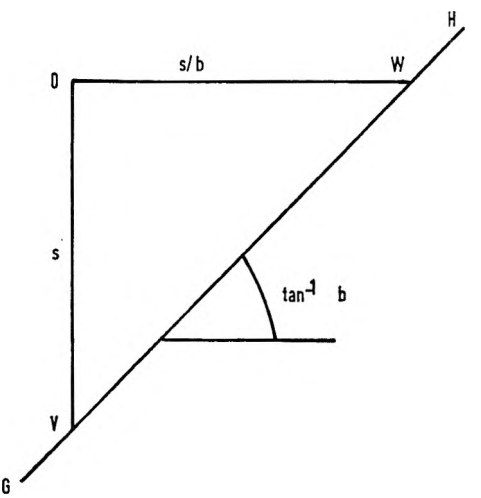

FIG. 5. Diagram to illustrate principle of determination of fiducial limits.

has the length s, so that OW has the length s/b. The fiducial limits (as a percentage of the relative potency) are approximated by a single-valued function of s/b, so that OW may be calibrated with these limits.

The logarithm of the relative potency is the ratio, m, given by

$$m = a/b$$

where, in a 2 + 2 assay,

$$a = \tfrac{1}{2}(T_1+T_2)-\tfrac{1}{2}(S_1+S_2)$$
$$b = \tfrac{1}{2}(T_2-T_1+S_2-S_1)$$

and, in a 3 + 3 assay,

$$a = \tfrac{1}{3}(T_1+T_2+T_3)-\tfrac{1}{3}(S_1+S_2+S_3)$$
$$b = \tfrac{1}{4}(T_3-T_1+S_3-S_1)$$

In either case, the exact fiducial limits, m_U and m_L, for m are

$$m_U, m_L = \left[m \pm \frac{ts}{b} \left\{ v_{11}(1-g) + m^2 v_{22} \right\}^{\frac{1}{2}} \right] \div (1-g)$$

where s^2 is the residual mean square estimate of σ^2 and has f degrees of freedom; v_{11} and v_{22} are constants such that the variances of a and b are $\sigma^2 v_{11}$ and $\sigma^2 v_{22}$ respectively; t is the t-deviate with f degrees of freedom corresponding to the fiducial probability being used; and $g = t^2 s^2 v_{22}/b^2$.

The logarithms of the exact limits, expressed as proportions of m, are p_U and p_L, given by

$$p_U, p_L = \frac{mg}{1-g} \pm \frac{ts}{b} \left\{ \frac{v_{11}}{1-g} + \frac{m^2 v_{22}}{(1-g)^2} \right\}^{\frac{1}{2}}$$

The approximate limits, p_U', p_L' used in the nomograms are obtained by putting m = 0, i.e.

$$p_U', p_L' = \pm \frac{ts}{b}\sqrt{\frac{v_{11}}{1-g}} = \pm t\sqrt{\frac{v_{11}}{(b/s)^2 - t^2 v_{22}}}$$

In a 3 + 3 assay where a and b are given in terms of the mean responses for each dose,

$$v_{11} = 2/3n, \quad v_{22} = 1/4n$$

Substituting these values in the formula for p_U', p_L' and writing $p'^2 = p_U'^2 = p_L'^2$, we find

$$\frac{s}{b} = \frac{\sqrt{4n}}{t}(1 + 8/3p'^2)^{-\frac{1}{2}}$$

If the percentage limits corresponding to p′ are plotted at a distance of

$$R(1+8/3p'^2)^{-\frac{1}{2}}$$

along the limits scale, the implied modulus of s/b is $Rt/\sqrt{4n}$. Then the modulus for s on a vertical scale should be $Rt/\sqrt{4n} \times n\lambda/d = Rt\sqrt{n}\lambda/2d$, i.e. the modulus for W should be $Rt\sqrt{n}\lambda/2d \times 1/6c = Rt\sqrt{n}\lambda/12cd$. The 3 + 3 nomogram is constructed with R = 12 inches, d = 3 inches; in Table 3, $K = t\sqrt{n}/3c$ when response sums from an assay with no block restrictions are plotted. Hence the modulus for K × W must be λ, i.e. K × W must be measured on the same scale as the response sum was plotted.

Tests of validity. The slope b is significant if $b/s\sqrt{v_{11}} > t$ i.e. if $g < 1$. As $g \to 1$, $p'^2 \to \infty$, so that if finite limits are found, the slope will be significant at the 5% level, and possibly at a much smaller level.

The parallelism effect L_1' determined from the response sums has variance $4n\sigma^2$. It is therefore significant at the 5% level if

$$|L_1'| > t.2s\sqrt{n} = 2t\sqrt{n}W/6c = K \times W.$$

The curvature effects may be found to have lengths given by

$$L_2 = \frac{\lambda}{3}(-T_1+2T_2-T_3-S_1+2S_2-S_3)$$

$$L_2' = \frac{\lambda}{3}(-T_1+2T_2-T_3+S_1-2S_2+S_3)$$

where the letters represent response sums or means, whichever have been plotted. For dose sums, the variance of L_2 or L_2' is $4\lambda^2n\sigma^2/3$. Hence L_2 is significant at the 5% level if $|L_2| > 2t\lambda\sqrt{ns}/\sqrt{3}$ i.e. if $|L_2| > \lambda KW/\sqrt{3}$. Hence, if the distance corresponding to $|L_2|$ on a scale with modulus $\sqrt{3}$ is greater than $K \times W$ plotted on the same scale as the response sums, then L_2 is significant at the 5% level. The same result holds for L_2'.

Alignment diagram. If R_2 and R_r are corresponding relative potencies assuming the ratios of successive doses are 2 and r respectively, then

$$\log_2 R_2 = \log_r R_r$$

$$\text{i.e. } \log_{10}\log_{10}R_2 - \log_{10}\log_{10}R_r - \log_{10}\log_{10}2 + \log_{10}\log_{10}r = 0$$

This equation may be expressed in determinantal form as

$$\begin{vmatrix} -1 & \log_{10}\log_{10}R_2 & 1 \\ 0 & \frac{1}{2}(\log_{10}\log_{10}2 - \log_{10}\log_{10}r) & 1 \\ 1 & \log_{10}\log_{10}R_r & 1 \end{vmatrix} = 0$$

and this is the basic determinant of the alignment diagram (see Allcock & Jones, 1950).

Derivation of Table 7. The exact limits of p_U, p may be expressed in terms of $(p')^2 = (p_U')^2 = (p_L')^2$ by noting that

$$(p')^2 = gv_{11}/(1-g)v_{22}$$

and p_U, $p_L = mg/(1-g) \pm [g(1-g)v_{11}/v_{22} + m^2g]^{\frac{1}{2}}/(1-g)$

Eliminating g from these formulae:

$$p_U,\ p_L = mv_{22}(p')^2/v_{11} \pm p'\sqrt{1 + m^2v_{22}/v_{11} + m^2v_{22}^2(p')^2/v_{11}^2}$$

This is the expression used to determine the exact fiducial limits in Table 7.

The fiducial probability corresponding to the approximate limits was determined by finding the values of g in the formula for the exact limits such that

$$p_U,\ p_L = \pm p'$$

After some algebraic manipulation, it is found that

$$g = \frac{(p')^2}{(m \pm p')^2 + v_{11}/v_{22}}$$

Then if we write $g^2 = t_E^2 s^2 v_{22}/b^2$ where t_E is the value of the t-deviate for the exact limit equal to one of the pair of approximate limits we find

$$\frac{t_E^2}{t^2} = \frac{v_{22}(p')^2 + v_{11}}{v_{22}(m \pm p')^2 + v_{11}}$$

where t is the t-deviate corresponding to the fiducial probability being used. With this formula, a value of t_E can be determined for each approximate limit, and hence the exact fiducial probability of the pair of approximate limits.

In Table 7, the exact limits are correct whatever the value of f. The exact fiducial probability varies however with f, the tabulated values being correct for $f = 24$. When $f < 24$, the exact fiducial probability differs less from the nominal value of 0·95; for $f > 24$, the difference is greater, but even when $f \to \infty$, the fiducial probability is never more than 0·01 less than the value in Table 7.

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References

Allcock, H. J. & Jones, J. R. (1950). *The Nomogram.* 4th ed. London: Pitman.
Askovitz, S. I. (1957). *J. Amer. Statist. Ass.*, **52**, 13–17.
De Beer, E. J. (1945). *J. Pharmacol.*, **85**, 1–13.
Finney, D. J. (1952). *Statistical Method in Biological Assay.* London: Griffin.
Giet, A. (1956). *Abacs or Nomograms.* London: Iliffe.
Gridgeman, N. T. (1951). *Biometrics*, **7**, 200–221.
Healy, M. J. R. (1949). *Ibid.*, **5**, 330–334.
Leech, F. B. & Grundy, P. M. (1953). *Brit. J. Pharmacol.*, **8**, 281–285.
Pearson, E. S. & Hartley, H. O. (1958). *Biometrika Tables for Statisticians*, Vol. **1**, 2nd ed. Cambridge: University Press.

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Absorption of ephedrine onto sulphonic acid cation-exchange resins

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Detailed studies of the kinetics of absorption of ephedrine onto the sulphonic acid cation-exchange resins have been made and most of the results have been interpreted by the particle diffusion theory of Boyd, Adamson & Myers (1947). The rate constant has been found to be inversely proportional to the square of particle radius and also dependent on the solution concentration, the temperature and the cross-linking of the resin. Absorption of ephedrine onto the 9% cross-linked (C.L.) resin proceeds to its capacity from ephedrine solution and not from ephedrine hydrochloride solution when experiments are made in closed tubes; with 13·5% C.L. resin, absorption does not proceed to capacity even after 2 months. The rate constant of absorption of ephedrine onto the sodium form of the resin is low as compared with the hydrogen form. In most cases diffusion coefficients have been calculated.

MANY attempts have been made to clarify the rate-controlling mechanism of exchange of inorganic ions but much less attention has been paid to the exchange of organic ions. Kressman & Kitchener (1949) working on a phenolsulphonic acid resin reported that exchange of large organic ions was controlled by particle diffusion. Saunders & Srivastava (1950, 1955) made a detailed study of absorption of several organic bases on to carboxylic acid resins and concluded that diffusion of these molecules into the resin particle was rate controlling. They found that rate of absorption onto an exchanger was dependent on the concentration of solute, the solvent, the temperature, the particle size and degree of cross-linking of the resin, and the dissociation constant of the organic base and its molecular size. Their experimental results however did not obey the particle diffusion equation of Boyd, Adamson & Myers (1947) and they arrived at their own conclusions by deriving appropriate equations. Kawabe, Sugimoto & Yanagita (1954) have studied the absorption of nicotine onto sulphonic acid and carboxylic acid exchangers and conclude that, in both, the rate is controlled by particle diffusion. We have already reported (1956) that the elution of ephedrine from sulphonic acid resins obeys the particle diffusion equation of Boyd & others (1947) when experiments are made by the infinite bath method.

In the present work the kinetics of absorption of ephedrine onto the sulphonic acid ion-exchange resins from aqueous solutions of ephedrine and ephedrine hydrochloride have been studied and conditions have been worked out for the absorption of ephedrine to the full capacity of the resin.

Experimental

Materials. Preliminary conditioning of the resins have been described elsewhere (Chaudhry & Saunders, 1956). A description of the resin

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fractions used is given in Table 1. Water absorptions are the quantities of water which are absorbed by the dry resins as determined by drying at 110°.

TABLE 1. DESCRIPTIONS OF RESIN FRACTIONS

Resin	*a*	*b*	*c*	*d*
Nominal divinyl-benzene content (%) i.e. degree of cross-linking (C.L.)	4·5	9*	9*	13·5
B.S.S. fraction of the air-dried hydrogen form	30–36	20–30	40–60	40–44
Moisture content (%) of the air-dried hydrogen form	28·0	26·4	26·2	24·7
Mean swollen particle diameter (mm)	0·70	0·71	0·45	0·45
Exchange capacity mg equiv/g dry resin (110°)	5·25	5·08	5·08	4·98
Water absorptions (mg)/mg equiv of dry resin (H)	342	169	—	124
Water absorption (mg) mg equiv of dry resin (ephedrine)	153	55	—	—
Linear expansion (%) of swollen particle of hydrogen form of resin on absorption of ephedrine	−1·4	+2·4	—	+3·4

* This is a usual degree of cross-linking in the commercial cation exchangers of this type.

ABSORPTION OF EPHEDRINE ONTO THE RESIN

Kinetic absorption studies were made with 50 ml portions of the aqueous solutions and 2·54 mg equiv of swollen particles of the resin. In all the experiments the amount of total ephedrine (ephedrine base and ephedrine hydrochloride) present in 50 ml of solution was more than the available capacity of the resin particles with which it was in contact.

The kinetic measurements were made by the closed tube method of Chaudhry & Saunders (1956). Four systems have been studied and the experimental details are described below.

A. *Absorption onto the hydrogen form of the resins from aqueous solutions of ephedrine.* In the first set of experiments, a comparison of rates of absorption (up to 6 hr) of ephedrine onto 2·54 mg equiv of the hydrogen form of resin *b* (Table 1) was made from two solutions. (i) 6 mg equiv of ephedrine in 50 ml water and (ii), 3 mg equiv of ephedrine base plus 3 mg equiv of ephedrine hydrochloride in 50 ml water. The results showed that the difference in uptake of the two was negligible.

The internal indicator method for initial stages of absorption. This method was used to study the initial stages of absorption. The hydrogen form of the resin was shaken with a solution of ephedrine hydrochloride and ephedrine, the amount of the latter being less than the available capacity of the resin. The solution, initially alkaline, became acidic when absorption of ephedrine onto the resin just exceeded the amount of ephedrine (free base) originally present in the solution. This change was shown by the indicator (methyl orange) present in the solution. The time elapsing between the addition of the solution to the resin and the colour transition of the indicator, was measured with a stop clock. Experiments for a number of stages of fractional absorption (F) at a particular solution concentration were made by varying the relative amounts of the free base and the hydrochloride, keeping the total ephedrine concentration constant.

F is defined by the equation:

$$F = Q_t/Q_e$$

Q_t is the amount in mg equiv of ephedrine absorbed onto the resin in time t, and Q_c is the total exchange capacity, in mg equiv, of the resin sample used in the measurement. The results have been interpreted by the particle diffusion theory of Boyd & others (1947). The values of rate constant, B, and the effective diffusion coefficient, D, have all been calculated in terms of F, as defined above.

According to this theory $F = 1 - \frac{6}{\pi^2} \sum_{n=1}^{n=\infty} \frac{\exp(-n^2Bt)}{n^2}$ and $B = \pi^2 D/r^2$

where r is the mean radius of the swollen resin particles. Numerical values of F for given values of B have been computed by Boyd, Adamson & Myers and are given in the reference quoted above. The table of functions has been extended by Reichenberg (1953).

The hydrogen form of the resin fraction (swollen particles) and the solution under study were put into tubes, one for each fractional stage of the absorption. The tubes were stoppered and rotated in the thermostat bath, and the time was noted when the colour of the indicator solution changed from yellow to orange-red. The results by this method were compared with the results obtained by the method for subsequent stages of absorption; good agreement was found.

Method for subsequent stages of absorption. The method consisted of rotating in closed tubes, swollen particles of the hydrogen form of the resin with 50 ml of a solution containing 3 mg equiv of ephedrine buffered with ephedrine hydrochloride to give an appropriate concentration as total ephedrine. After each time interval, a tube was removed from the thermostat bath and its contents analysed for ephedrine (base) by titrating aliquot portions with 0·05N hydrochloric acid using methyl orange as indicator.

B. *Absorption onto the hydrogen form of resin from aqueous solutions of ephedrine hydrochloride.*

Closed tube method. The technique was the same as for the subsequent stages of absorption, described above, except that 50 ml portions of solutions of ephedrine hydrochloride were used and the contents of the tubes were analysed for free hydrochloric acid by titration with carbonate free 0·05N sodium hydroxide solution using methyl red as indicator. The absorption does not proceed to capacity and an equilibrium is set up.

Column method. 0·12N ephedrine hydrochloride solution was slowly passed through a column (20 cm × 1 cm) containing 2·54 mg equiv of hydrogen form of resin *b*, for seven days until the pH of the overnight effluent was the same as that of the influent. The amount of *free* hydrochloric acid found in the combined effluents was taken as equivalent to the amount of ephedrine absorbed onto the resin. The absorption in this case proceeds to the capacity of the resin sample because hydrochloric acid formed is removed by the downwards flow of the solution in the column.

C. *Absorption onto the sodium form of the resin from aqueous solutions of ephedrine hydrochloride.* A series of 2·54 mg equiv portions of hydrogen form of resin *b* were converted to the sodium form by shaking with 50 ml portions of a mixed solution of 0·2N sodium hydroxide and 0·8N sodium chloride. Each resin portion was washed and transferred to a closed tube to which was added 50 ml of 0·12N solution of ephedrine hydrochloride. The tubes were rotated in thermostat bath and their contents analysed for ephedrine by a modification of the colorimetric method of Allport & Jones (1942).

D. *Absorption of ephedrine onto the sodium form of the resin from aqueous solution of ephedrine.* The method was identical with that used for the preceding system, except that instead of 0·12N ephedrine hydrochloride solution, 0·12N ephedrine (free base) solution was used.

The amount of ephedrine absorbed onto the resin after three days was found to be 0·18 mg equiv, that is the F value was 0·07, showing that only a small amount of ephedrine was taken up by the sodium form of the resin, probably by a physical solution rather than by an exchange process.

Results and discussion

Absorption of ephedrine onto the hydrogen form of resin from ephedrine solutions. The Bt–t plots (Chaudhry & Saunders, 1956) for the initial stages of absorption (internal indicator method) were linear passing through the origin and it was possible to calculate the rate constant, B, in sec^{-1} and also the effective diffusion coefficient, D.

TABLE 2. EFFECT OF SOLUTION CONCENTRATION WITH RESIN *b* AT 25°, FOR THE INITIAL STAGES OF ABSORPTION

Concentration of ephedrine solution (N)	Rate constant, B $sec^{-1} \times 10^5$	Effective diffusion coefficient D $cm^2/sec^{-1} \times 10^8$
0·06	8·56	1·09
0·09	9·09	1·16
0·12	9·72	1·24
0·20	11·20	1·43
0·30	12·70	1·62
0·40	13·90	1·77
0·50	14·75	1·88
0·60	15·52	1·98
0·70	16·20	2·06
0·80	16·65	2·12

Effect of change in solution concentration. The values of the rate constant B, and also effective diffusion coefficient D (Table 2), increase with increase in solution concentration. The graph of D against solution concentration (C in g equiv/litre) could be fitted by an equation:

$$10^8D = 0{\cdot}942 + 2{\cdot}47C - 1{\cdot}24C^2$$

The plots of F (combined for both initial and subsequent stages of absorption) against time (t) show that though the rate of absorption onto the resin *b* is higher with 0·66N solution than with 0·12N solution for

the initial stages of absorption (ratio of B for 0·66N/0·12N = 1·58) the two curves join after 6 hr and absorption to capacity proceeds in six days with both the solutions. It can therefore be concluded that (i) the rate of absorption of ephedrine is affected by the solution concentration at the initial stages of absorption but not at the subsequent stages of absorption, (ii) the rate of absorption depends on the amount of ephedrine already absorbed onto the resin and (iii) the rate controlling mechanism is the diffusion of ephedrine into the resin particles which is a slow process particularly in the later stages when ephedrine is entering the innermost channels.

TABLE 3. EFFECT OF RESIN PARTICLE ON ABSORPTION (INITIAL STAGES) OF EPHEDRINE AT 25°

Resin sample	Observed swollen diameter, mm	0·12N ephedrine concentration		0·66N ephedrine concentration	
		B $sec^{-1} \times 10^6$	D $cm^2 sec^{-1} \times 10^8$	B $sec^{-1} \times 10^6$	D $cm^2 sec^{-1} \times 10^8$
a	0·71	9·86	1·26	15·65	1·99
c	0·45	23·60	1·48	35·78	1·85

Effect of resin particle size (Table 3). The rate constant, B, increases with the decrease in the resin particle size but the effective diffusion coefficient, D, remains almost constant at a particular solution concentration.

The value of B should be inversely proportional to the square of the particle radius, therefore the square root of the ratio of the values of B for a given ephedrine solution concentration should equal the inverse ratio of the mean particle diameters. This inverse ratio of diameters of resin fractions *b* and *c* is 1·57; with 0·12N ephedrine solution concentration, the square root of the ratio of B values is 1·7; with 0·66N solution concentration it is 1·52. This represents a reasonable agreement with the theory (Boyd & others, 1947; Reichenberg, 1953; Chaudhry & Saunders, 1956) considering the uncertainty in the mean particle diameter estimates. The ratio of values of D is 0·94 for the 0·66N solution and 1·18 for the 0·12N solution; this ratio should be equal to one in an ideal case.

Effect of degree of cross-linking. Keeping the particle size constant, the rate constant is found to increase with decrease in percentage cross-linking (C.L.) (Table 4). The effective diffusion coefficients D $cm^2 sec^{-1}$ ($\times 10^9$) are 636, 13·7 and 3·4 for the 4·5, 9 and 13·5% C.L. resins respectively;

TABLE 4. EFFECT OF DEGREE OF CROSS-LINKING OF THE RESIN ON ABSORPTION (INITIAL STAGES) OF EPHEDRINE FROM 0·12N SOLUTION CONCENTRATION AT 25°

Resin	*a*	*b*	*c*	*d*
Cross-linking (%) D.V.B.	4·5	9	9	13·5
Swollen particle diameter (mm)	0·70	0·71	0·45	0·45
Water absorptions (mg/mg equiv of dry resin H)	342	169	—	124
B $sec^{-1} \times 10^6$	512	9·86	28·60	6·62
D $cm^2 sec^{-1} \times 10^9$	636	12·56	14·82	3·43

the value of 13·7 for the 9% C.L. resin has been taken as the mean of 12·56 and 14·82 found with resins *b* and *c*. The water absorptions per mg equiv of the dry resins (hydrogen form) have been found to be 342, 169 and 124 mg for the 4·5, 9 and 13·5% C.L. resins, respectively. The rate of absorption of ephedrine, the value of D, and the water absorptions of the dry resins are all found to decrease with increase in the percentage of cross-linking of the resins.

TABLE 5. EFFECT OF SOLUTION CONCENTRATION, PARTICLE SIZE AND CROSS-LINKING OF THE RESIN ON PERCENTAGE ABSORPTION OF EPHEDRINE, i.e. F × 100 AT 25°

Time	0·12N solution concentration				0·66N solution concentration resin *b*
	Resin *a*	Resin *b*	Resin *c*	Resin *d*	
0·5 hr	100·1	38·0	67·6	33·2	47·2
1 hr	—	50·0	68·3	40·8	58·6
2 ,,	—	62·4	78·3	48·6	67·9
3 ,,	—	70·2	82·9	—	73·7
4 ,,	—	74·5	85·7	54·8	77·6
5 ,,	—	78·8	88·0	—	80·3
6 ,,	—	81·8	89·3	57·7	81·8
24 ,,	—	93·5	96·8	65·8	93·4
48 ,,	—	96·5	99·1	—	96·6
72 ,,	—	98·4	99·7	—	98·4
96 ,,	—	99·2	100·2	—	99·3
120 ,,	—	99·5	100·1	—	99·7
144 ,,	—	99·8	—	80·6	99·9
2 weeks	—	—	—	83·3	—
1 month	—	—	—	86·8	—
2 months	—	—	—	87·2	—

It is seen from Table 5 that although absorption proceeds to capacity with 4·5% C.L. resin in less than 30 min, it takes nearly seven days to do so in the case of 9% C.L. resin (same particle size). This difference is accentuated with the 13·5% C.L. resin where after two months the absorption proceeded to 87·2% of the capacity (F = 0·872).

TABLE 6. EFFECT OF TEMPERATURE ON ABSORPTION (INITIAL STAGES) OF EPHEDRINE; RESIN *b*; SOLUTION CONC. 0·12N

Temperature	25°	30°	35°	40°
Initial rate constant	9·86	12·77	16·61	21·10
Effective diffusion coefficient, D cm^2 sec^{-1} × 10^6	1·26	1·63	2·11	2·69

Effect of temperature. Increasing the temperature (Table 6) results in an increase of the rate constant and the effective diffusion coefficient. The plot of $\log_{10}$ B sec^{-1} against 1000/T, was linear and a heat of activation calculated from the slope of this plot, was found to be 9 (±1) kcal $mole^{-1}$. This value is rather high for a diffusion process, which may be partly due to hindrances experienced by ephedrine in its movements through the pores of the resin. In addition, the fact that the entering ephedrine has to displace nearly its own volume of water from the resin against the influence of the water attracting sulphonic acid groups, will increase the activation energy for absorption.

Rate controlling mechanism. The fact that plots of Bt-time are linear and pass through the origin, as expected by the particle diffusion theory of Boyd & others (1947), establishes that the rate controlling mechanism is primarily diffusion of ephedrine in the resin particles. Also Kressman & Kitchener's test (1949) for particle diffusion was applied to these results by plotting F values against the square root of time (in min). Linear curves passing through the origin were obtained, as was to be expected if the particle mechanism was operative. Further support for particle mechanism is given by the study of different resin particle sizes.

Absorption of ephedrine onto the hydrogen form of the resin, from aqueous solutions of ephedrine hydrochloride. Absorption of ephedrine, when carried out by the closed tube method, does not proceed to the capacity of the resin, as after 24 hr an equilibrium is set up in which the sulphonic acid groups in the resin and the free hydrochloric acid liberated in the solution compete for the available ephedrine. The Bt-time plots are not linear due to the presence of free hydrochloric acid which tends to elute ephedrine from the resin. The plots of Bt calculated from the values of F′ ($F' = Q_t/Q_\infty$) against the experimental value of time were also non-linear.

Absorption of ephedrine onto the sodium form of the resin from aqueous solution of ephedrine hydrochloride. The absorption does not proceed to the capacity of the resin as after about a week, an equilibrium is set up in which the liberated sodium ions and the ephedrine ions present in the solution compete for the sulphonic acid groups of the resin. The Bt-time plots are linear passing through the origin, the values being as follows:

$$B\ sec^{-1} \times 10^5 = 1{\cdot}48;\ D\ cm^2/sec \times 10^9 = 1{\cdot}88$$

It is seen that the rate constant and the effective diffusion coefficient are very low (about one-seventh of that of absorption on to the hydrogen form of the resin—Table 3) which is probably due to relatively greater work required to be done by the diffusing ephedrine ions to drive out the sodium ions. There was hardly any change in the resin particle size to account for this slow rate.

References

Allport, N. L. & Jones, N. R. (1942). *Quart. J. Pharm.*, **15**, 238–250.

Boyd, G. E., Adamson, A. W. & Myers, L. S., Jr. (1947). *J. Amer. chem. Soc.*, **69**, 2836–2848.

Chaudhry, N. C. & Saunders, L. (1956). *J. Pharm. Pharmacol.*, **8**, 975–983.

Kawabe, H., Sugimoto, S. & Yanagita, M. (1954). *Repts Sci. Res. Inst. Tokyo*, **30**, 155–160, through *Chem. Abstr.*, 1955, **49**, 10003*b*.

Kressman, T. R. E. & Kitchener, J. A. (1949). *Disc. Faraday Soc.*, **7**, 90–104.

Reichenberg, D. (1953). *J. Amer. chem. Soc.*, **75**, 589–597.

Reichenberg, D. (1957). In *Ion Exchangers in Organic and Biochemistry*, Editors Calmon, C. & Kressman, T. R. E., p. 67. New York: Interscience.

Saunders, L. & Srivastava, R. S. (1950). *J. chem. Soc.*, 2915–2919.

Saunders, L. & Srivastava, R. S. (1955). *Ion Exchange and its Applications*, p. 170. London: Society of Chemical Industry.

J. Pharm. Pharmacol., 1964, **16**, 241–249 Received October 28, 1963

Method for recording respiratory changes induced in guinea-pigs by aerosols of histamine or of specific antigen, and for assessing drugs which antagonise bronchoconstriction

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A lightly-anaesthetised guinea-pig is made to breathe an aerosol of histamine or of specific antigen pumped into a breathing chamber. The guinea-pig's respiration produces pressure changes which are recorded on a smoked drum. Isoprenaline and the antihistamine thenyldiamine, administered as aerosols or by injection, have been used to inhibit the reduction in "tidal volume" due to the bronchoconstrictor agents.

MANY investigations of the inhibition of bronchoconstriction produced by aerosols of histamine (Kallós & Pagel, 1937; Halpern, 1942; Bovet & Walthert, 1944; Loew, Kaiser & Moore, 1945) or specific antigen (Kallós & Pagel, 1937; Herxheimer, 1949; Feinberg, Malkiel, Bernstein & Hargis, 1950; Friebel, 1953) have been based on the ability of a drug to prevent anoxic death in guinea-pigs. In 1952, however, Herxheimer developed a "microshock" method for evaluating the effect of drugs on "asthma" in guinea-pigs. He showed that when a sensitised guinea-pig was exposed at intervals to an aerosol of the specific antigen, the time taken to induce dyspnoea (the "preconvulsion time") remained fairly constant when sufficient time elapsed between each exposure. Lengthening of the "preconvulsion time" by a drug has been used as an index of its anti-asthmatic activity by Herxheimer (1952, 1953, 1955, 1956), Armitage, Herxheimer & Rosa (1952), Herxheimer & Rosa (1953), Herxheimer & Stresemann (1960) and others. Spirometric techniques have been widely used in the examination of the relief of bronchospasm in man, but do not appear to have been used in guinea-pigs.

A reduction of tidal volume and of the force of inspiration and expiration, hereafter referred to collectively as "tidal volume", occur during bronchoconstriction. A technique for measuring changes in the "tidal volume" of guinea-pigs exposed to aerosols of histamine or specific antigen is described below. The method is used to assess the ability of drugs to inhibit bronchoconstriction.

Experimental

APPARATUS

In principle, the apparatus consists of a breathing chamber which accommodates the head of a lightly-anaesthetised guinea-pig. Aerosol is pumped into the chamber through an inlet tube and leaves by an outlet tube to which is connected a tambour and a frontal writing lever (see Fig. 1).

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The breathing chamber consists of a glass cylinder 7 cm in diameter and 10 cm long sealed at one end. A rubber diaphragm 0·8 mm thick, with a central, slightly elliptical hole into which fits the head of a guinea-pig, is secured to the open end of the chamber. A cylinder of strong flexible polythene, into which are fixed a hand pump and a water manometer, is attached to the front of the chamber.

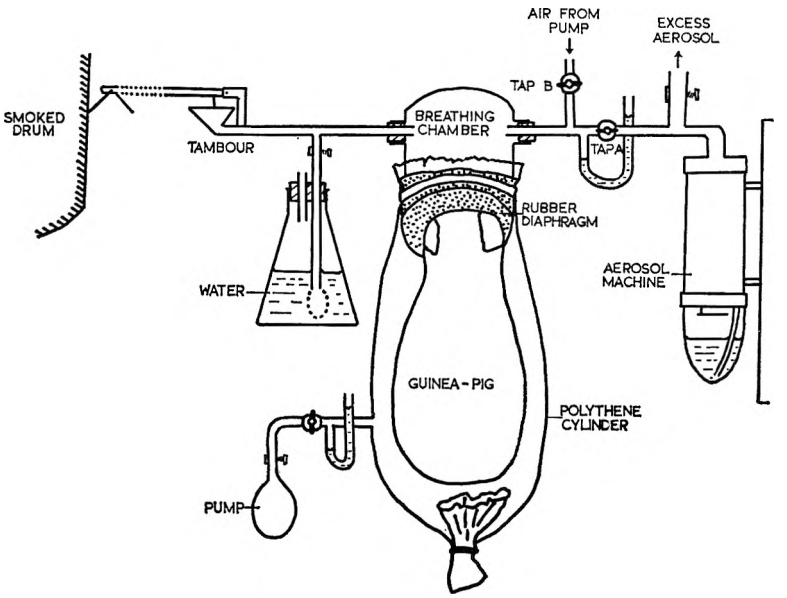

FIG. 1. Diagram of the apparatus used to measure changes in the 'tidal volume' of a guinea-pig exposed to broncho-constrictor aerosols.

The aerosol is produced by a model SB4 Aerolyzer (Aerosol Products Limited). The volume of liquid in the reservoir is adjusted to a fixed amount so that the weight of liquid vapourised per min is kept constant. The aerosol is led into a T-junction and forced through a glass tap (A) at a pressure of 5 cm of water above atmospheric pressure and enters the breathing chamber. The excess aerosol not forced through tap A passes through the other arm of the T-junction, and is removed from the laboratory by a wide-bore rubber tube controlled by an adjustable screw clip.

As shown in Fig. 1, a small tambour covered with a loose, thin rubber membrane is connected to the outlet from the breathing chamber. The respiration of the guinea-pig alters the pressure and causes changes in the distension of the tambour which are recorded on a smoked drum by means of a light frontal writing lever (magnification 40:1).

The aerosol leaving the breathing chamber passes through a rubber tube controlled by an adjustable screw clip and bubbles into water through a perforated rubber teat. The perforations in the teat ensure the formation of small bubbles which do not interfere with the movement of the tambour.

METHOD FOR RECORDING RESPIRATORY CHANGES

Air, instead of aerosol, can be blown into the breathing chamber through tap B at a pressure of 5 cm of water above atmospheric pressure, by means of an Es-Es pump.

DRUGS

Histamine acid phosphate, isopropylnoradrenaline sulphate and thenyldiamine hydrochloride were dissolved in 0·9% w/v aqueous NaCl before each experiment. Doses have been expressed as the weights of the salts.

METHOD

Guinea-pigs were sensitised by injecting 100 mg egg albumin intraperitoneally and 100 mg subcutaneously, at least 14 days before the experiments.

A guinea-pig was weighed and anaesthetised lightly with sodium pentobarbitone (0.5 ml/kg) injected intraperitoneally. Occasionally more anaesthetic was required. The aerosol delivery tube was closed (tap A) and air was pumped into the breathing chamber through tap B. Five to 6 min later, the head of the guinea-pig was pushed through the hole in the rubber diaphragm, and the polythene cylinder closed and inflated to give a pressure of 5 cm of water above atmospheric pressure. Ten min after the injection of pentobarbitone the kymograph and the aerosol machine containing 0·9% w/v aqueous NaCl were switched on. Tap B was closed, tap A opened, and a recording made of the respiratory pattern of the animal exposed to the saline aerosol. Five min later the guinea-pig was removed from the apparatus and allowed to recover from the anaesthetic. Any aerosol remaining in the breathing chamber was removed by air blown through tap B.

Subsequently, the normal guinea-pigs were anaesthetised lightly with pentobarbitone sodium and exposed to an aerosol of 0·5% histamine acid phosphate on alternate days. Sensitised guinea-pigs were anaesthetised and exposed to an aerosol of a centrifuged solution of 1% egg albumin every five days. These procedures were continued until in each animal the time taken to induce a marked reduction in "tidal volume" was approximately constant. When a severe, unrelieved bronchoconstriction occurred, or after 4–5 min when bronchoconstriction was slight or absent, the aerosol machine was switched off and the guinea-pig removed quickly from the apparatus. Guinea-pigs were artificially respirated if necessary and all exposed to aerosols of antigen were then made to breathe an aerosol of 1% isoprenaline sulphate. Animals not responding satisfactorily to the bronchoconstrictor aerosols were discarded.

When each animal showed a constant respiratory pattern, thenyldiamine hydrochloride or isoprenaline sulphate were either mixed in solution with the bronchoconstrictor substance or were injected intramuscularly into the conscious animals 30 min before exposure to aerosol. After each experiment in which a guinea-pig was protected by a drug, the animal was next time exposed to the bronchoconstrictor agent alone so that any change in

the sensitivity of the animal could be recognised and not erroneously attributed to a drug. In the few instances where the unprotected guinea-pig produced a control pattern significantly different from before, exposures to histamine or antigen alone were repeated until two consistent responses were obtained.

Results

Aerosols of histamine acid phosphate (0·5% w/v) and of specific antigen (1 % w/v egg albumin) each reduced the "tidal volume" of the guinea-pigs (see Fig. 2). Fairly constant reductions of "tidal volume" were produced in 15 of 21 guinea-pigs exposed to aerosols of histamine and in 11 out of

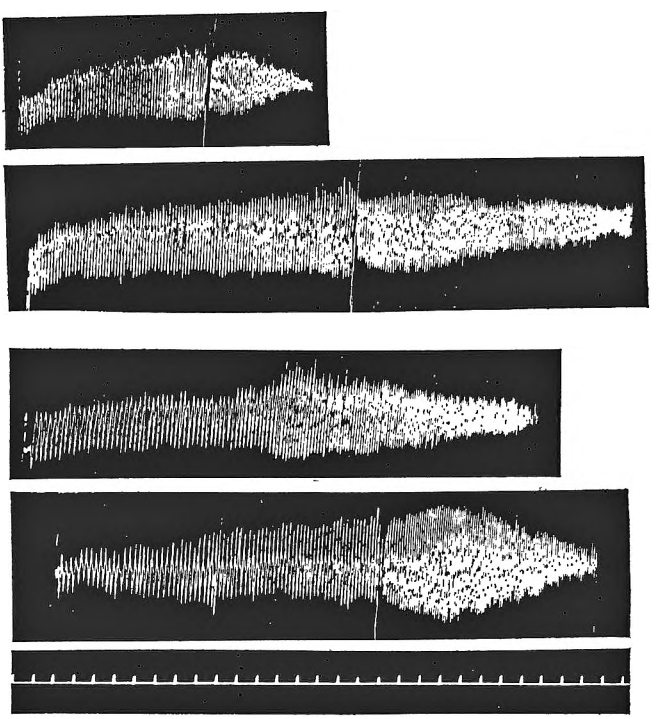

FIG. 2. Typical records of the respiratory patterns of guinea-pigs exposed to aerosols of histamine (top 2 traces) and of specific antigen (lower 2 traces). Time trace, 5 sec.

16 guinea-pigs exposed to aerosols of specific antigen. The remaining animals gave inconsistent responses and were discarded. When isoprenaline sulphate or thenyldiamine hydrochloride were present in the aerosols or had been injected intramuscularly into the guinea-pigs, the reduction in "tidal volume" was either postponed or did not occur at all (Table 1 and Fig. 3). In some animals the length of postponement varied on different occasions.

TABLE 1. PROTECTION AGAINST BRONCHOCONSTRICTION PRODUCED BY AEROSOLS OF HISTAMINE AND EGG ALBUMIN, USING ISOPRENALINE AND THENYLDIAMINE

Bronchoconstrictor aerosol	Protecting drug	Dose*	Degree of protection†
egg albumin 1%	Isoprenaline sulphate	25 μg/ml	o
		50 "	+
		100 "	+ + +
		200 "	+ + +
		0·1 μg i.m.	o
		5 "	+
		20 "	+ + +
	Thenyldiamine hydrochloride	1·0 mg/ml	o to + +
		2·0 "	+ to + +
		4·0 "	+ + +
Histamine Acid Phosphate 0·5%	Isoprenaline sulphate	25 μg/ml	o
		50 "	+
		100 "	+ + +
		200 "	+ + +
		0·1 μg i.m.	o
		0·2 "	o
		2·0 "	+
		20·0 "	+ + +
		50 "	+ + +
	Thenyldiamine hydrochloride	0·5 mg/ml	o to + +
		1·0 "	+ to + +
		1·5 "	+ +
		2·0 "	+ + +
		5 μg i.m.	+
		20 "	+ + +
	5 μg Thenyldiamine i.m. + 2 μg Isoprenaline i.m.		+ + +
	0·5 mg/ml Thenyldiamine + 50 μg/ml Isoprenaline		+ + +
	5 μg Thenyldiamine i.m. + 50 μg/ml Isoprenaline		+ + +

* Doses are expressed as μg or mg/ml in the bronchoconstrictor solution aerosolised, or as μg injected intramuscularly 30 min before exposure to aerosol.
† Protection ranges from nothing (o) to a maximum at + + +.

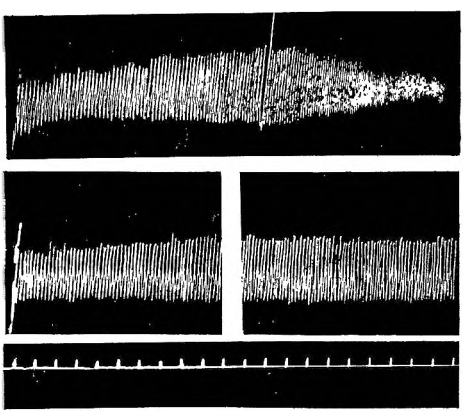

FIG. 3. The reduction in the "tidal volume" of a guinea-pig exposed to an aerosol of histamine (top trace), and the protection afforded to the same guinea-pig two days later by 2 mg/ml thenyldiamine in the histamine aerosol (lower trace). Time trace, 5 sec.

The reductions of "tidal volume" were plotted against time taking the measurement at 20 sec as 100% with the histamine aerosol, and the measurement at 40 sec as 100% with the antigen aerosol (Figs 4–7). In several experiments the rate and depth of respiration were also measured

but showed no relationship to the time taken to induce bronchoconstriction in the same animal on different occasions. Table I shows that 50 μg/ml of isoprenaline sulphate or 0·5–1 mg/ml of thenyldiamine hydrochloride in the aerosol retarded the onset of bronchoconstriction induced by histamine or antigen, while 100 μg/ml of isoprenaline sulphate or 2–4 mg/ml of thenyldiamine hydrochloride completely prevented it. The intramuscular injection of 2 μg isoprenaline sulphate or of 5 μg thenyldiamine hydrochloride retarded the onset of histamine-induced bronchoconstriction while 20 μg of either drug completely protected guinea-pigs from the effects of histamine; 5 μg and 20 μg isoprenaline sulphate injected into

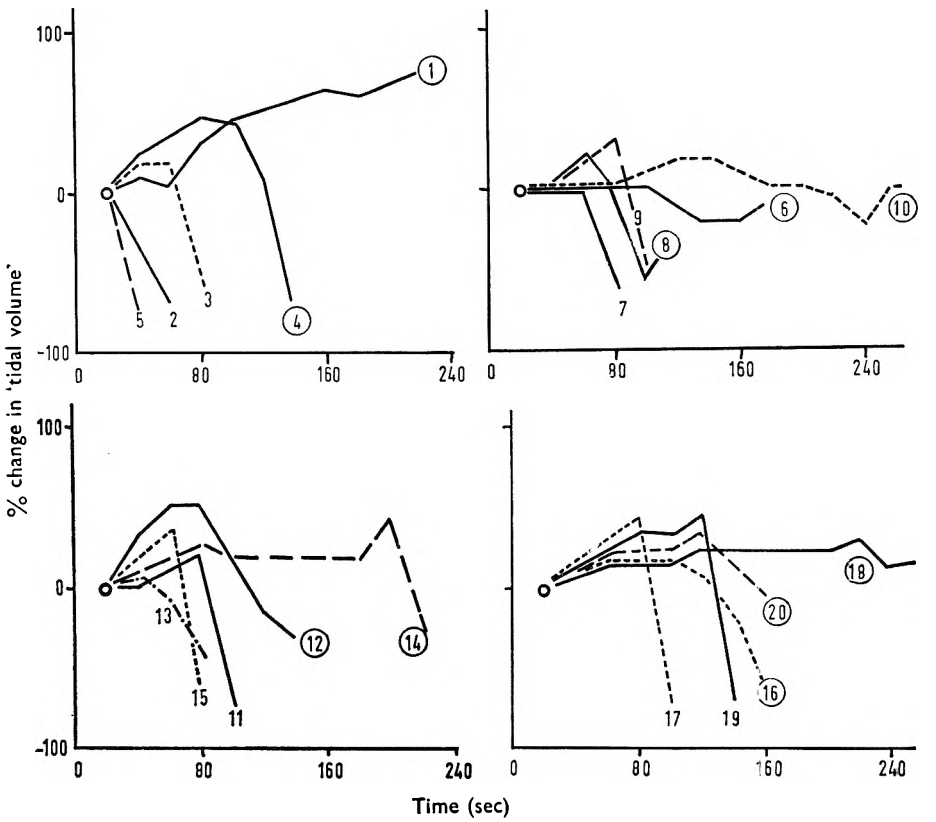

FIG. 4. 19 exposures of a normal guinea-pig to an aerosol of 0·5% histamine acid phosphate on every second day. The sequence of experiments is indicated by a number. When another drug is administered in addition to histamine, the number is ringed and the explanation is given below:

1. 0·9% NaCl aerosol only.
4. Thenyldiamine, 100 μg/ml.
6. Thenyldiamine, 2 mg/ml.
8. Thenyldiamine, 1 mg/ml.
10. Thenyldiamine, 2 mg/ml.
12. Thenyldiamine, 1 mg/ml.

Break of 12 days between 12 and 13.

14. Thenyldiamine, 5 μg i.m. + Isoprenaline 2 μg i.m.
16. Thenyldiamine, 5 μg i.m.
18. Thenyldiamine, 5 μg i.m. + Isoprenaline 2 μg i.m.
20. Isoprenaline 2 μg i.m.

sensitised guinea-pigs afforded similar protection against aerosols of egg albumin. These results show that thenyldiamine is almost as active as isoprenaline when injected intramuscularly, but is much less active when administered as an aerosol. Low doses of isoprenaline and thenyldiamine administered together markedly inhibit histamine-induced broncho-constriction.

Discussion

The method records changes of pressure produced in a breathing chamber by the respiration of a guinea-pig. The measurements obtained are a function of the tidal volume and of the force of inspiration and

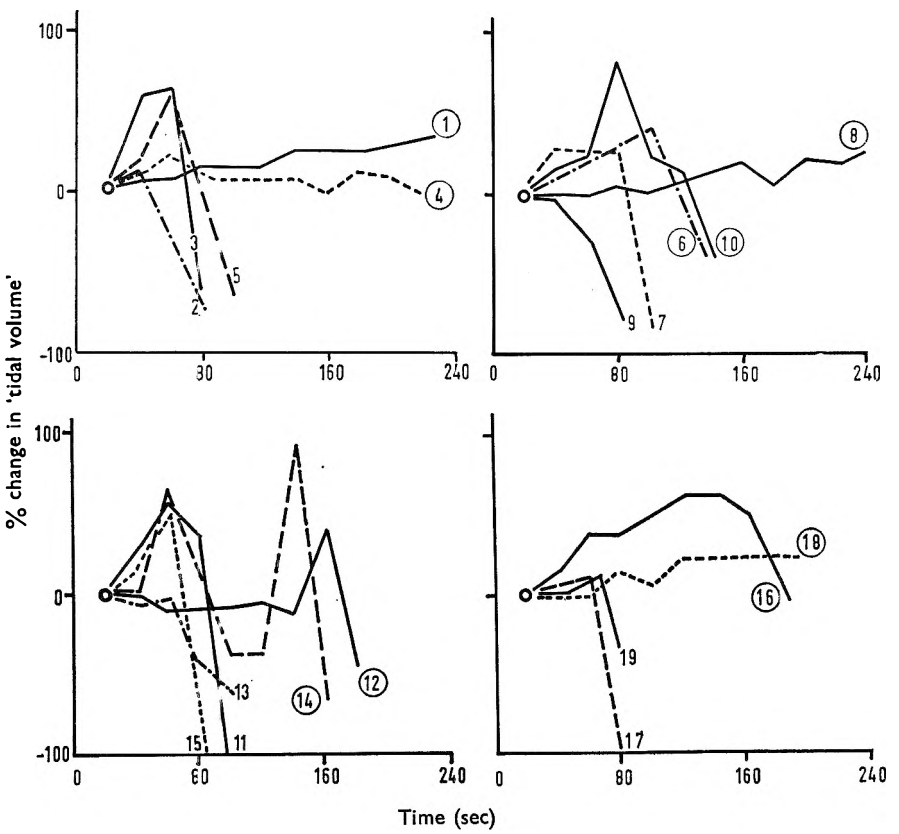

FIG. 5. Eighteen exposures of a normal guinea-pig to an aerosol of 0·5% histamine acid phosphate on every second day, as in Fig. 4.

1. 0·9% NaCl aerosol only.
4. Thenyldiamine, 20 μg i.m.
6. Thenyldiamine, 5 μg i.m.
8. Thenyldiamine, 20 μg i.m.
10. Thenyldiamine, 5 μg i.m.
12. Isoprenaline, 50 μg/ml.
14. Isoprenaline, 50 μg/ml + thenyldiamine, 5 μg i.m.
16. Isoprenaline, 50 μg/ml.
18. Isoprenaline, 50 μg/ml + thenyldiamine, 5 μg i.m.

expiration. The apparatus, which is easy to construct and use, can be employed to examine the effects of aerosols on the respiration of a guinea-pig and to assess the ability of drugs to inhibit bronchoconstriction induced by aerosols of histamine or specific antigen.

The advantages of this method for assessing anti-asthmatic drugs are that each animal can be used many times and each acts as its own control.

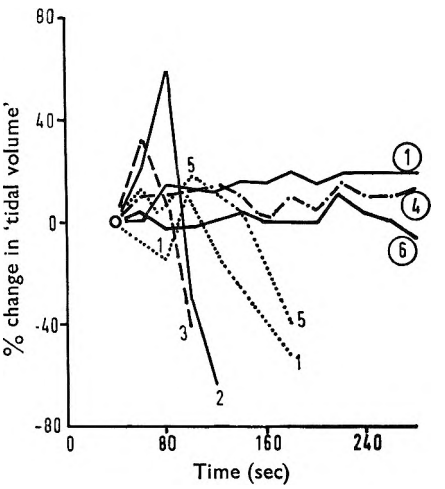

FIG. 6. Six exposures of a sensitised guinea-pig to an aerosol of a 1% solution of egg albumin every fifth day. The sequence of experiments is indicated by a number. When a drug is administered in addition to the egg albumin, the number is ringed and the explanation is given below: 1. 0·9% NaCl aerosol only. 4. Isoprenaline, 20 μg i.m. 6. Isoprenaline, 200 μg/ml.

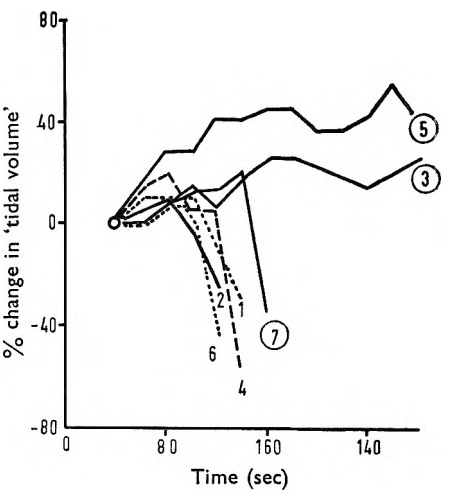

FIG. 7. Seven exposures of a sensitised guinea-pig to an aerosol of a 1% solution of egg albumin every fifth day, as in Fig. 6. 3. Thenyldiamine, 4 mg/ml. 5. Thenyldiamine, 2 mg/ml. 7. Thenyldiamine, 1 mg/ml.

Further, the onset of bronchoconstriction is clearly seen and easily measured. It is possible, however, that the light anaesthesia which is necessary may affect the response of the guinea-pig to the bronchoconstrictor aerosol. Koontz & Shackelford (1940) and Katz (1940) found that ether anaesthesia afforded some protection against anaphylactic shock in guinea-pigs, but Loew, Kaiser & Moore (1946) found that 10–20 mg/kg pentobarbitone sodium had little effect on the mortality of guinea-pigs exposed to aerosols of histamine. On the other hand, conscious animals may become conditioned by repeated experimentation whereas anaesthesia would prevent this.

The results obtained with this method are usually consistent and reproducible but large variations in the response of the animals sometimes occur. Therefore, in common with other methods of screening and assessing anti-asthmatic compounds, it enables only approximate estimates of drug potency to be made. In contrast to these other methods, however, changes in respiration are recorded and the onset of a marked bronchoconstriction can be measured directly.

References

Armitage, P., Herxheimer, H. & Rosa, L. (1952). *Brit. J. Pharmacol.*, **7**, 625–636.
Bovet, D. & Walthert, F. (1944). *Ann. pharm. franç.* **2**, Suppl. to No. 4, pp 3–43.
Feinberg, S. M., Malkiel, S., Bernstein, T. B. & Hargis, B. J. (1950). *J. Pharmacol.*, **99**, 195–201.
Friebel, H. (1953). *Arch. exp. Path. Pharmak.*, **217**, 35–42.
Halpern, B. N. (1942). *Arch. int. Pharmacodyn.*, **68**, 339–409.
Herxheimer, H. (1949). *Brit. med. J.*, **2**, 901.
Herxheimer, H. (1952). *J. Physiol.*, **117**, 251–255.
Herxheimer, H. (1953). *Ibid.*, **122**, 49P–50P.
Herxheimer, H. (1955). *Brit. J. Pharmacol.*, **10**, 160–162.
Herxheimer, H. (1956). *Arch. int. Pharmacodyn.*, **106**, 371–380.
Herxheimer, H. & Rosa, L. (1953). *Brit. J. Pharmacol.*, **8**, 177–180.
Herxheimer, H. & Stresemann, E. (1960). *Arch. int. Pharmacodyn.*, **125**, 265–271.
Kallós, P. & Pagel, N. (1937). *Acta. med. scand.*, **91**, 292–305.
Katz, G. (1940). *Amer. J. Physiol.*, **129**, 735–43.
Koontz, A. R. & Shackelford, F. T. (1940). *Curr. Res. Anesth.*, **19**, 196–201.
Loew, E. R., Kaiser, M. E. & Moore, V. (1945). *J. Pharmacol.*, **83**, 120–219.
Loew, E. R., Kaiser, M. E. & Moore, V. (1946). *Ibid.*, **86**, 1–6.

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The identification and determination of lysergic acid diethylamide in narcotic seizures

K. GENEST AND C. G. FARMILO

Methods are described by which lysergic acid diethylamide (LSD) can be identified and determined in the presence of heroin, other narcotics and controlled drugs. Thin layer chromatography and an Ehrlich's-reagent spray is applied to the LSD-containing material. For confirmation, thin layer chromatography after ultra-violet irradiation and acid hydrolysis should be carried out where ergot alkaloids are present. Spectrophotofluorometric analysis of LSD can be used in the presence of heroin, other narcotics and controlled drugs with a standard error of ±2%. If ergot alkaloids are present, thin layer separation, elution and subsequent fluorometric analysis are recommended.

LYSERGIC acid diethylamide (LSD) has pharmacological properties (Rothlin, 1957) which have made it the subject of legislation. In Canada, under the Food and Drugs Act (1963), the sale of LSD is prohibited except to authorised persons for experimental research purposes. Rumours recently reached the Food and Drug Directorate that LSD was being used clandestinely and it became necessary to have methods for the detection and determination of LSD in narcotic seizures. Since LSD is a potent drug (normal dose 50–100 μg) it was necessary to develop methods on a microscale. Methods available for the identification of LSD and related drugs include colour tests (Pharmacopeia of the United States of America, 1955), paper chromatography (Foster, Macdonald & Jones, 1949; Stoll & Rüegger, 1954; Macek, 1954; Pöhm, 1958; Rochelmeyer, Stahl & Patani, 1958; Reio, 1960; Alexander, 1960, 1962; Heacock & Mahon, 1961; Taber & others, 1963a,b) and thin-layer chromatography (TLC) (Stahl, 1959; Hofmann, 1961; Stahl & Kaldewey, 1961; Waldi, Schnackerz & Munter, 1961; Gröger & Erge, 1963; Taber & others, 1963a,b).

We describe the development of a rapid, sensitive micro-identification procedure, and the adaptation of a spectrophotofluorometric method (Axelrod, Brady, Witkop & Ewarts, 1956, 1957) for the assay of LSD in presence of narcotics, such as heroin and controlled drugs, i.e., barbiturates and amphetamines.

Experimental

THIN LAYER CHROMATOGRAPHY

This was on Silica Gel G-layers (about 250μ) produced with Desaga-equipment according to Stahl (1959) on 20× 20 cm glass plates.

System A: Chloroform: methanol (9:1) on plates prepared with Silica Gel G (30 g) and 0·1 N sodium hydroxide (60 ml). Time for a 10 cm-chromatogram is about 20 min. This solvent is applied to basic compounds and their salts before and after irradiation.

System B: Chloroform: methanol: ammonium hydroxide (28%) (40:40:20) on plates prepared with Silica Gel G (30 g) and water (60 ml)

From the Food & Drug Directorate, Ottawa, Ontario, Canada

(Fahmy, Niederwieser, Pataki & Brenner, 1961). Time for a 10 cm-chromatogram is about 40 min. This solvent is used to examine the acid hydrolysis products of seizure material containing LSD and ergot alkaloids.

System C: Morpholine : isopropylether : chloroform (10 : 80 : 10) on Silica Gel G Eosin Y (30 g + 30 mg + 30 ml water)-plates. The time for a 10 cm-chromatogram is about 35 min. This solvent is for barbiturates.

All Rf values reported were measured on sets of two plates chromatographed simultaneously in the same jar.

CHROMOGENIC REAGENTS

1. *p*-Dimethylaminobenzaldehyde (DMBA) (0·5 g) in hydrochloric acid (37%) (5 ml) and ethanol (95 ml) (Hellberg, 1957). LSD and ergot-alkaloids give blue-violet spots. The highest intensity is reached about 10 min after spraying. This reagent is applied to material chromatographed in system A.
2. Ninhydrin (0·2%) in n-butanol (95 ml) and 2 N acetic acid (5 ml) (Farmilo & Genest, 1961). After spraying, the plates were heated for 5 min at 110°. This reagent is applied to acid hydrolysis products chromatographed in system B.
3. Potassium iodoplatinate (Farmilo & Genest, 1961). This reagent is applied to spots containing heroin and controlled drugs.

HYDROLYSIS

25 μl of the test solution is mixed with an equal amount of hydrochloric acid (37%) and sealed into two melting-point capillaries. The capillaries are then placed in a boiling water-bath for 2 hr, after which they are broken, drawn out and their contents applied directly to the thin layer plate to be chromatographed in system B.

IRRADIATION

The test material is spotted in the thin layer plate, irradiated with ultraviolet light from an Aristogrid lamp (3,660 Å; distance 1·7 cm) for 2 hr and chromatographed in system A.

SPECTROPHOTOFLUOROMETRY

A Bowman-Aminco spectrophotofluorometer, 1p28 phototube; multiplier position 0·01 and 0·03; slit arrangement No. 3 is used. The seizure material (10 mg, containing 10–20 μg LSD) is dissolved in water (10 ml) and diluted with 0·004 N hydrochloric acid (1 : 10). The fluorescent maximum is measured at 435 mμ after activation at 335 mμ and compared with values obtained for the standard curve (0·01–0·4 μg LSD* ml). All quantitative experiments were in darkened rooms.

* LSD-25 Sandoz used throughout.

ELUTION OF SPOTS

Seizure material is dissolved in water and an aliquot to contain 1–10 μg LSD per spot is chromatographed in system A. The spots are detected by ultra-violet light, encircled and transferred by suction into a small (5 ml) light-protected wash bottle containing 2 ml methanol. The eluate is shaken (5 min) and diluted with 0·004 N hydrochloric acid so that it contains 0·01–0·4 μg LSD/ml. Spectrofluorometric analysis against a blank eluate is as described above.

Results

Separation of LSD from heroin, other narcotics, and controlled drugs can be achieved by chromatography in system A. LSD (0·05 μg) can be identified by the light blue fluorescence in ultra-violet light and by the DMBA-spray. Because of the close relation of drugs of the ergot group to LSD they were also included in this study (Table 1); a good distribution of the group over the Rf range is given by system A.

TABLE 1. Rf VALUES OF LYSERGIC ACID DERIVATIVES, NARCOTICS AND CONTROLLED DRUGS

Compound	Rf values	Compound	Rf values
LSD	0·60	Dihydroergocristine	0·57
Ergonovine maleate	0·18	Ergotoxine	0·45–0·69–0·81
Ergometrinine	0·43	Heroin	0·48
Ergotamine tartrate	0·52	Morphine	0·10
Ergotaminine	0·72	Codeine	0·30
Ergocristine	0·71	Thebaine	0·53
Ergocristinine	0·77	Narcotine	0·77
Lysergic acid	0·05	Papaverine	0·77
Dihydroergotamine	0·35	Amphetamine	0·16
		Methamphetamine	0·17

TABLE 2. Rf VALUES OF LYSERGIC ACID DERIVATIVES AFTER IRRADIATION WITH ULTRA-VIOLET LIGHT

Compound	Rf values	
	DMBA-spray	Ultra-violet fluorescence
LSD	0·61	0·80 GB*
	0·38	0·73 Y
		0·61 B
		0·47 YB
Ergotamine tartrate	0·54	0·54 B
	0·51	0·35 Y
	0·31	
Ergocristine	0·72	0·72 B
	0·45	0·43 Y
Dihydroergocristine	0·57	0·66 YG
		0·35 YG
Ergotaminine	0·72	0·72 Gy
	0·52	0·52 Or
		0·20 Gy
		0·17 Ol
Heroin	0·48	0·48 B
		0·13 B

* G, green; Y, yellow; B, blue; Gy, grey; Or, orange; Ol, olive.

Since the positive DMBA reaction is given by all and a light blue fluorescence by most lysergic acid derivatives of pharmaceutical importance, further methods were developed to distinguish LSD from the more important ergot alkaloids. Table 2 shows chromatography results of LSD and related lysergic acid derivatives after irradiation with ultra-violet light. Compounds which travel close to LSD before irradiation develop additional spots after treatment with ultra-violet light and the DMBA spray. LSD, ergocristine and ergotaminine give one, dihydrocristine none and ergotamine three DMBA-positive spots. The fluorescent spots do not always coincide with the DMBA spots.

Both methods for the analysis of LSD in presence of other lysergic acid derivatives are based on Rf measurements after DMBA-treatment. Use was made of the fact that after acid hydrolysis of ergot alkaloids the lysergic acid moiety is decomposed completely, whereas the peptide part at C(8) is hydrolysed to yield amino-acids (Stoll & Hofmann, 1950). If a strongly alkaline solvent is chosen for the chromatography of the hydrolysis products, the amino-acids, after a ninhydrin spray, give reddish spots in amongst the ergot alkaloids, whereas LSD gives no reaction (Table 3).

TABLE 3. Rf VALUES OF AMINO-ACIDS AND ACID HYDROLYSIS PRODUCTS OF LYSERGIC ACID DERIVATIVES

Compound	Rf values
LSD	No spot
Dihydroergotamine	0·49–0·78
Ergotamine	0·48–0·78
Ergotaminine	0·49–0·78
Dihydroergocristine	0·49–0·79
Ergocristine	0·49–0·77
Ergotoxine	0·49–0·60–0·68 (faint) –0·78
Proline	0·49
Valine	0·61
Leucine	0·69
Phenylalanine	0·79

TABLE 4. Rf VALUES OF BARBITURATES, HEROIN AND LSD

Compound	Rf value
Pentobarbitone	0·40
Amylobarbitone	0·44
Quinalbarbitone	0·49
Phenobarbitone	0·23
Butabarbital	0·44
Barbitone	0·39
Hexobarbitone	0·46
LSD	0·34
Heroin	0·35*

* Elongated

The presence of barbiturates is anticipated in LSD-containing seizure material. If preliminary colour and crystal tests indicate barbiturates, chromatography in system C is suggested. In this system (for Rf values see Table 4) on eosin-impregnated plates (Eberhardt, Freundt & Langbein, 1962) the barbiturates show as dark spots on a light yellowish fluorescent

background, whereas LSD gives the usual blue fluorescence. Quinine, occasionally found in heroin seizures (Fulton, 1953), also gives a light blue fluorescence with slightly different maxima and is easily separable from LSD in system A (Rf quinine, 0·31).

For quantitative analysis we prepared seizure-like material by adding known amounts of LSD to heroin seizures and in one instance to a cube of sugar. The material was dissolved in water and, after appropriate dilution, a determination of LSD was made by spectrophotofluorometry (Table 5). Other substances were tested for interference in the fluorometric procedure. As well as heroin, barbitone, phenobarbitone, pentobarbitone, morphine, codeine, amphetamine and methamphetamine in a 1000-fold excess were measured in presence of LSD and found not to interfere. Most ergot alkaloids show a similar fluorescence curve to LSD.

TABLE 5. RECOVERY OF LSD ADDED TO SEIZURE MATERIAL

Seizure No.	LSD added μg	LSD found μg
Heroin 6	20	19·9
Sugar cube	20	19·8
Heroin 6	10	10·1
Heroin 122	10	10·1
Heroin 481 B	10	10·1

In presence of lysergic acid derivatives, LSD had to be chromatographed in system A, eluted and measured as described on page 252. Methanol gave an average recovery of 91% from 1–10 μg spots of LSD and is superior to methylene chloride : methanol (9 : 1) reported by Gröger & Erge (1963). These recoveries are better than those obtained in trials with a partition method (Axelrod & others, 1956, 1957).

Discussion

The method described is useful forensically because it is simple and rapid. For the qualitative identification of LSD in the presence of narcotics, other controlled drugs and pharmaceutically important ergot alkaloids, thin layer chromatography is preferred to pharmacopoeial colour tests or paper chromatography because the general colour tests for ergot alkaloids require 1 mg of substance while paper chromatography often needs pretreatment of the paper, and is more time consuming. Of the thin layer systems previously reported, that of Hofmann (1961) was closest to the one needed. Of the spray reagents, the DMBA spray was more specific and sensitive than potassium iodoplatinate (Farmilo & Genest, 1961), ninhydrin (Berg, 1952), vanillin (Nigam, Saharabudhe & Levi, 1963) *p*-toluene sulphonic acid (Leemann & Weller, 1960), and concentrated sulphuric acid (Stoll & Schlientz, 1955).

To obtain more values for identification, chromatography in a series of solvents, as described for narcotics by Genest & Farmilo (1961), or 2-dimensional chromatography, as reported by Heacock (personal communication) for lysergic acid derivatives, could be used. Thin layer

chromatography after ultra-violet irradiation in the same solvent is preferable to either procedure, because additional basic compounds characteristic for each lysergic acid derivative are produced. Through the work of Stoll & Schlientz (1955) and Hellberg (1957) it is known that lumi-ergot alkaloids are produced if acid aqueous solutions of ergot alkaloids are exposed to ultra-violet light. Water is added to the C(9–10) double bond thus creating another asymmetric C-atom at C(10). The lumi-alkaloids should exist, therefore, in four series designated as lumi-lysergic and lumi-isolysergic I and II. Assuming that no epimerisation takes place at C(8)—there should be two spots if lumi I- and lumi II-compounds are formed under our experimental conditions. This assumption is strengthened by the observation that no additional DMBA spots were found with dihydro-ergocristine. Two additional spots were found in ergotamine, but only one additional spot in LSD, ergocristine and ergotaminine. Either, one of the possible lumi-compounds is not formed, or, if they are both formed, the second one cannot be separated from the original alkaloid by our system. Stoll & Schlientz (1955) report that in aqueous solution preferably lumi-LSD I and to a much smaller degree lumi-LSD II are produced. Hellberg's data indicate that the "β-lumi"*-compound produced by irradiation could not be separated from the original alkaloid in the case of ergotaminine and ergocristinine. From ultra-violet observation of the thin layer plates chromatographed after irradiation, it is evident that under these experimental conditions transformation products other than lumi compounds I and II are formed. Schlientz, Brunner, Hofmann, Berle & Stürmer (1961) have reported aci-ergot alkaloid formation which was obtained under other experimental conditions. The finding that lumi-alkaloids give a characteristic reaction with concentrated sulphuric acid (Stoll & Schlientz, 1955) could not be confirmed, when applied to thin layer plates, since LSD and the natural ergot alkaloids gave spots of the same colour before irradiation. The fact remains that after ultra-violet treatment, characteristic DMBA- and ultra-violet patterns are produced which are helpful for micro-identification purposes. Microhydrolysis was used to confirm the identity of LSD in presence of related lysergic acid derivatives. Foster & others (1949) described the acidic hydrolysis of ergot alkaloids on a mg scale, and subsequent analysis of the hydrolysate on paper chromatograms. Diethylamine, the hydrolysis product of LSD, does not show up in system B as do the amino-acids produced from most ergot alkaloids. In the instance of ergonovine, which is easily separable from LSD in system A, a spot with an Rf between valine and leucine was found. This is probably 2-amino-1-propanol having the same Rf in system B as 1-amino-2-propanol.

Ultra-violet spectrometric assay of LSD in presence of heroin is possible but is not sensitive enough (log $\epsilon = 3{\cdot}99$ at 313 mμ) and is subject to interferences. The van Urk-reaction (Vining & Taber, 1959; Taber & others, 1962, 1963), is not as sensitive as spectrophotofluorometry (Axelrod & others, 1956, 1957; Vining & Taber, 1959; Taber & others,

* Stoll and Hellberg use different nomenclature.

1963a,b). Udenfriend, Duggan, Vasta & Brodie (1957) report 0·002 μg/ml to be the "practical" sensitivity for LSD as defined by Duggan, Bowman, Brodie & Udenfriend (1957). The range from 0·01 to 0·4 μg/ml was preferred because less background fluctuation of the fluorometer was encountered than at more sensitive photomultiplier positions. A straight-line relationship between concentrations and fluorometer-readings was confirmed for this range (Sprince, Rowley & Jameson, 1957). The method for LSD analysis in heroin seizure material is simple because there is no interference by heroin itself or by excipients normally found in such material. Although fluorometric methods for morphine, codeine (Brandt, Erlich, Rogosinky & Cheronis, 1961; Brandt, Olsen & Cheronis, 1963) and barbiturates (Duggan & others, 1957) have been reported in different media and at different activation and fluorescent wavelengths, these compounds, and the amphetamines, do not interfere in the direct LSD analysis.

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References

Alexander, T. G. (1960). *J. Ass. Off. agric. Chem., Wash.*, **43**, 224–229.
Alexander, T. G. (1962). *J. pharm. Sci.*, **51**, 702–703.
Axelrod, J., Brady, R. O., Witkop, B. & Evarts, E. V. (1956). *Nature, Lond.*, **178**, 143–144.
Axelrod, J., Brady, R. O., Witkop, B. & Evarts, E. V. (1957). *Ann. N.Y. Acad. Sci.*, **66**, 435–444.
Berg, A. M. (1952). *Pharm. Weekbl.*, **87**, 69.
Brandt, R., Ehrlich-Rogosinsky, S. & Cheronis, N. D. (1961). *Microchem. J.*, **5**, 215–223.
Brandt, R., Olsen, M. J. & Cheronis, N. D. (1963). *Science*, **139**, 1063–1064.
Duggan, D. E., Bowman, R. L., Brodie, B. B. & Udenfriend, S. (1957). *Arch. Biochem. Biophys.*, **68**, 1–14.
Eberhardt, H., Freundt, K. J. & Langbein, J. W. (1962). *Arzneimitt.—Forsch.*, **12**, 1087–1089.
Fahmy, A. R., Niederwieser, A., Pataki, G. & Brenner, M. (1961). *Helv. chim. acta*, **44**, 2022–2026.
Farmilo, C. G. & Genest, K. (1961). *Toxicology, Mechanisms and Analytical Methods*, Editors, Stewart, and Stolman, p. 573, New York: Academic Press.
Farmilo, C. G. & Genest, K. (1961). *Ibid.*, p. 576.
Food and Drugs Act (1963). Schedule H, p. 11B, The Queen's Printer, Ottawa, Canada.
Foster, G. E., Macdonald, J. & Jones, T. S. G. (1949). *J. Pharm. Pharmacol.*, **1**, 802–812.
Fulton, C. C. (1953). *Bull. Narcotics*, UN, Dep. Social Affairs, **5** (2), 27–35.
Genest, K. & Farmilo, C. G. (1961). *J. Chromatog.*, **6**, 343–349.
Gröger, D. & Erge, D. (1963). *Pharmazie*, **18**, 346–349.
Heacock, R. A. & Mahon, M. E. (1961). *J. Chromatog.*, **6**, 91–92.
Hellberg, H. (1957). *Acta chem. scand.*, **11**, 219–229.
Hofmann, A. (1961). *Planta med.*, **9**, 354–366.
Leemann, H. G. & Weller, H. (1960). *Helv. chim. acta*, **43**, 1359–1364.
Macek, K. (1954). *Pharmazie*, **9**, 420–424.
Nigam, I. C., Sahasrabudhe, M. & Levi, L. (1963). *Can. J. Chem.*, **41**, 1535–1539.
Pharmacopeia of the United States of America (1955). 15th revision, p. 1094, Easton, Pennsylvania: Mack.
Pöhm, M. (1958). *Arch. Pharm., Berl.*, **291**, 468–480.
Reio, L. (1960). *J. Chromatog.*, **4**, 458–476.
Rochelmayer, H., Stahl, E. & Patani, A. (1958). *Arch. Pharm., Berl.*, **291**, 1–3.
Rothlin, E. (1957). *Ann. N.Y. Acad. Sci.*, **66**, 668–676.

Schlientz, W., Brunner, R., Hofmann, A., Berle, B. & Stürmer, E. (1961). *Pharm. Acta Helvet.*, **36**, 472–488.
Sprince, H., Rowley, G. R. & Jameson, D. (1957). *Science*, **125**, 442–443.
Stahl, E. (1959). *Arch. Pharm., Berl.*, **292**, 411–416.
Stahl, E. & Kaldeway, H. (1961). *Hoppe-Seyl. Z.*, **323**, 182–191.
Stoll, A. & Hofmann, A. (1950). *Helv. chim. acta*, **33**, 1705–1711.
Stoll, A. & Rüegger, A. (1954). *Ibid.*, **37**, 1725–1732.
Stoll, A. & Schlientz, W. (1955). *Ibid.*, **38**, 585–594.
Taber, W. A. & Heacock, R. A. (1962). *Can. J. Microbiol.*, **8**, 137–143.
Taber, W. A., Vining, L. C. & Heacock, R. A. (1963a). *Phytochemistry*, **2**, 65–70.
Taber, W. A., Heacock, R. A. & Mahon, M. E. (1936b). *Ibid.*, **2**, 99–101.
Udenfriend, S., Duggan, D. E., Vasta, B. M. & Brodie, B. B. (1957). *J. Pharmacol.*, **120**, 26–32.
Vining, L. C. & Taber, W. A. (1959). *Can. J. Microbiol.*, **5**, 441–451.
Waldi, D., Schnackers, K. & Munter, F. (1961). *J. Chromatog.*, **6**, 61–73.

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Sorption of water vapour and surface activity of ghatti gum

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The sorption of water vapour by three fractions of sodium ghattate has been studied at 25° and 40°. The results were fitted by Brunauer, Emmett & Teller plots at low relative vapour pressures. Calculations of the differential heats and entropies of sorption gave initially exothermic $\Delta\bar{H}$ values and negative $\Delta\bar{S}$ values. As the sorption proceeded the signs of the thermodynamic properties became positive The mechanism of sorption onto different parts of the macromolecules was discussed.

The surface tension of one sodium ghattate fraction was measured in water, 0·05 and 0·5 N sodium chloride solution, as a function of concentration, using the sessile drop method. The presence of salt enhanced the surface activity. A structure for the adsorbed film is suggested.

PREVIOUSLY (Elworthy & George, 1963) sodium ghattate was studied in water and in dilute sodium chloride solutions by light-scattering and viscosity techniques. The molecular weight was in the $2–3 \times 10^6$ region depending on which of the three fractions (Elworthy & George, 1963) used; a rod shaped molecule fitted the experimental results best. For fraction 2, the length of the rod was 3,680 Å in water, and 2,660 Å in 0·5N sodium chloride solution. The molecular contraction found on adding salt is characteristic polyelectrolyte behaviour.

Some idea of the interaction of water with the sodium ghattate molecules has now been gained by studying the sorption of water vapour on to the solid gum. It was also interesting to determine the effect of added salt on the surface activity at the air: water interface.

Experimental

MATERIALS

The three fractions of sodium ghattate have been described by Elworthy & George (1963). Analar sodium chloride was used, and water was twice distilled from permanganate.

SORPTION OF WATER VAPOUR

About 0·4 g of the three samples of sodium ghattate were contained in weighing bottles which fitted into wells cut in a thick brass plate. The plate rested on a glass support, 4 cm. high, inside a wide mouthed glass bottle. A layer of sulphuric acid solution, 1–2 cm deep was placed at the bottom of the bottle. The bottle was connected to a three way tap by means of ground glass joints; vacuum could be gently applied to the samples. The samples were placed in the apparatus after drying at 40° in a vacuum oven over P_2O_5, and weighing them accurately, Both the sulphuric acid and the samples were degassed at 0·01 mm Hg for 2 hr, during this process the bottle was cooled in a solid carbon dioxide/acetone mixture, although it was allowed to warm up occasionally. The apparatus

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was immersed in a thermostated bath at 25° or 40° (±0·01°). The samples were removed and weighed every few days until their weights were constant. The concentration of sulphuric acid was determined by titration with standard alkali after equilibrium had been reached. Relative vapour pressures were obtained from the acid concentration using Stokes & Robinson's (1959a) tables for 25°, and International Critical Tables for 40°.

Adsorption experiments were commenced with samples which had been dried and degassed, while for desorption experiments degassed samples were placed over water for 2–3 days before use.

SURFACE TENSION MEASUREMENTS

Preliminary experiments using a Wilhelmy plate apparatus (Elworthy & Macfarlane, 1962) gave irreproducible results, possibly due to adsorption of solute on the platinum plate. This method was discarded, and a sessile drop apparatus based on the designs of Shotton (1955) and Andreas, Hauser & Tucker (1938) was set up. The drop of solution was placed on a glass plate made hydrophobic with a coating of hard paraffin, and the plate placed in a glass box thermostated to 20±0·1°. The drop was illuminated with light from a sodium vapour lamp (Mazda 80/H), which passed through a stop controlling the area of illumination, and a condensing lens. An ebonite rod of known diameter was suspended above the drop to enable the magnification factor to be calculated. Several small tubes filled with water were present in the glass box to give a saturated atmosphere around the drop.

The drop could be photographed with a half plate camera to give a permanent record, using a Cambridge Universal Measuring Machine to measure the image. By removing the camera lens, the drop could also be viewed through the body of the camera by a cathetometer. Both drop height and equatorial diameter could be measured. Wheeler, Tartar, & Lingafelter's (1945) corrections were used in the calculation of surface tension.

The apparatus was tested by measuring the surface tension of water, giving 72·9 dynes cm^{-1} at 20° and 72·0 dynes cm^{-1} at 25°, compared with literature figures of 72·8 and 72·0 dynes cm^{-1} respectively (Harkins, 1959). The readings on water were constant over the time period necessary for equilibrium of solutions.

Results and Discussion

SORPTION OF WATER VAPOUR

The adsorption and desorption isotherms for sodium ghattate are shown in Fig. 1a, b, and c, as plots of 'a' (g water/100g material) against 'x' (relative vapour pressure). No hysteresis loops were observed for any of the samples at either temperature. Each sample sorbed more water vapour at low x values at 25° than at 40°, but at higher relative vapour pressures the situation was reversed.

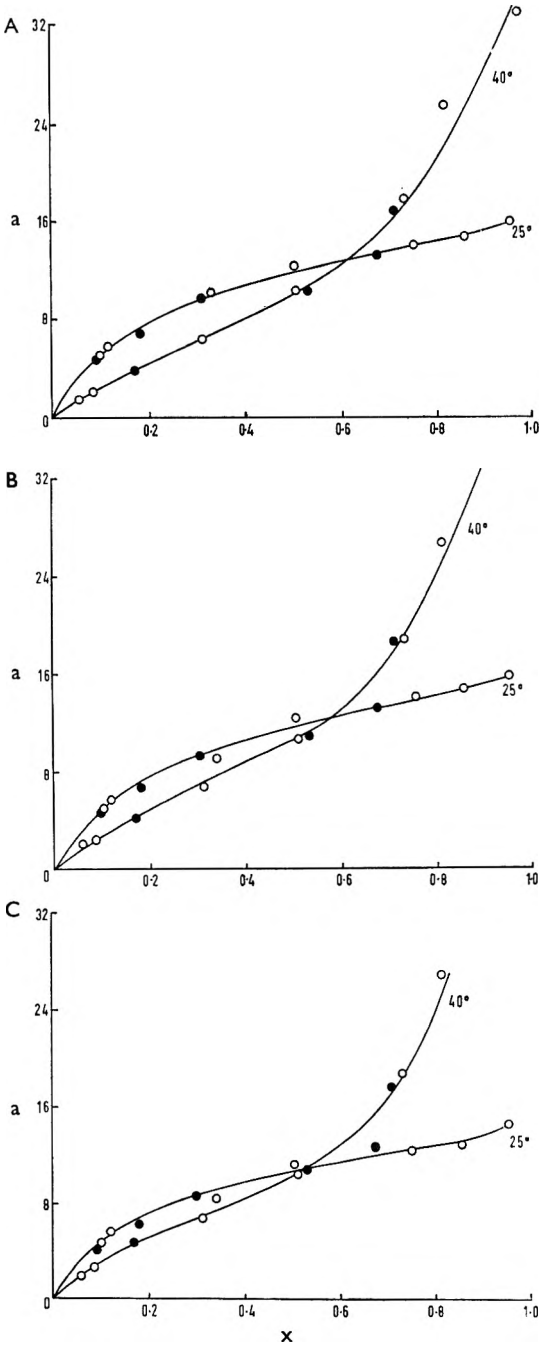

FIG. 1. Sorption isotherms for water vapour on sodium ghattate. A. Fraction 1. B. Fraction 2. C. Fraction 3. ○ = adsorption. ● = desorption. See p. 259 for symbols.

Good B.E.T. plots (Brunauer, Emmett & Teller, 1938) were obtained for all samples below x = 0·4. The values of the amount of water sorbed in the first layer, a_1, and the B.E.T. constant, C, were calculated from the slopes and intercepts of the plots. The values of a_2 and a_s, the amount of water sorbed in the second layer and at saturation respectively, were determined from graphs of a/x against x; a_2 being calculated from the minimum in the curves, and a_s from the intercept on the a/x axis at x = 1 (see Elworthy, 1961).

TABLE 1. SORPTION OF WATER VAPOUR ON SODIUM GHATTATE

Fraction	Temp. ° C	a_1	a_2	a_s	C	a_1 moles/polar group
1	25	7·69	15·35	19·70	13·0	7·13
1	40	6·58	8·99	36·75	4·75	
2	25	7·66	15·44	18·75	12·32	7·80
2	40	7·35	10·08	39·30	4·39	
3	25	7·50	13·77	18·00	11·80	7·73
3	40	7·95	9·00	45·00	5·09	

At 25° the values of a_2 for all fractions were approximately twice the a_1 values. In general there is little difference between the a_1 values for the three fractions. By analogy with the sorption of water vapour on proteins and phosphatides (Bull, 1944; Mellon, Horn & Hoover, 1949; Altman & Benson, 1960; Elworthy, 1961, 1962) it would be expected that the polar groups in the sodium ghattate molecule would be responsible for the initial sorption; there is also the possibility of interactions between additionally sorbed water and the hydroxy groups of the sugar residues present in the macromolecules. The thermodynamics of sorption gives some aid in distinguishing between these effects.

The differential heats and entropies of sorption were determined from the isotherms, assuming $\Delta\bar{H}$ to be linear over the 25–40° temperature interval, using (Barrer & Kelsey, 1961):

$$\Delta\bar{H} = \bar{H}_s - \tilde{H}_1 = R\left(\frac{d\ln.x}{d(1/T)}\right)_{p,\,N_1,\,N_2}$$

$$\Delta\bar{S} = \bar{S}_s - \tilde{S}_1 = (1/T)(\Delta\bar{H} - RT\ln.x)$$

where $\bar{H}_s$ and $\bar{S}_s$ are the partial molar enthalpies and entropies of sorbate, $\tilde{H}_1$ and $\tilde{S}_1$ are the molar enthalpies and entropies of water, and N_1 and N_2 are the number of moles of sorbate and sorbent respectively. The values of $\Delta\bar{H}$ and $\Delta\bar{S}$ are plotted against a in Figs 2 and 3.

All three fractions show roughly the same thermodynamic behaviour. Both $\Delta\bar{H}$ and $\Delta\bar{S}$ are negative at low values of a, but become increasingly positive as more sorption takes place. This implies that during the initial stages of sorption there is a distinct ordering of the water molecules on the sodium ghattate compared with their state in liquid water. This could well be a result of interactions with the polar groups and counter ions of the macromolecules. For $\Delta\bar{S}$ to become positive with further sorption a second process must be postulated, which may well be a mixing process between the sorbent and the hydroxy groups of the macromolecule. The

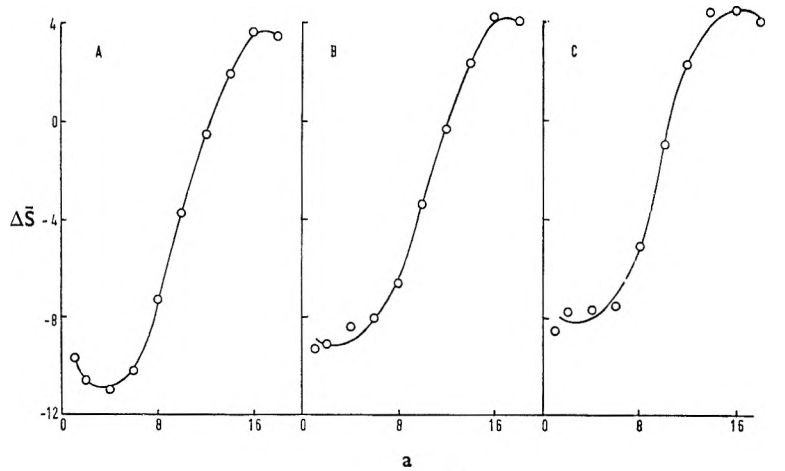

FIG. 2. Differential entropy of sorption, $\Delta\bar{S}$, against amount of water vapour sorbed, a. A. Fraction 1. B. Fraction 2. C. Fraction 3.

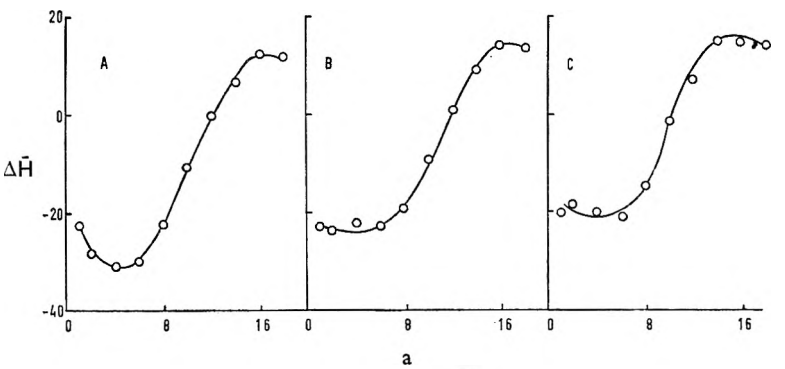

FIG. 3. Differential enthalpies of sorption, $\Delta\bar{H}$, against amount of water vapour sorbed, a. A. Fraction 1. B. Fraction 2. C. Fraction 3.

observed value of $\Delta\bar{S}$ will be a resultant of both processes, and presumably at large relative vapour pressures, the mixing process predominates.

The values of $\Delta\bar{H}$ and $\Delta\bar{S}$ at a_1 are given in Table 2.

TABLE 2. $\Delta\bar{H}$ (K.CAL. MOLE^{-1}) AND $\Delta\bar{S}$ (CAL. MOLE^{-1} DEG^{-1}) AT MONOLAYER COVERAGE

Fraction	$\Delta\bar{H}$	$\Delta\bar{S}$	Equiv wt
1	−8·7	−22·9	1,750
2	−6·8	−18·3	1,800
3	−5·4	−16·4	2,040

Fraction 1, containing the largest number of polar groups, gives the greatest negative values of $\Delta\bar{H}$ and $\Delta\bar{S}$, indicating that the polar groups might be responsible for the first stage of the sorption. The lowest

points in Figs 2 and 3 occur before monolayer coverage, so the mixing process begins before sorption on to the polar group is complete. Table 1 shows that 7–8 water molecules were sorbed to each -COONa group, (assuming in the calculation that all water molecules at a_1 are associated with the polar groups). This number is larger than that required to solvate one sodium plus one carboxyl ion, estimates varying from 3–5 molecules (Stokes & Robinson 1959b; Elworthy, 1963); again there is the indication that several processes are responsible for sorption.

The polar groups are present on the side-chains in the sodium ghattate molecule, and would thus be expected to readily sorb water vapour. The hydroxyl groups are distributed throughout the molecular structure; at high temperatures the molecule may be more expanded and permit greater interactions to take place between hydroxyl groups and sorbate, provided x is large enough. This may explain why the 40° isotherm crosses the 25° one as x is increased.

In previous work (Elworthy, 1961: Elworthy & Macfarlane, 1964), water vapour sorption isotherms have been used to give an idea of particle hydration, the method being to determine the value of a at $x = 1$ by extrapolation, i.e. a_8. The method is empirical but has been succesful for a number of systems. The results obtained at 25° for fractions 1 and 2 (Table 1) were used in the calculation of molecular volume (Elworthy & George, 1963).

SURFACE ACTIVITY

The surface tensions of fractions 2 in water, 0·05 and 0·5 N sodium chloride solution were studied. Considerable surface-ageing effects were noted, 24–36 hr being required for the equilibrium surface tension to develop (see Fig. 4). The ageing effects may be due to slow diffusion of solute into the surface region, and to the possibility that considerable orientation of the macromolecules is necessary at the surface. Equilibrium was obtained more rapidly when salt was present in the solutions. The results are given in Fig. 5, the surface tension decreasing with increasing sodium ghattate concentration until a roughly constant value was reached,

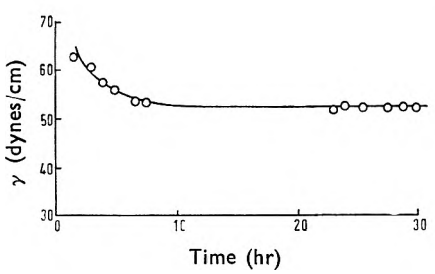

FIG. 4. Surface-ageing for 1·66 % sodium ghattate in water.

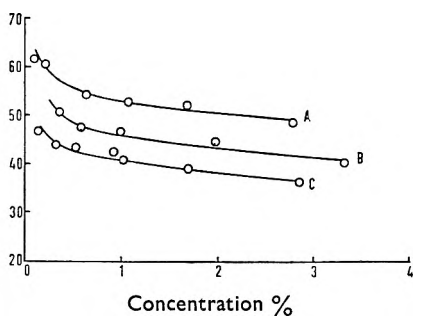

FIG. 5. Surface tension, γ, against concentration of sodium ghattate (fraction 2) in: A. Water. B. 0·05 N NaCl. C. 0·5 N NaCl.

at which the surface layer should be fairly closely packed with adsorbed molecules.

Jorgensen & Strauss (1961) studied the surface tension of solutions of coiled polyelectrolytes, and found very little surface activity. Sodium ghattate and sodium arabate are both surface-active. It may be significant that the two molecules have fairly stiff structures, with the polar groups on flexible side chains. Adsorption of a detergent at the air: water interface gives a film structure with the hydrocarbon chains remote from the water and the polar groups in contact with it. An explanation of the surface activity of sodium ghattate may lie in the molecule arranging itself in two regions at the interface, with the flexible side-chains and polar groups in the water, and the main backbone excluded from the surface. Elworthy & George (1963) have shown that the backbone was capable of some flexibility. This type of arrangement is difficult to visualise for the coiled type of polyelectrolyte, as the energy required to untangle the coil and orientate it at an interface would be considerable.

Like sodium arabate (Shotton & Wibberly, 1959), sodium ghattate has been found to give denatured films at the benzene/water interface; the films formed could be manipulated with a platinum wire. No evidence of a denatured film could be found at the air: water interface.

The presence of sodium chloride in the solutions reduces the surface tension at any particular sodium ghattate concentration e.g. for a 2·5% solution, γ fell from 50·6 dynes cm^{-1} in water to 37·2 dynes cm^{-1} in 0·5 N sodium chloride. The previous paper also showed that an increase in ionic strength causes the molecules to contract, and this effect may give rise to a more closely packed film at the interface.

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References

Altman, R. L. & Benson, S. W. (1960). *J. phys. Chem.*, **64**, 851–855.
Andreas, J. M., Hauser, E. A. & Tucker, W. B. (1938). *Ibid.*, **42**, 1001–1019.
Barrer, R. M. & Kelsey, K. E. (1961). *Trans. Faraday Soc.*, **57**, 452–462.
Brunauer, S., Emmett, P. H. & Teller, E. (1938). *J. Amer. chem. Soc.*, **60**, 309–319.
Bull, H. B. (1944). *Ibid.*, **66**, 1499–1507.
Elworthy, P. H. (1961). *J. chem. Soc.*, 5385–5389.
Elworthy, P. H. (1962). *Ibid.*, 4897–4900.
Elworthy, P. H. & Macfarlane, C. B. (1964). *Ibid.*, 311–315.
Elworthy, P. H. & Macfarlane, C. B. (1962a). *J. Pharm. Pharmacol.*, **14**, 100*T*–102*T*.
Elworthy, P. H. (1963). *J. chem. Soc.*, 388–392.
Elworthy, P. H. & George, T. M. (1963). *J. Pharm. Pharmacol.*, **15**, 781–793.
Harkins, W. D. (1959) in *Physical Methods of Organic Chemistry*, Editor, Weissberger, A., Vol. 1., Part 1, p. 772. New York: Interscience.
International Critical Tables, (1928). Vol. 3, p. 303. New York: McGraw-Hill.
Jorgensen, H. E. & Strauss, U. P. (1961). *J. phys. Chem.*, **65**, 1873–1877.
Mellon, E. F., Horn, A. H. & Hoover, S. R. (1949). *J. Amer. chem. Soc.*, **71**, 2761–2764.
Shotton, E. (1955). *J. Pharm. Pharmacol.*, **7**, 990–1003.
Shotton, E. & Wibberly, K. (1959). *Ibid.*, **11**, 120*T*–126*T*.
Stokes, R. H. & Robinson, R. A., (1959). *Electrolyte Solutions*, (*a*) p. 477; (*b*) p. 126. 2nd ed., London: Butterworths.
Wheeler, O. L., Tartar, H. V. & Lingafelter, E. C. (1945). *J. Amer. chem. Soc.*, **67** 2115–2119.

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Seven fatal cases involving imipramine in man

A. S. CURRY

The results of the distribution of imipramine in various organs are reported for four cases of acute overdosage and three cases in which the dose was probably therapeutic. Much lower concentrations of the drug have been found in the blood than in the liver brain and kidney.

ALTHOUGH a number of instances of imipramine poisoning have been recorded (Lancaster & Foster, 1959; Manners, 1960; Somunocuoglu, 1960; Bateman, 1961), reports of the distribution of the drug in the tissues are rare. Denton (1962) describes three cases in which the concentration of the drug in the liver was determined in two (1·9 and 1·25 mg/100 g) but he also showed that the method used for isolation was inefficient. Using a 40% v/v hydrochloric acid digestion for 5 min at 100° and an ultra-violet method of assay he was able to show a recovery of 60–66% of imipramine added to tissue.

There has also been a report of blood levels of 10–60 μg/100 ml in patients receiving 150–300 mg of the drug per day (Gillette, Dingell & Quinn 1960) and Yates, Todrick & Tait (1963), giving 150 mg of the imipramine metabolite, desipramine, to patients, found blood levels of 59–138 μg/100 ml, and also reported an inverse relationship of blood concentration to body weight.

The relation between tissue levels and ingested dose is of great importance to the forensic toxicologist, and the following four cases of fatal poisoning (1–4) and three cases (5–7) in which the dose was most probably at a high (therapeutic) level are reported in an effort to assist in solving such problems.

Cases 1 and 2 were found dead; case 3 had convulsions; case 4 died in coma; case 5 had also taken barbiturates and a phenothiazine in therapeutic dosage and died after about 8 hr in sleep or coma; cases 6 and 7 were found dead, both after being seen alive within 24 hr of death—the postmortem examination showed bronchopneumonia in both. Case 7 was prescribed 100 mg of imipramine per day; the dosage in case 6 was unknown.

Cases 6 and 7 also had monoamine oxidase inhibitors in their possession (Parstelin with phenelzine, and phenelzine respectively). Although deaths from the combination of monoamine oxidase inhibitors and imipramine have been described (Davies, 1960; Babiak, 1961; Luby, 1961) the manufacturer's literature warns against the taking of imipramine within ten days of the cessation of monoamine oxidase therapy.

Methods

The hydrochloric acid method of isolation has been used. In our hands this gives a recovery of approximately 76% of imipramine added to

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blood. After extraction into ether from alkaline solution, washing and re-extraction into 0·1 N sulphuric acid, the drug has been assayed either by measurement of its ultra-violet spectrum or colorimetrically by Forrests' reagent (Forrest & Forrest, 1960) or by both methods. Full details have been described elsewhere (Curry, 1963).

TABLE 1. DETAILS OF CASES

Case number	Age	Sex	Estimated weight lb	Time between ingestion and death
1	22	F	122	not more than 4 hr.
2	46	M	140	found dead after several days
3	4	M	35	3½ hr
4	28	F	133	not more than 4¾ hr
5	45	F	105	not more than 8 hr
6	53	F	119	not more than 6 hr
7	55	F	77	not more than 24 hr

TABLE 2. THE AMOUNT OF IMIPRAMINE IN MG OBTAINED BY ULTRA-VIOLET OR COLORIMETRIC ASSAY

Case number	Stomach contents	Small intestine contents	Blood	Liver	Kidney	Brain	Bile
1	22 (20·4)	17 (7·6)	— (0·28)	22·7 (21)	4·0 (3·8)	3·08 (3·0)	
2	— (18·2)	— —	— (0·57)	— (12)			
3	240 (200)		0·6 (0·56)	10·3 (8·6)	6·5 (5·6)	7·2 (7·4)	(2·16)
4	227 (213)		approx. 1·2 (0·7)	(25)			
5	— (2)		—	— (0·5)			
6	3·25 (1·5)		(0·033)	3·2 (2·06)			
7	4·0 —		— —	4·3, 2·4 (1·2)	1·15 (0·5)		

The figures in brackets obtained by colorimetric assay.

Results

Table 1 shows the relevant details of the deaths. Table 2 shows the results of assays on various organs as determined by the ultra-violet and the colorimetric methods of assay.

In case 7 it was found that the concentration in the liver varied with the sampling of the liver. The results are given for two samples. It was then discovered that the drug was firmly bound to the tissue, and the result depended on the proportion of tissue taken to the fluid that had drained from liver into the container.

Discussion

Neither of the methods of assay will distinguish imipramine from its metabolite desipramine. In the cases of fatal intoxication, correlation of

the colorimetric method and the ultra-violet assay is good, but in the two cases 6 and 7 in which the dose was thought to be therapeutic the ultra-violet method gave significantly higher values. This is probably because of the presence of other metabolites; demethylation and hydroxylation have been found to be involved in imipramine metabolism (Herrmann & Pulver, 1960). It is clear that a consideration of the concentration of the drug in the blood will enable a diagnosis of imipramine intoxication to be made. However, it is not always possible to obtain adequate samples from a decomposed body or from young children, and the interpretation of tissue levels may be necessary. The realisation that liver levels can exceed the blood concentration by a factor of 60 (case 6) will avoid erroneous diagnosis. It may also assist to perform both methods of assay. There is a prima facie reason, supported by this experimental study, that in acute poisoning most of the drug will be in a relatively unchanged condition with a minor proportion of metabolites.

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References

Babiak, W. (1961). *Canad. Med. Ass. J.*, **85**, 377.
Bateman, C. R. (1951). *Ibid.*, **85**, 759.
Curry, A. S. (1963). *Poison Detection in Human Organs*, Springfield, Ill., U.S.A., Charles C. Thomas.
Davies, G. (1960). *Brit. med. J.*, **2**, 1019.
Denton, S. (1962). *Analyst*, **87**, 234–236.
Forrest, I. & Forrest, F. (1960). *Amer. J. Psy.*, **116**, 1021–1022.
Gillette, J. R., Dingell, J. N. & Quinn, G. P. (1960). *Fed. Proc.*, **19**, 137.
Herrmann, B. & Pulver, R. (1960). *Arch. int. Pharmacodyn.*, **126**, 454–469.
Lancaster, N. P. & Foster, A. R. (1959). *Brit. med. J.*, **2**, 1458.
Luby, E. D. (1961). *J. Amer. med. Ass.*, **177**, 68.
Manners, T. (1960) *Lancet*, **2**, 932–933.
Somunocuoglu, S. (1960). *Med. Welt.*, **47**, 2509.
Yates, C. M., Todrick, A. & Tait, A. C. (1963). *J. Pharm. Pharmacol.*, **15**, 432–439.

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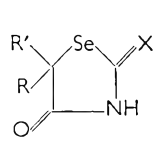
Some 2-Iminoselenazolidin-4-ones and related compounds

A. M. COMRIE, D. DINGWALL AND J. B. STENLAKE

A series of 2-iminoselenazolidin-4-ones, selenazolidine-2,4-diones and some 2-alkylidenehydrazones have been synthesised. Wide-range screening for biological activity failed to reveal any compounds of promise.

OXAZOLIDINONES, thiazolidinones and the closely related rhodanines have been extensively investigated for their biological properties, including antibacterial and antifungal activity (Clarke-Lewis, 1958; Brown, 1961). A recent review (Dingwall, 1962) has shown that comparatively little is known about the properties of their selenium isosteres.

Among the selenazolidin-4-ones, the 2-imino-derivative (I, X=NH; R=R′=H), its 5-methyl homologue (I, X=NH; R=Me; R′=H) (Hofmann, 1889; Frerichs, 1903) and a number of 2-dialkylamino-2-selenazolin-4-ones (II) have already been described (Zingaro, Bennett & Hammar, 1953) but not examined for biological activity. A series of 5-alkyl-2-iminoselenazolidin-4-ones (I, X=NH; R=alkyl; R′=H) and the corresponding 5-alkylselenazolidine-2,4-diones (I, X=O; R=alkyl; R′=H) were therefore prepared for examination. Since the ultra-violet



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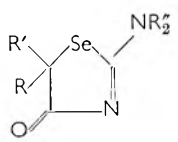

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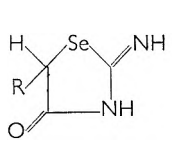
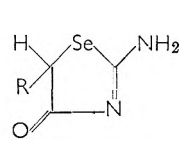


III



IV

and infra-red absorption spectra of the former, which are potentially capable of imino–amino tautomerism (III ⇌ IV), favours the imino-structure (Comrie, Dingwall & Stenlake, 1963), nomenclature consistent with this evidence is used throughout.

5-Alkyl(or aryl)-2-iminoselenazolidin-4-ones were prepared by condensing α-halogenocarboxylic acids with selenourea in ethanol (Comrie, Dingwall & Stenlake, 1963). Acid hydrolysis of the parent 2-imino-compound conveniently gave the 5-alkylselenazolidine-2,4-diones (I, X=O, R=alkyl; R′=H), which could be prepared alternatively by condensing the α-halogenocarboxylic acid with selenourea in water. Nucleophilic reactivity of the methylene carbon atom in selenazolidine-2,4-dione (I; X=O; R=R′=H) was shown by the ready formation of 5-arylidene derivatives (I; X=O; RR′=ArCH:) with aromatic aldehydes in the presence of base.

Selenazolidine-2,4-dione 2-alkylidene(or arylidene)hydrazones (I; X = N·N:CHR) were easily prepared by a base-catalysed condensation of α-halogenocarboxylic acids and the selenosemicarbazones of acetone or

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benzaldehyde. The relatively greater insolubility of 5-ethylselenazolidine-2,4-dione 2-benzylidenehydrazone (I, X = N·N:CHPh; R=Et; R′=H) compared with that of the corresponding 2-isopropylidenehydrazone (I, X=N·N:CMe_2; R=Et; R′=H) probably accounts for its formation from the latter and benzaldehyde.

Alkyl halides and selenourea gave the appropriate isoselenouronium halide. With alkali, *Se*-benzylselenouronium chloride was transformed in air into dibenzyl diselenide and it would appear, therefore, that hydrolysis to benzyl selenomercaptan takes place, since this substance is readily oxidised to the diselenide (Painter, 1941). Attempted cyclisation of *Se*-benzylselenouronium chloride as a step in a reaction sequence designed to obtain the unknown isomeric selenohydantoins failed, as did other methods which have been successfully applied to hydantoins (Jack, 1959) and thiohydantoins. Condensation of 2-iminoselenazolidin-4-one with sulphonyl chlorides in pyridine gave the corresponding 2-sulphonamido-2-selenazolin-4-ones (Roy & Guha, 1945).

Experimental

Selenium was determined spectrophotometrically (Dingwall & Williams 1961).

TABLE 1. 5-ALKYL-2-IMINOSELENAZOLIDIN-4-ONES (I; X=NH; R′=H)

Compound	R	M.p.°C	Yield %	Formula	Found %		Requires %	
					N	Se	N	Se
2-Imino-5-propyl-selenazolidin-4-one*	Pr^n	184–6	80	$C_6H_{10}N_2OSe$	13·9	38·3	13·7	38·5
2-Imino-5-isopropyl-selenazolidin-4-one	Pr^i	211–3	61	$C_6H_{10}N_2OSe$	13·7	38·4	13·7	38·5
5-Butyl-2-iminoselenazolidin-4-one hydrobromide	Bu	179–81 (decomp.)	43	$C_7H_{13}BrN_2OSe$†	9·5	26·0	9·3	26·6
2-Imino-5-phenyl-selenazolidin-4-one	Ph	200–4	60	$C_9H_8N_2OSe$	11·6	32·6	11·7	33·0

* Recrystallised from ether.
† Found: C, 28·2; H, 4·7; Br, 26·4; requires C, 28·0; H, 4·4; Br, 26·6%. *Base* m.p. 178–9° (decomp.) (from ethanol) Found: N 12·85; Se, 35·6; $C_7H_{12}N_2OSe$ requires N, 12·8; Se, 36·0%. *Picrate* m.p. 126–30° (from methanol) Found: N, 15·4; Se, 17·2; $C_{13}H_{16}N_6O_8Se$ requires N, 15·6; Se, 17·6%.

2-*Iminoselenazolidin*-4-*ones* (Table 1). Equimolecular quantities of α-halogenocarboxylic acids and selenourea were refluxed together in ethanol for 30 min to give the product as the hydrohalide (Comrie, Dingwall & Stenlake, 1963). The *bases* were liberated by neutralizing aqueous solutions of the salts and purified by recrystallising from ethanol unless otherwise stated.

Selenazolidine-2,4-*diones* (Table 2). (*a*) The α-halogenocarboxylic acid (0·01 mole) and selenourea (0·01 mole) were heated together in water (10 ml) for 2 hr on a boiling water-bath. Cooling the reaction mixture followed by ether extraction (5 × 10 ml) gave the *product*, usually as a pale-yellow oil, on removing the solvent. Purification was achieved by crystallisation from ethanol or aqueous ethanol, by distillation or by sublimation under a high vacuum.

TABLE 2. 5-SUBSTITUTED SELENAZOLIDINE-2,4-DIONES (I; X=O)

Compound	R	R′	M.p.°C	Yield %	Formula	Found %		Requires %	
						N	Se	N	Se
5-Methylselenazolidine-2,4-dione	Me	H	74–5	82	$C_4H_5NO_2Se$*	7·9	43·9	7·9	44·35
5-Ethylselenazolidine-2,4-dione	Et	H	69–71	75	$C_5H_7NO_2Se$†	7·4	41·6	7·3	41·1
5-Propylselenazolidine-2,4-dione	Pr^n	H	55–6	67	$C_6H_9NO_2Se$	6·7	38·1	6·8	38·3
5-Isopropylselenazolidine-2,4-dione	Pr^i	H	73–4	73	$C_6H_9NO_2Se$	6·9	37·9	6·8	38·3
5-Butylselenazolidine-2,4-dione	Bu	H	92–3	89	$C_7H_{11}NO_2Se$	6·3	35·3	6·4	35·9
5-Phenylselenazolidine-2,4-dione	Ph	H	160–2	60	$C_9H_7NO_2Se$	5·9	32·6	5·8	32·7
5,5-Dimethylselenazolidine-2,4-dione	Me	Me	82·5–83·5	68	$C_6H_7NO_2Se$	7·4	40·9	7·3	41·1

* Found: C, 26·6; H, 3·1; requires C, 27·0; H, 2·8%.
† Found: C, 31·6; H, 4·1; requires C, 31·3; H, 3·7%.

(*b*) The corresponding 2-iminoselenazolidin-4-one (*ca* 1 g) was refluxed in a mixture of hydrochloric acid (2 ml) and water (25 ml) for 2 hr. Isolation and purification of the *product* was carried out as in method (*a*).

5-*Benzylideneselenazolidine*-2,4-*dione*. Selenazolidine-2,4-dione (1·64 g), benzaldehyde (1·48 g), anhydrous sodium acetate (1·3 g) and acetic anhydride (10 drops) were refluxed together in glacial acetic acid (4 ml) at 160–70° for 3 hr. The *product* (1·93 g) which separated on cooling gave pale-yellow needles, m.p. 250–2° (decomp.) (from ethanol). Found: C, 47·5; H, 3·1; N, 5·6; Se, 30·75; $C_{10}H_7NO_2Se$ requires C, 47·6; H, 2·8; N, 5·6; Se, 31·3%.

5-*Salicylideneselenazolidine*-2,4-*dione* was similarly obtained as a yellow amorphous solid, m.p. 214–6° (from ethanol) in 61% yield from the dione and salicylaldehyde. Found: N, 5·3; Se, 29·15; $C_{10}H_7NO_3Se$ requires N, 5·3; Se, 29·45%.

Selenazolidine-2,4-*dione* 2-*isopropylidenehydrazone*. Acetone selenosemicarbazone (0·82 g) and chloroacetic acid (0·64 g) were refluxed together in 95% ethanol (15 ml) for 1 hr. The *product* (0·66 g) which separated on cooling was washed with ethanol and hot water and recrystallised from aqueous ethanol to give plates, m.p. 181–3°. Found: Se, 35·95; $C_6H_9N_3OSe$ requires Se, 36·2%.

5-*Ethylselenazolidine*-2,4-*dione* 2-*isopropylidenehydrazone*, m.p. 103–6° (from ethanol), was similarly obtained in 69% yield from acetone selenosemicarbazone and α-bromobutyric acid. Found: C, 38·3; H, 5·6; N, 16·8; Se, 31·8; $C_8H_{13}N_3OSe$ requires C, 39·0; H, 5·3; N, 17·1; Se, 32·1%.

5-*Phenylselenazolidine*-2,4-*dione* 2-*isopropylidenehydrazone*. Acetone selenosemicarbazone and α-chloro-α-phenylacetic acid were condensed as above. Water was added to the reaction mixture, which was then neutralised to pH 8 with concentrated ammonia solution and extracted with ether. Removal of the solvent gave the *product* (39%), m.p. 208° (from ethanol). Found: C, 48·5; H, 4·8; N, 13·9; Se, 26·8; $C_{12}H_{13}N_2OSe$ requires C, 49·0; H, 4·45; N, 14·3; Se, 26·8%.

Selenazolidine-2,4-dione 2-benzylidenehydrazone. Benzaldehyde selenosemicarbazone (0·26 g), chloroacetic acid (0·11 g) and anhydrous sodium acetate (0·2 g) were refluxed together in 95% ethanol (10 ml) for 10 min. The *product* (0·23 g, m.p. 254–6° decomp.) (from glacial acetic acid) separated on cooling. Found: Se, 30·1; $C_{10}H_9N_3OSe$ requires Se, 29·7%.

5-*Ethylselenazolidine*-2,4-*dione 2-benzylidenehydrazone* (64%, m.p. 200–2°, from ethanol) was similarly obtained from benzaldehyde selenosemicarbazone and α-bromobutyric acid. (Found: Se, 26·9; $C_{12}H_{13}N_3OSe$ requires Se, 26·8%). The same product was formed from 5-ethylselenazolidine-2,4-dione 2-isopropylidenehydrazone and benzaldehyde in glacial acetic acid,

Se-*Benzylselenouronium chloride*, Selenourea (0·88 g) and benzyl chloride (1·0 g) were refluxed together for 30 min in dry ethanol (10 ml), access of moisture being prevented by a calcium chloride guard tube. The reaction mixture was filtered, concentrated and cooled, giving the *product* (1·42 g), m.p. 194–6° (from ethanol, charcoal). Found: N,11·2; Se, 31·4; $C_8H_{11}ClN_2Se$ requires N, 11·2; Se, 31·6%. *Picrate*, m.p. 173–4° (from water). Found: N, 14·65; Se, 16·3; $C_{14}H_{13}N_5O_7Se{:}2H_2O$ requires N, 14·6; Se, 16·4%. *Toluene*-p-*sulphonate*, m.p. 172–3° (from ethanol). Found: N, 7·1; Se, 21·2; $C_{15}H_{18}N_2O_3SSe$ requires N, 7·4; Se, 20·8%.

Se-*Methylselenouronium iodide*, m.p. 113–5° (from ethanol-ether), was similarly obtained in 79% yield from selenourea and methyl iodide. Found: N, 10·7; Se, 30·2; $C_2H_7IN_2Se$ requires N, 10·6; Se, 29·8. *Picrate*, m.p. 218–20° (from ethanol). Found: N, 18·8; Se, 21·3; $C_8H_9N_5O_7Se$ requires N, 19·1; Se, 21·6%.

2-*Toluene*-p-*sulphonamido*-2-*selenazolin*-4-*one*. 2-Iminoselenazolidin-4-one hydrochloride (0·64 g) and toluene-*p*-sulphonyl chloride were heated together in pyridine (5 ml) for 2 hr. Extraction gave the *sulphonamide* (0·35 g), m.p. 209–11° (from ethanol). Found: N, 8·8; Se, 24·8; $C_{10}H_{10}N_2O_3SSe$ requires N, 8·8; Se, 24·9%. 2-Iminoselenazolidin-4-one hydrochloride and benzenesulphonyl chloride similarly gave 2-*benzenesulphonamido*-2-*selenazolin*-4-*one*, m.p. 168° (from ethanol), in 46% yield. Found: N, 9·0; Se, 26·4; $C_9H_8N_2O_3SSe$ requires N, 9·3; Se, 26·1%.

Biological results

In view of the diverse biological activity of the oxygen and sulphur analogues, general pharmacological screening of selected compounds was carried out (Table 3). For test procedures in mice the compounds were suspended in a 5% acacia mucilage at various concentrations depending on the dose employed; the dose volume was 0·5 ml/20 g weight. The only activity observed was weak antihistamine and parasympatholytic activity.

Antibacterial activity of 5-ethyl-2-iminoselenazolidin-4-one hydrobromide, 5-ethylselenazolidine-2,4-dione and its 2-isopropylidenehydrazone, and 5-benzylideneselenazolidine-2,4-dione was negligible against *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Proteus vulgaris*.

TABLE 3. BIOLOGICAL RESULTS

Test	Animal	Route	5-Ethylselenazolidine-2,4-dione	5-Ethyl-2-iminoselenazolidin-4-one hydrobromide
Dose range	Mouse	Oral	LD50: 24 mg/kg Severe clonic convulsions before death.	LD50: 250 mg/kg Depression 125 mg/kg. Death after several hours.
		s.c.	LD50: 24 mg/kg. Severe clonic convulsions before death.	LD50: 750 mg/kg. Depression 125 mg/kg
Analgesic	Mouse	s.c.	−ve at 10 mg/kg	−ve at 10 mg/kg
Max. electro-shock seizure ..	Mouse	Oral	−ve at 10 mg/kg	−ve at 100 mg/kg
Conditioned response ..	Rat	Oral	−ve at 9·6 mg/kg	−ve at 100 mg/kg
Max. leptazol seizure	Mouse	Oral	sl. +ve at 10 mg/kg (3/5 protected)	−ve at 100 mg/kg
Antihistamine	Guinea-pig (ileum)		+ve at 100 μg in 20 ml	+ve at 100 μg in 20 ml
Anti-amphetamine	Mouse	Oral	−ve at 1–9 mg/kg	−ve at 10–90 mg/kg
Anti-Tremorine	Mouse	Oral	−ve at 9·6 mg/kg	−ve at 100 mg/kg
Sympathetic block	Cat	s.c.	sl. +ve at 5 mg/kg	−ve at 50 mg/kg
Diuretic	Rat	Oral	−ve at 1–9 mg/kg	−ve at 10–90 mg/kg
Parasympatholytic	Guinea-pig (ileum)		−ve at 100 μg in 20 ml	+ve at 100 μg in 20 ml

Conclusion

The 2-iminoselenazolidin-4-ones and selenazolidine-2,4-diones tested exhibited negligible biological activity.

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References

Brown, F. C. (1961). *Chem. Rev.*, **61**, 463–521.
Clark-Lewis, J. W. (1958). *ibid.*, **58**, 63–69.
Comrie, A. M., Dingwall, D. & Stenlake, J. B. (1963). *J. chem. Soc.*, 5713–5717
Dingwall, D. (1962). *J. Pharm. Pharmacol.*, **14**, 765–775.
Dingwall, D. & Williams, W. D. (1961). *Ibid.*, **13**, 12–19.
Frerichs, H. (1903). *Arch. Pharm.*, **241**, 177–222.
Hofmann, G. (1889). *Ann.*, **250**, 294–332.
Jack, D. (1959). *J. Pharm. Pharmacol.*, **11**, 108*T*–114*T*.
Painter, E. P. (1941). *Chem. Rev.*, **28**, 179–213.
Roy, A. N. & Guha, P. C. (1945). *J. Ind. chem. Soc.*, **22**, 82–84.
Zingaro, R. A., Bennett, F. C. & Hammar, G. W. (1953), *J. org. Chem.*, **18**, 292–296.

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The stability, staining and corrosive properties of an iodine-non-ionic surface-active agent complex

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The stability of concentrated and dilute solutions of an iodine-cetomacrogol complex and the staining of fabrics and the corrosion of metals it produced, have been compared at the same available iodine concentration, with a system based on weak iodine solution B.P. The available iodine content of the complex decreased on storage, but this loss was associated with the production of an equivalent amount of hydrogen iodide, such that the total iodine content of the system remained constant. The reaction was accelerated by an increase in temperature, but was neither a photochemical nor an oxidative decomposition. The complex was not subject to the large losses of solvent which occurred with the potassium iodide-ethanol system, and in dilute solution was generally more stable; both systems being more stable in acid conditions. Dilute solutions of electrolytes, as found in hard waters, had little effect on the stability of either type of dilute solution except where they caused the pH to rise, but potassium salts produced a characteristic precipitation of the complex, which resulted in a loss of available iodine. The technique of testing the permanency of staining indicated that the complex often produced the less permanent stain, but it was not completely devoid of staining properties. The complex was less corrosive towards certain metals when tested by a static total immersion test, but when the attack was severe as with aluminium and copper, there was little difference between the two iodine systems in their corrosive action.

AMONGST the advantages claimed for iodophors are increased stability, especially in dilute solution, and decreased staining of fabrics and corrosion of metals (Lazarus, 1955, Wetzler, 1959; Wilson, Mizuno & Bloomberg, 1961). The work reported in an earlier paper has shown that an aqueous iodine system can be prepared by addition of cetomacrogol 1,000 B.P.C. The stability, staining of fabrics and corrosion of metals of such a system has been compared with weak iodine solution B.P. Both systems contained the same amount of available iodine.

Experimental

The limits of the amount of iodine which can be incorporated into an aqueous solution of cetomacrogol are set by the solubility of iodine in the cetomacrogol solutions and the concentration at which cetomacrogol solutions form a gel. Thus, allowing for batch variation, an aqueous system containing 2% iodine can be prepared in a 20% solution of any of the three batches of cetomacrogol described in an earlier paper (Hugo & Newton, 1963).

The iodine-cetomacrogol system was prepared by melting the cetomacrogol (batch C) in a closed vessel at 50–60°, adding finely ground iodine and agitating until all the crystals of iodine had disappeared, this taking 2 to 3 hr. Water at about 50° was then added with constant agitation to disperse the gel which formed. Final adjustment to volume was made when the system had cooled to room temperature. The

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analytical details of the system and of the dilution of the weak iodine solution B.P. (henceforth referred to as iodine solution) were for the complex (g/litre: available iodine, 20·32; total iodine, 23·70; HI, 3·36; iodide, 4·21; pH, 1·93. For the solution they were: available iodine, 20·08; iodide, 20·04 (KI); pH, 6·20.

THE STABILITY OF IODINE SYSTEMS

Stability of undiluted solutions. The effect of light, the protective effect of amber glass; the effect of air and of temperature were investigated after eliminating the factor of closure by storing in ampoules sealed by fusion. The effectiveness as closures of corks, aluminium screw caps with rubber liners and glass stoppers was assessed using 4 fl oz clear glass bottles to store the systems. The storage in open containers was studied by placing 10 ml of solution into a series of 100 ml beakers, weighing after known time intervals and determining the iodine content.

Stability of dilute solutions. In this series the stability of systems containing 400, 200 and 35 μg/ml of iodine was estimated over a period of 80 days when contained in glass stoppered volumetric flasks and stored in darkness. Similar series were also set up but dilution was with citric acid: disodium hydrogen phosphate buffers of pH 2·2, 4·0 and 6·0. The effect of pH on the availability of iodine and stability of solutions of 2,000 μg/ml was ascertained under the same conditions, with sodium acetate: hydrochloric acid, disodium hydrogen phosphate: citric acid and glycine sodium chloride: sodium hydroxide buffers.

The effect of water hardness on dilutions ranging from 5,000 to 10 μg/ml, over 9 weeks storage in glass stoppered volumetric flasks, in darkness, was determined. Two types of synthetic hard waters were employed those of (i) Klimek & Bailey (1956) and of (ii) Kravetz & Stedman (1957).

These give a water hardness of 320 ppm calcium carbonate for (i) and 328 ppm calcium carbonate for (ii). They were used undiluted to give a final water hardness of these values and at a 1 : 10 dilution to give 32 and 32·8 ppm calcium carbonate.

The effect of electrolytes was determined qualitatively by adding equal volumes of saturated solution of the electrolyte and iodine-cetomacrogol systems and quantitatively by adding known molar concentrations of electrolyte solutions.

THE STAINING OF FABRICS

Two inch square pieces of selected BPC dressings were completely immersed in the undiluted iodine preparations for 2 min. The dressing was removed and allowed to drain for 30 sec, before washing by placing in 2 litres of cold water in a screw cap bottle fitted with plastic baffles to ensure turbulence in the water when the bottles were rotated at 56 rev/min. After washing for the required time, the samples were removed and dried between filter paper and examined for staining.

THE CORROSION OF METALS

The corrosion produced by 5,000 and 100 μg/ml iodine was investigated in both types of solution by a static total immersion test. Cleaned and degreased samples of metals of known size were suspended by a glass hook at a depth of 2 cm below the surface of the solution contained in a screw cap glass bottle. At known times the metals were removed, washed in running distilled water, rinsed in acetone, dried between filter paper and weighed. Macro- and microscopical observations were made before replacing the samples in the solution. The solutions were replaced when no iodine could be detected or when they had reached a constant low level.

TABLE 1. METALS USED IN CORROSION TESTS

Metal	Source
Galvanised mild steel	See text
Aluminium	B.D.H. reagent grade foil
Copper	B.D.H. analytical reagent grade foil
Nickel	B.D.H. reagent grade foil
Tin	B.D.H. " "
Tin and lead alloy	B.D.H. " " 50% tin and 50% lead
Lead, Mild steel, Zinc	Commercial sheet supplied by the Metallurgy Department of Nottingham University
Stainless steel	Supplied by the Metallurgy Department of Nottingham University
Monel	Monel Nickel Co. Ltd., 63% nickel and 28–34% copper

The metals tested are listed in Table 1. The galvanised mild steel was prepared by cutting, drilling, cleaning, degreasing and drying a sample of mild steel of the required size. After coating with a flux (consisting of 70% zinc chloride and 30% ammonium chloride made to a paste with water) and drying in a bunsen flame, the sample was immersed in molten zinc, at 500° removed, drained and cooled before smoothing the surface with OO emery paper. The film was 0·03 mm thick.

Results

THE STABILITY OF THE IODINE SYSTEMS

The iodine and hydrogen iodide content and pH of the iodine systems after 52 weeks storage under varying conditions is given in Table 2.

TABLE 2. THE EFFECT OF STORAGE CONDITIONS AFTER 52 WEEKS ON THE STABILITY OF IODINE PREPARATIONS

Type of glass	Sealed under	Temp.	Conditions of light	Iodine-cetomacrogol complex			Iodine solutions		
				Iodine conc. g/litre	Hydrogen iodide conc. g/litre	pH	Iodine conc. g/litre	Hydrogen iodide conc. g/litre	pH
Clear	Air	Room*	Daylight	17·88	5·87	1·68	20·11	—	4·66
"	Nitrogen	"	"	18·03	5·95	1·70	20·25	—	4·66
Amber	Air	"	"	17·95	5·92	1·72	20·17	—	4·66
Clear	"	"	Darkness	18·14	6·02	1·64	20·17	—	4·67
"	Nitrogen	"	"	18·14	5·78	1·72	20·38	—	4·51
"	Air	4° C.	"	18·68	5·75	1·66	20·20	—	5·04
"	"	37° C.	"	15·67	8·44	1·52	19·53	0·76	3·06

* 14–30°

Temperature is the factor producing the greatest effect, although there was a reduction in the iodine content of cetomacrogol systems under all conditions of storage.

The production of acid was not due to the decomposition of the cetomacrogol alone, as no significant changes in the pH of a 20% solution when stored at 4, 37 and 50° were observed. In containers stored in daylight at room temperature (14–30°) for 52 weeks, the results for iodine concentration, hydrogen concentration and pH were similar for the three kinds of closure examined; the aluminium cap with rubber liner allowing the least loss of solvent. The % weight and iodine lost when stored in open beakers is illustrated in Fig. 1.

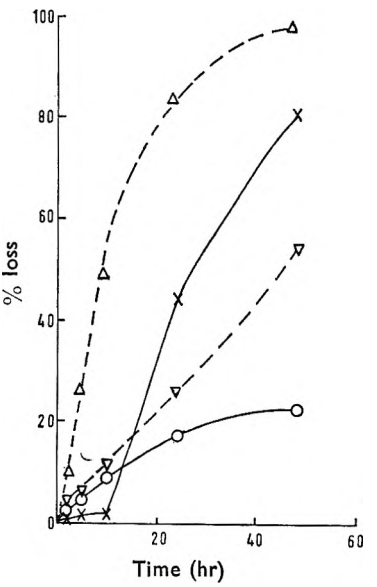

Fig. 1. Changes in the weight and iodine content of iodine preparations when stored in open beakers at room temperature. △ % weight of iodine solution, ▽ % weight of iodine-cetomacrogol complex, × %weight of iodine from iodine solution lost, ○ % weight of iodine from iodine-cetomacrogol complex lost.

TABLE 3. THE EFFECT OF pH ON THE STABILITY OF DILUTIONS OF THE IODINE PREPARATIONS

	Iodine concentrations μg/ml							
	Iodine-cetomacrogol complex				Iodine solution			
Time in weeks	(a)	(b)	(c)	(d)	(a)	(b)	(c)	(d)
0	35·5	33·6	35·8	36·8	33·1	34·5	35 5	36·8
12	11·5	7·9	11·7	17·9	2·7	0·3	1 5	1·3
0	203·2	204·6	204·5	232·5	181·2	180·2	188·0	183·5
12	106·9	120·0	134·4	130·2	0·0	0·0	2·3	2·3
0	423·5	421·6	425·0	428·0	385·0	385·0	383·5	386·0
12	325·0	365·5	360·2	362·2	5·1	7·6	13·0	12·5

(a) no buffer, (b) pH 6·0 buffer, (c) pH 4·0 buffer and (d) pH 2·2 buffer. Solutions stored in stoppered volumetric flasks at room temperature (16–20°) in darkness.

Table 3 shows the change in iodine content of 35, 200 and 400 μg/ml dilutions in water and in buffers of pH 2·2, 4·0 and 6·0, after 12 weeks storage in stoppered volumetric flasks, in darkness.

The effect of pH on the availability and stability of 2,000 μg/ml dilutions is shown in Fig. 2. The iodine content of dilutions in two types of hard waters, after 9 weeks storage under the same conditions is given in Table 4. The pH of the bicarbonate-containing hard water dilutions increased as the extent of dilution increased, but never exceeded 8.0.

In the presence of electrolytes which were strongly alkaline, both iodine preparations were decolourised due to formation of iodide. In the

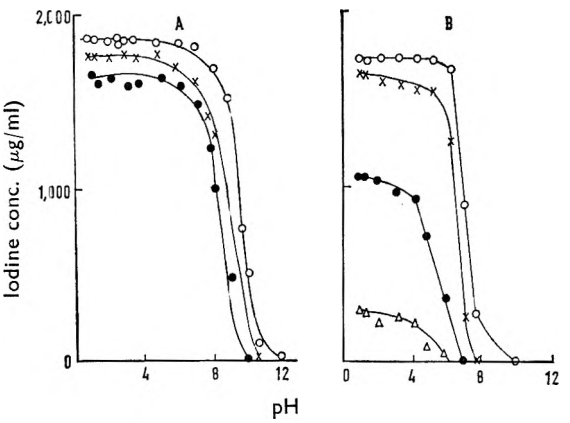

FIG. 2. The effect of pH on the availability and stability of dilutions of iodine preparations containing 2,000 μg/ml of iodine A, Iodine-cetomacrogol complex: ○ after 0 and 5 days; × after 58 days; ● after 185 days.
B, Iodine solution: ○ initial; × after 4 days; ● after 38 days; △ after 163 days.

TABLE 4. THE EFFECT OF WATER HARDNESS ON THE STABILITY OF (A) THE COMPLEX AND (B) THE SOLUTION STORED IN STOPPERED VOLUMETRIC FLASKS AT ROOM TEMPERATURE (16–20°) IN DARKNESS

		Iodine concentration μg/ml				
Time in weeks		(a)	(b)	(c)	(d)	(e)
A						
	0	5128·0	5128·0	5105·0	5100·0	5100·0
	9	5030·0	4890·0	4900·0	4870·0	4868·0
	0	1016·0	1019·0	1013·0	1013·0	992·0
	9	1004·0	392·0	881·0	902·0	883·0
	0	481·8	477·8	481·0	474·0	474·0
	9	432·0	356·9	363·0	275·0	297·6
	0	92·6	90·8	96·0	90·8	92·7
	9	79·6	40·8	46·3	45·1	48·9
	0	42·6	42·6	43·2	42·8	42·7
	9	10·0	12·2	14·0	12·1	12·0
	0	7·6	7·6	6·7	7·6	7·0
	5	2·7	3·2	2·3	1·7	0·0
B						
	0	90·7	98·9	98·9	90·0	89·3
	9	74·8	76·1	78·1	5·4	0·0
	0	43·1	40·9	40·5	41·2	39·2
	9	36·1	35·6	33·7	0·0	0·0

(a) Distilled water, (b) and (c) water containing calcium and magnesium chlorides, to give (b) 32 and (c) 320 ppm calcium carbonate and (d) and (e) water containing calcium chloride and sodium bicarbonate to give (d) 32·8 and (e) 328 ppm calcium carbonate.

presence of potassium salts, a characteristic precipitation of the iodine-cetomacrogol complex occurred, the extent of which depended on various factors including potassium content and the iodine content of the complex.

THE STAINING OF FABRICS

The condition of the various dressings after the staining and washing process is described in Table 5.

TABLE 5. THE STAINING OF DRESSINGS BY IODINE FORMULATIONS

	Iodine-cetomacrogol complex		Iodine solution	
Dressing	10 min washing time	60 min washing time	10 min washing time	60 min washing time
Bandages: White open weave	Pale blue staining of some fibres	No stain remaining	Blue staining of fibres	Slight blue stain
Calico	Pale blue irregular stain	No stain remaining	Dark blue irregular stain	Bright blue stain remains
Domette	Bright yellow staining of the weft, and blue staining of the warp	Slight yellow stain remaining	Dark blue staining of the warp, slight yellow staining of the weft	Blue staining of the warp, light staining of the weft
Flannel	A slight brown tinge added to the original green colour	A slight yellow stain	Definite brown colouring of the original green colour	A slight yellow stain remains
Cotton crepe	Slight staining of the rubber threads	No stain remains	Brown staining of the rubber threads and slight blue staining of the cotton	A slight brown stain remains
Crepe	Bright yellow staining of the wool warp, no effect on the cotton	Bright yellow stain of the wool remains	Bright yellow staining of the wool warp, little effect on the cotton	Bright yellow stain of the wool remains
Rayon and rubber elastic	Slight brown staining of the cotton rubber yarn, but no effect on the rayon	No stain remains	Dark brown staining of the cotton and rubber yarn, and a slight brown staining of the rayon	No stain remains
Absorbent gauze	No stain	remains	No stain	remains
Lint	General pale green blue stain with a few areas coloured brown. Both colours fade on standing	No stain remains	Bright blue stain which fades on standing	No stain remains
Rayon lint	A few pale yellow	No stain remains	A few pale yellow	No stain remains
Silk	No stain	remains	No stain	remains

THE CORROSION OF METALS

A full description of the effect of the two strengths of the iodine systems on the different metals and the weight change/time curves for periods of 40 to 80 weeks are given by Newton (1962). Here the results have been condensed to give the corrosion rates after the initial and final sampling (Table 6).

TABLE 6. THE CORROSION RATES OF METALS BY A STATIC TOTAL IMMERSION IN SOLUTIONS OF IODINE OF STRENGTH 5,000 μg/ml FOR VARYING TIMES AND 100 μg/ml FOR 280 DAYS

Metal	Rate of corrosion in mg/sq. dm/day			
	At initial sampling, at 7 days		At final sampling*	
	A	B	A	B
5000 μg/ml solutions				
Aluminium	−8·16	−26·40	−6·81 (91)	−17·48 (14)
Copper	−168·10	−128·40	−107·90 (35)	−71·52 (49)
Galvanised mild steel	−96·00	−96·20	−6·80 (434)	−23·22 (158)
Lead	−42·60	+21·55	−0·52 (441)	+0·72 (441)
Mild steel	−72·90	−87·60	−20·44 (189)	−54·18 (119)
Monel	−8·63	−60·40	−2·84 (287)	−37·28 (287)
Nickel	−75·20	−38·85[a]	−1·17 (518)	−43·55 (49)
Stainless steel	0·0	−6·59[b]	0·0 (560)	−0·34 (434)
Tin	0·0	−0·02[a]	+0·08 (560)	0·0 (420)
Tin and lead	0·0	+32·60[a]	−0·63 (560)	+1·07 (385)
Zinc	−179·60	−153·00	−2·67 (441)	−11·73 (441)
100 μg/ml solutions				
Aluminium	−0·37	−0·27	−0·09	−0·10
Copper	+3·77	+8·38	+3·62	+6·54
Galvanised mild steel	−0·09	−2·29	−0·42	−0·56
Mild steel	−6·40	−6·90	−4·82	+7·24
Monel	−0·67	−0·21	−0·13	−0·22
Nickel	−1·10	−1·42	−0·05	−0·54
Stainless steel	0·0	0·0	−0·02	+0·01
Tin	+0·17	+1·02	+0·01	−0·01
Tin and lead	−4·47	−3·29	−0·85	−4·44 (196)
Zinc	+0·77	−1·66	+0·35	−0·98

A Iodine-cetomacrogol complex. B Iodine solution. * After 280 days or figures in parenthesis.
a After 14 days. b After 21 days.

Discussion

The failure of existing iodine formulations to meet all the requirements for antibacterial applications of iodine has limited the use of this halogen. Nevertheless, the ethanol-potassium iodide solution, as weak iodine solution B.P. provides a stable formula with which to compare the iodine-cetomacrogol complex. The inclusion of an alkali iodide has been shown to increase the stability of ethanolic solutions (Courtot, 1910a; Carter, 1926). This was confirmed for storage at room temperature (14–30°), but at 37° the solution was prone to decomposition in the same manner as the simple ethanolic solution, with the production of an equivalent amount of hydrogen iodide by the oxidation of the ethanol to the corresponding aldehyde or acid (Courtot, 1910b). The decomposition of the complex also results in the production of an equivalent quantity of hydrogen iodide and hence one would similarly postulate an oxidation reaction. Table 1, however, shows that replacing the air in the ampoules with nitrogen does not decrease the amount of decomposition which occurs at room temperature. Hence, as with the production of hydrogen iodide on dissolving iodine in solutions of cetomacrogol, an oxidation reaction must be discounted, the decomposition presumably being a continuation of the "solution" phenomenon reported by Hugo & Newton (1963). The decomposition, as with the iodine solution, was not accelerated by light. Brost & Krupin (1957) recommend the inclusion of acid to prevent the decomposition and thus stabilise such complexes.

The type of the closure did not grossly affect stability but loss of solvent was more important in the case of the iodine solution than the complex.

This loss is further illustrated by reference to the storage in open beakers (Fig. 1), where approximately 50% of the solvent was lost before there was a significant loss of iodine. This loss of solvent eventually resulted in the appearance of iodine crystals and an 80% loss of iodine in 48 hr. With the complex, the final product was a dark brown water soluble paste, from which 20% of iodine had been lost in 48 hr. Of the closures tested the aluminium screw cap allowed least loss of solvent, but the aluminium was attacked by the iodine solution and the rubber liner by the iodine-cetomacrogol complex, and therefore none of the closures was entirely suitable.

The results with more dilute solutions indicate that, under the conditions of storage, dilutions of the complex were more stable than those of the solution, in that they retained their availability and stability over a wider pH range (Fig. 2); dilutions of both systems were more stable in acid solution. The effect of water hardness on stability was probably closely linked to the presence of sodium bicarbonate rather than to the presence of other electrolytes. The pH, however, never rose above 8·0 and therefore there was no major effect on stability associated with high values of pH (Fig. 2). In the case of the iodine solution the increase in pH was sufficient to affect the stability.

The precipitation produced by the addition of potassium salts to the complex does not appear explicable by any simple theory. Van der Hoeve (1948) noted that a solution of iodine in potassium iodide produced a precipitate with polyoxyethylene ethers, and Higuchi & Guttman (1955) noted that an insoluble complex was formed between iodine in aqueous potassium iodide and polyethylene glycols. In neither case was a full explanation of the phenomenon given.

Holness & Ross (1951; 1952) and Ross (1952) have shown that non-ionic surface-active agents do not attack copper, mild steel or tin. It is assumed therefore that any corrosion by the complex is due to the presence of iodine. No attempt will be made to explain the processes involved in the attack of the various metals by the iodine systems. The results in Table 6 show that where the attack of the metals was severe, there was little difference between the two iodine systems at the 5,000 μg/ml level. Where the attack was less, and in the case of the 100 μg/ml solutions, the complex was generally less corrosive than the solution.

Johns (1954) reported that iodophors were non-corrosive to metals, but no details of the test procedures were given. Anderson (1957) did note that iodophors attacked aluminium, iron, copper, brass and phosphor bronze, but as the rate of corrosion did not exceed 0·1 g/m^2/hr (24 mg/dm^2/day), he did not consider the attack of any practical significance. He also reported that there was no attack of stainless steel and tinplate, but again there were no details of test procedure, nor iodine concentration. If the level of practical significance of attack is taken from the work of Anderson (1957) it would be possible to classify the metals tested into those suitable and those unsuitable for use with these iodine solutions. The danger of using corrosion rates alone is shown by reference to the attack of aluminimum, which could be accepted on the above rating yet marked

pitting of the samples occurred in all the solutions. Similarly, corrosion rates indicate that there is no significant attack of the tin/lead sample by the 5,000 μg/ml iodine solution but the physical effects were clearly visible. With the low rates of corrosion which occurred in the 100 μg/ml solution, micro- and macroscopical observations become more important. Only in the case of the stainless steel and the tin samples was there no observed effect produced on the metals. Whether or not these effects were of practical significance would depend on the conditions of practical application. It may also be noted that, in the 100 μg/ml solutions there was a greater tendency for the sample to increase in weight, due to the formation of a film on the surface of the sample.

An additional feature was that in all cases the iodine solution needed replacing more frequently than the iodine-cetomacrogol complex. This, however, was not entirely due to the reaction with the metal, for when a control was carried out without the inclusion of a metal sample, it was found that a 100 μg/ml dilution of the iodine solution was decolourised in 6 weeks, whereas the iodine-cetomacrogol complex of the same concentration was still yellow after 40 weeks.

From constant handling of the two preparations the general impression obtained was that the complex stained skin and fabrics to a far less extent than did the iodine solution. The attempts to verify this experimentally produced less difference than anticipated. Nevertheless, the results in Table 5 do indicate that the iodine-cetomacrogol complex was less inclined to stain, but was not completely devoid of staining properties, e.g. on woollen fabrics. This decrease in staining power could be linked with the smaller amount of iodine adsorbed from the iodine-cetomacrogol complex than the iodine solution by yeast, serum and bacteria, which has been described (Hugo & Newton, 1964).

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References

Anderson, L. (1957). *Svenska Mejeritidn.*, **49**, 309–312, 323–326.
Brost, G. A. & Krupin, F. (1957). *Soap Chem. Spec.*, **33**, 93–95. 97, 105 and 107.
Carter, R. M. (1926). *Indust. Engng Chem.*, **18**, 827–828.
Courtot, C. (1910a). *J. Pharm. Chim., Paris*, **1**, 297–300.
Courtot, C. (1910b). *Ibid.*, 354–359.
Guttman, D. E. & Higuchi, T. (1955). *J. Amer. pharm. Ass.* (Sci. Ed.), **44**, 668–678.
Holness, H. & Ross, T. K. (1951). *J. appl. Chem.*, **1**, 158–169.
Holness, H. & Ross, T. K. (1952). *Ibid.*, **2**, 520–526.
Hugo, W. B. & Newton, J. M. (1963). *J. Pharm. Pharmacol.*, **15**, 731–741.
Hugo, W. B. & Newton, J. M. (1964). *Ibid.*, **16**, 49–55.
Johns, C. K. (1954). *Canad. J. Technol.*, **32**, 71–77.
Klimek, J. W. & Bailey, J. H. (1956). *Appl. microbiol.*, **4**, 53–59.
Kravitz, E. & Stedman, R. L. (1957). *Ibid.*, **5**, 34–35.
Lazarus, N. E. (1954). *J. Milk Tech.*, **17**, 144–147.
Newton J. M. (1962). Ph.D. Thesis, Nottingham.
Ross, T. K. (1952). *J. appl. Chem.*, **2**, 526–531.
Van der Hoeve, J. A. (1948). *Rec. Trav. Chim., Pays-Bas*, **67**, 649–664, through *Chem. Abst.*, **43**, 1,584i (1949).
Wetzler, T. F. (1959). *Soap Chem. Spec.*, **35**, 115–117, 119 and 135.
Wilson, J. L., Mizuno, W. G. & Bloomberg, C. S. (1961). *Ibid.*, **36**, 121–124.

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Letters to the Editor

Evaluation of changes of capillary permeability*

SIR,—The effects of drugs on capillary permeability are usually studied by means of dyes which bind to plasma proteins. Intradermal administration of agents increasing capillary permeability, such as 5-hydroxytryptamine (5-HT), histamine and bradykinin, results in the formation of coloured areas at the sites of injection when a dye is administered intravenously. The degree and extent of the colour accumulation are considered an index of the capillary lesion and are estimated by various methods. Thus, Parratt & West (1957) used the visual grading of the blueing, Ungar, Kobrin & Sezesny (1959) cut out and then weighed the pieces of skin, Judah & Willoughby (1962) determined the degree of dye leakage by extraction and photometric determination of the dye, whilst other authors presented their results in terms of the "mean lesion diameter" (Miles & Wilhelm, 1955; Sparrow & Wilhelm, 1957; Bonaccorsi & West, 1963). We have usually evaluated the degree of dye leakage into the coloured areas by measuring the two diameters (length and width) and utilising the product of these two measurements (Jori, Bentivoglio & Garattini, 1961; Bonaccorsi, Jori, & Garattini, 1963).

We have now found that the product of the two diameters of the coloured area parallels the effective movement of blue dye from the circulation into the skin, when 5-HT is used as a permeability-increasing agent.

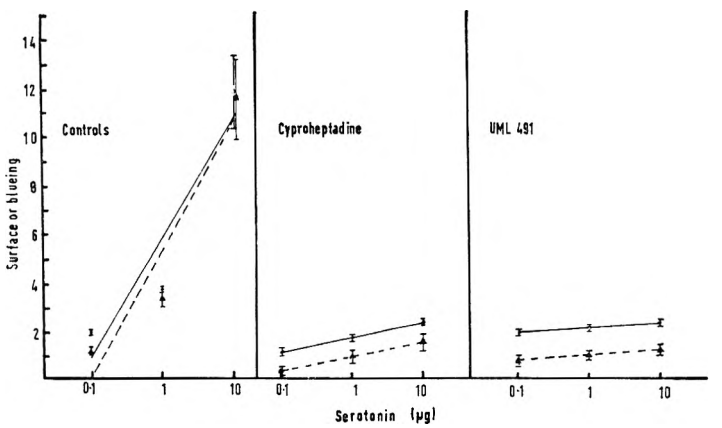

FIG. 1. Measurement of the coloured surface area (●) or of dye concentration (▲) induced by 5-HT in control animals (a) or in animals pretreated with cyproheptadine (b) or UML 491 (c). On the abscissae are shown (log scale) the concentrations of 5-HT (serotonin) injected intradermally; on the ordinates, the products of the two main diameters of the coloured spots in cm^2 or the μg ($\times$ 1/5) of azovan blue dye.
The analysis of the variance shows the following probability: regression for (a) $<0{\cdot}005$; (b) $<0{\cdot}01$; (c) $<0{\cdot}01$; deviation from linear regression for (a) 0·025; (b) 0·90; (c) 0·90; deviation from parallelism for (a) 0·50; (b) 0·70; (c) 0·40.

●—● surface (a) $y = 0{\cdot}84 + 5x$ (b) $y = 1{\cdot}10 + 0{\cdot}61x$ (c) $y = y\ 1{\cdot}74 + 0{\cdot}17x$

- - ▲ blueing $y = 0{\cdot}22 + 5{\cdot}22x$ $y = 0{\cdot}32 + 0{\cdot}57x$ $y = 0{\cdot}60 + 0{\cdot}23x$

* Supported by U.S. Army, European Research Office, Frankfurt, Germany (Contract DA-91-591-EUC-2686).

Sprague-Dawley rats weighing about 180 g were used. After depilation of the abdominal skin, the animals were anaesthetized with ethyl ether and injected intravenously with azovan blue (3 ml/kg of a 0·4% solution). Immediately, 0·1, 1 and 10 μg of 5-HT were injected intradermally, each in 0·1 ml of saline, into each animal. 30 min later, the rats were killed, the diameters of the coloured areas measured, and the pieces of skin removed and submitted to the azovan blue extraction and determination, according to the method of Beach & Steinetz (1961). Two antagonists of 5-HT, 1-methyl-lysergic acid butanolamide (UML 491—Sandoz) (Berde, Doepfner & Cerletti, 1960) and cyproheptadine (Periactin—Merck) (Stone, Wenger, Ludden, Stavorski & Ross, 1961), which had been shown to have a strong inhibitory activity in this test (Jori, Bentivoglio & Garrattini, 1961), were injected intravenously 30 min before azovan blue, at doses of 3 μg/kg and 100 μg/kg respectively. Fig. 1 shows that the curves of the dye concentration (blueing) and of the coloured surface area measurements are congruent. Therefore, at least in our experimental conditions, measurement of the surface of the coloured area is adequate to evaluate the degree of the dye infiltration.

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References

Beach, V. L. & Steinetz, G. B. (1961). *J. Pharmacol.*, **131**, 400–406.
Berde, B., Doepfner, W. & Cerletti, A. (1960). *Helv. Physiol. Acta*, **18**, 537–544.
Bonaccorsi, A., Jori, A. & Garattini, S. (1963). *La Settimana Medica*, **51**, 46, 51—Suppl. No. 1.
Bonaccorsi, A. & West, G. B. (1963). *J. Pharm. Pharmacol.*, **15**, 372–378.
Jori, A., Bentivoglio, A. & Garattini, S. (1961). *Ibid.*, **13**, 617–619.
Judah, J. D. & Willoughby, D. A. (1962). *J. Path. Bact.*, **83**, 567–572.
Miles, A. A. & Wilhelm, D. L. (1955). *Brit. J. exp. Path.*, **36**, 71–81.
Parratt, J. R. & West, G. B. (1957). *J. Physiol.*, **139**, 27–41.
Sparrow, E. M. & Wilhelm, D. L. (1957). *Ibid.*, **137**, 51–65.
Stone, C. A., Wenger, H. C., Ludden, C. T., Stavorski, J. M. & Ross, C. A. (1961). *J. Pharmacol.*, **131**, 73–84.
Ungar, C., Kobrin S. & Sezesny, B. R. (1959). *Arch. int. Pharmacodyn.*, **123**, 71–77.

Paper chromatography and identification of *Magnolia acuminata* L. alkaloids

SIR,—In a survey of plants for steroidal sapogenins and other constituents Wall, Fenske, Garwin, Willaman, Jones, Schubert & Gentry (1959) screened some plants of the magnoliaceae family. Of the four species of the American magnolias examined, presence of alkaloids was reported in stems and leaves of *Magnolia acuminata* L. (Cucumber-tree). However, these compounds have not been isolated and characterized.

We have extracted the stems of *M. acuminata* and isolated fractions containing alkaloids. Descending paper chromatography of the quaternary chlorides was performed using three different solvent systems. Five quaternary bases (A to E) were detected (Table 1) after development with solvent system 1 and spraying with reagent I. Four bases have been identified and checked by running them with authentic specimens. A is choline chloride; B is magnoflorine chloride, D is salicifoline chloride and E is magnocurarine chloride.

Chromatograms developed with solvent systems 2 and 3 showed distinct coloured spots of salicifoline and magnocurarine chlorides when both IIa and IIb were sprayed. Thus reagent II affords a useful means of identification of each of the three quaternary alkaloids magnoflorine, magnocurarine and salicifoline, which are known to occur in some species of magnolias (Tomita & Nakano, 1957).

TABLE 1. Rf VALUES AND COLOUR REACTIONS OF QUATERNARY BASES FROM STEMS OF *M. acuminata*

Quaternary base	Rf values system			Colour reaction after systems	
	1	2	3	Reagent I	Reagent II *
A	0·14	0·10	0·27	Violet	—
B†	0·16	0·21	0·58	Orange	Red then yellow-orange
C†	0·26	0·31	0·62	Orange	Red then yellow-orange
D	0·41	0·22	0·57	Orange	Yellow then orange
E	0·70	0·51	0·67	Orange	Light brown then dark brown

Solvent systems 1 = Ethyl acetate : pyridine : water (750 : 310 : 165).
2 = n-Butanol : water : acetic acid (100 : 100 : 5).
3 = n-Butanol : water : acetic acid (50 : 40 : 10).
Reagent I = Modified Dragendorff reagent (Munier & Macheboeuf, 1951).
Reagent II = Modified Pauly's reagent (Ames & Mitchell, 1952).

* Colour changes indicate shades observed when sprayed with IIa (diazotized sulphanilic acid) followed by IIb (20% sodium carbonate solution). Distinct changes were seen only on papers developed with sytem 1.

† Shows purple fluorescence under ultra-violet light.

Chromatographic identification of compound D and E was confirmed by isolating crystalline picrates of the two bases. Mixed melting point determination of picrate D with salicifoline picrate showed no depression and its infra-red spectrum was identical with that of the latter. Compound E was similarly characterized as magnocurarine chloride.

Quaternary alkaloidal fraction of leaves of *M. acuminata* has been isolated and studied by chromatographic technique. Results suggest the presence of only one major phenolic quaternary compound with Rf values identical with magnoflorine chloride.

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References

Wall, M. E., Fenske, C. S., Garwin, G. W., Willaman, J. J., Jones, Q., Schubert, B. G. & Gentry, H. S. (1959). *J. Amer. pharm. Ass., Sci. Ed.*, **48**, 695–722.
Munier, R. & Macheboeuf, M. (1951). *Bull. Soc. Chim. Biol.*, **33**, 846–56.
Ames, B. N. & Mitchell, H. K. (1952). *J. Amer. chem. Soc.*, **74**, 252.
Tomita, M. & Nakano, T. (1957). *Planta Med.*, **5**, 33–43.

Kinetics of histaminase

SIR,—Spencer (1963) outlined a method for the determination of relative concentrations of histaminase. In his experimental work he found that the plot of $\log_{10}(x_0/x_t)$ against t is not strictly linear (x_0 initial concentration of substrate, x_t concentration at time t). If the reaction involved is of the form:

$$S + En \underset{k_2}{\overset{k_1}{\rightleftharpoons}} S.En \underset{k_4}{\overset{k_3}{\rightleftharpoons}} P.En \underset{k_6}{\overset{k_5}{\rightleftharpoons}} En + P$$

where S denotes substrate molecule, P product molecule and En enzyme molecule, it can be shown that $-1/e.d[S]/dt = (k_a[S]-k_b[P])/(k_c[S] + k_d + k_e[P])$ where k_a, k_b, k_c, k_d, and k_e are each composites of some of the velocity constants above, square brackets denote molar concentrations and e is the concentration of enzyme. But $-1/e.d[S]/dt = 1/e.d[P]/dt$ and $[S] = [S_0]$ when $t = 0$, so $[P] = [S_0]-[S]$ and the rate equation takes the form $-1/e.d[S]/dt = (k[S]-k^1[S_0])/([S] + K)$ where $k + k^1$ are constants, and for a particular value of $[S_0]$, K also is constant. The reaction may be effectively irreversible if any of the backward velocity constants are sufficiently small since k^1 is directly proportional to $k_2.k_4.k_6$ and hence the rate equation becomes $-1/e.d[S]/dt = k[S]/([S] + K)$ which is formally the same as the Michaelis equation (1913) though the constants have different meanings. Integration and evaluation of the integration constant by insertion of $[S_0]$ for [S] when $t = 0$ gives the equation

$$k\,e\,t = ([S_0]-[S]) + 2{\cdot}303\ K \log_{10}([S_0]/[S]) \quad .. \quad .. \quad .. \quad .. \quad (1)$$

which differs from that used in the original paper solely in the presence of the term $([S_0]-[S])$ and thus accounts for the lack of linearity. The dependance of K on $[S_0]$ except when $k_6 = 0$ may account partially for dependance of the results on $[S_0]$ particularly after long times of incubation.

If t_α is the time at which α % of the substrate has been consumed

$$k\,e\,t_\alpha - \alpha[S_0] = 2{\cdot}303\ K \log_{10}(1/1-\alpha) = \text{a constant for a given value of } \alpha \quad (2)$$

Thus if $t_{\alpha 1}$, and $t_{\alpha 2}$ are the corresponding times in incubation mixtures containing enzyme concentrations of e_1 and e_2 respectively but the same initial concentration $[S_0]$ of substrate

$$k\,e_1\,t_{\alpha 1} - \alpha[S_0] = k\,e_2\,t_{\alpha 2} - \alpha[S_0] \text{ or } e_1/e_2 = t_{\alpha 2}/t_{\alpha 1} \quad .. \quad .. \quad .. \quad .. \quad (3)$$

and hence the method of determination in the original paper is theoretically sound despite the non-linearity of the plot used which is purely empirical.

The data of the paper provides a further test of the equations: if two different percentages of consumption are used the direct effect of variations of $[S_0]$ can be eliminated. From (2) for incubation mixtures containing initially $[S_1^0]$ of substrate, e_1 of enzyme; and $[S^0_2]$ of substrate and e_2 of enzyme.

$$\text{For percentage } \alpha \text{ consumption } k\,e_1\,t_{\alpha 1} - \alpha[S_1^0] = k\,e_2\,t_{\alpha 2} - \alpha[S_2^0] \quad .. \quad .. \quad (4)$$

$$\text{For percentage } \beta \text{ consumption } k\,e_1\,t_{\beta 1} - \beta[S_1^0] = k\,e_2\,t_{\beta 2} - \beta[S_2^0] \quad .. \quad .. \quad (5)$$

whence $\beta \times (4) - \alpha \times (5)$ gives

$$k\,e_1(\beta t_{\alpha 1} - \alpha t_{\beta 1}) = k\,e_2(\beta t_{\alpha 2} - \alpha t_{\beta 2})$$
$$\therefore e_1/e_2 = (\beta t_{\alpha 2} - \alpha t_{\beta 2})/(\beta t_{\alpha 1} - \alpha t_{\beta 1}) \quad .. \quad .. \quad .. \quad .. \quad .. \quad (6)$$

Table 1 gives the application of this equation to the data of Fig. 4 of the original paper.

TABLE 1

$[S_0]$, (μg/ml)	1·53	2·01	2·49
$\alpha = 50$, t_α (min.)	25	26	27
$\beta = 25$, t_β (min.)	10	10·5	11
$(\beta t_\alpha - \alpha t_\beta)$	125	125	125
Ratio of enzyme concentrations (calc.)	1·00	1·00	1·00

Equation (6) may be used also for incubation mixtures containing the same $[S_0]$. Table 2 shows its application to the data in Fig. 3 of the original paper.

TABLE 2

Ratio of enzyme concentrations (pre-arranged)	1·30	1·00	0·30	0·50
$\alpha = 50$, t_α (min.)	22	28	34	53
$\beta = 25$, t_β (min.)	9	11·5	14	22
$(\beta t_\alpha - \alpha t_\beta)$	100	125	150	225
Ratio of enzyme concentrations (calc.)	1·25	1·00	0·83	0·55

In practice using equation (6) to determine relative concentrations of enzyme would require greater experimental accuracy than keeping $[S_0]$ constant and using equation (3). There is a trend of deviation in the results of the calculations in Table 2 and in those of Table 1 of the original paper which may be due to a dependence of K on $[S_0]$ so that equation (2) is not strictly equal to a constant: the effect is rather greater when (6) is used. This can be allowed for by replacing K by $(K - \theta[S_0])$, where θ is a constant, in equation (2) with the result that equation (3) becomes $(e_1 + \psi[S_0])/(e_2 + \psi[S_0]) = t_{\alpha 2}/t_{\alpha 1}$, where ψ is a constant. Substituting the data of Table 1 of the original paper for incubation mixtures A and B ($e_2 = 1{\cdot}30$ inits, $e_1 = 1{\cdot}00$ units, $t_{\alpha 2} = 22$, $t_{\alpha 1} = 28$, $\alpha = 50$) gives $\psi[S_0] = 0{\cdot}10$. Thus, for another incubation mixture using the same $[S_0]$, e may be calculated in the same units from $(e_1 + 0{\cdot}10)/(e + 0{\cdot}10) = t_\alpha/t_{\alpha 1}$ or, in this case $e = (30{\cdot}8/t_\alpha) - 0{\cdot}10$. This gives $e = 0{\cdot}80$ and $0{\cdot}48$ respectively for incubation mixtures C and D of the original paper: they were set up to be $0{\cdot}80$ and $0{\cdot}50$ respectively and the uncorrected calculation gives $0{\cdot}82$ and $0{\cdot}53$ respectively. Thus the accuracy of the results can be improved by using two control incubation mixtures in which the concentrations of the enzyme are in a known ratio.

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References

Michaelis, L. & Menten, M. L., (1913), *Biochem. Ztochr.*, **49**, 333.
Spencer, P. S. J., (1963), *J. Pharm. Pharmacol.*, **15**, 225—232

Pharmacological properties of tetrahydropapaveroline and their relation to the catecholamines

SIR,—Recent reports from this laboratory (Santi, Contessa & Ferrari, 1963; Santi, Ferrari & Contessa, 1964) have shown that papaverine is a powerful inhibitor of the aerobic oxidation of substrates linked to nicotinamide adenine dinucleotide (NAD) in rat liver mitochondria. The inhibition of oxidative phosphorylation which could be localised in the electron transfer step between NAD and cytochrome b might be important in understanding the mechanism of the spasmolytic effect of the drug. Since some effects of papaverine resemble those generally referred to stimulation of the so-called β-receptors of adrenaline, Santi (1963) has put forward a working hypothesis based on the possibility that the adrenaline-like drugs produce an impairment of cellular energy sources.

In this context, the conclusion by Holtz, Stock, & Westerman (1963) that a substance similar in structure to papaverine, tetrahydropapaveroline, could be formed by the condensation of the well-known precursor of adrenaline, dopamine, and dihydroxyphenylacetic aldehyde, is of interest.

We have confirmed the results of Holtz & others (1963) and we believe that the pharmacological properties of tetrahydropapaveroline are in themselves very interesting in as much as this drug behaves in some respects like papaverine, in others like the catecholamines and particularly, isoprenaline. Some pharmacological properties of the drug were described several years ago by Laidlaw (1910).

The spasmolytic activity of tetrahydropapaveroline, as seen on the isolated guinea-pig ileum, resembles that of eupaverin (1-benzyl-3-ethyl-6,7-dimethoxyisoquinoline) rather than that of papaverine. It differs from papaverine in not inhibiting mitochondrial respiration.

Tetrahydropapaveroline stimulates the myocardium as was seen *in vivo* by measuring the contractile strength by means of the strain gauge technique described by Boniface, Brodie & Walton (1953), as well as *in vitro* on isolated guinea-pig atria. The latter effect is antagonised by dichloroisoprenaline (DCI). The action of tetrahydropapaveroline on the heart is presumably important in understanding its pharmacological activities. In the dog, 0·1 mg/kg injected intravenously greatly increases the contractile strength of the heart and also its frequency. At 0·02 μg/ml, it has a positive inotropic and chronotropic action; a similar effect may be shown on isolated atria of the previously reserpinised guinea-pig. In dogs and cats, tetrahydropapaveroline reduces blood pressure at concentrations 20–30 times lower than papaverine and 50 times greater than isoprenaline. The hypotensive effect mainly concerns diastolic pressure, whereas the systolic values remain unchanged, the differential increasing accordingly. The decrease of blood pressure must be presumed to be due to a peripheral vasodilatation, since when 5–10 μg of the drug was introduced into the femoral artery, a strong increase of blood flow was measured with a Shipley-Wilson rotameter (1951). On the other hand, the vasodilator response of the blood vessels of the isolated rabbit ear according to the technique of Pissemski (1914) was present, although not intense. When administered intravenously to dogs and cats, the drug greatly stimulated respiration.

Tetrahydropapaveroline, similarly to adrenaline (Ussing, 1960), and in contrast to papaverine, increases the short circuit current of the isolated frog skin as measured by the technique of Ussing & Zerahn (1951), modified by Vescovini & Marro (1960). Finally, the drug injected intraperitoneally produces an increase in the plasma level of free fatty acids in rats; this effect is considered to be

specific for adrenaline and related drugs. The mobilisation by tetrahydropapaveroline of lipids from adipose tissue was also observed *in vitro*.

From these observations it is concluded that the drug acts like papaverine on respiration and like eupaverin on the isolated ileum whereas the pharmacological effects on isolated rat liver mitochondria, isolated mammalian heart or atria, blood vessels, free fatty acids and frog skin resemble those of the catecholamines and particularly isoprenaline. Whether tetrahydropapaveroline might play a role as a chemical transmitter in the central nervous system is the subject of current enquiry in our laboratories.

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References

Boniface, K. J., Brodie, O. J. & Walton, R. P. (1953). *Proc. Soc. exp. Biol. N.Y.*, **84**, 263–269.
Holtz, P., Stock, K. & Westermann, E. (1963). *Arch. exp. Path. Pharmak.*, **246**, 133–146.
Laidlaw, P. P. (1910). *J. Physiol.*, **40**, 480–491.
Pissemski, S. A. (1914). *Arch. Phys.*, **156**, 426.
Santi, R. (1963). *Arch. It. Sci. Farmacol.* (in the press).
Santi, R., Contessa, A. R. & Ferrari, M. (1963). *Biochem. Biophys. Res. Comm.*, **11**, 156–159.
Santi, R., Ferrari, M. & Contessa, A. R. (1964). *Biochem. Pharmacol.*, **13**, 153–158.
Shipley, R. R. & Wilson, C. (1951). *Proc. Soc. exp. Biol. N.Y.*, **78**, 724–728.
Ussing, H. H. (1960). The alkali metal ions in isolated systems and tissues. In *Handbuch der Experimentellen Pharmacologie*, Bd. XIII p. 1–576 Berlin: Springer.
Ussing, H. H. & Zerahn, K. (1951). *Acta physiol. scand.*, **13**, 110–127.
Vescovini, G. & Marro, F. (1960). *Boll. Soc. Ital. Biol. Sper.*, **36**, 1831–1835.

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