SYMPOSIUM*

THE INFLUENCE OF ANIMAL STRAIN SELECTION AND CONDITIONING ON BIOLOGICAL EXPERIMENTS AND ASSAYS

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INTRODUCTION

THE method suggested in the British Pharmacopoeia for the biological assay of pertussis vaccine begins with the words:—"Healthy white mice drawn from a uniform stock . . .". As I have said elsewhere, the possession of a white coat is no more a guarantee of purity in mice than it is in those who use them.

In a paper by Elizabeth Russell, of the Jackson Memorial Laboratory in Bar Harbor, which appeared in the *British Medical Journal* (1955, **1**, 826–829), reference was made to work published in 1929 by Wright and Eaton, in which they listed four types of inbred strain difference. On re-reading the paper I do not think that these are very clear-cut types, but they do indicate that the differences may be *either* of the all or none variety; for example, BALB/c mice have white coats, C57B1 black, and CBA animals agouti: and DBA mice are unlike most other strains in being extremely susceptible to audiogenic seizures; *or* the differences may be graded; for example, the incidence of spontaneous mammary tumours will vary from the highly susceptible C_3H mice to the moderately susceptible DBA/1 and the insusceptible C57B1/6 strain. Gowan and Schneider, working independently in the U.S.A., have shown a grading from strains highly susceptible to mouse typhoid to those that are resistant. Russell in her paper mentions many other differences between strains; the nature of disease (for example, the same infecting organism may produce in one strain septicaemia, and in another an upper respiratory infection, and in another pneumonia): differences in the survival time after an infection; antibody production; cold tolerance; susceptibility to the vapour of chloroform (which will certainly kill male DBA/2 mice; DBA/1 mice are nearly as sensitive; C_3H are less so; BALB/c are less sensitive again; and most other strains are resistant to chloroform. This is a sex-linked difference, in that the females do not readily die from small amounts of chloroform vapour); sensitivity to hormones; content of hormones; reactions to the removal of endocrine glands; enzyme activity; blood picture; and longevity.

As these differences between strains exist also in other species of laboratory animal, why is it that inbred strains are not more commonly used? The reasons that have been advanced against the use of inbred

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strains, I think, are four. (1) It has been said that the differences are not great enough to be of any practical interest. That is not true; the differences in many cases are very large. (2) They are said to be difficult to breed. That again is largely untrue. Many inbred strains have a productivity that compares reasonably well with the productivity of non-inbred strains. Even under the far from ideal conditions that we have at Carshalton, we find that the best of our inbred strains are not much less productive than the best of our non-inbred strains. (3) The technique of inbreeding is laborious, and (4) if colonies of inbred strains are established in different places, genetic divergence will arise between them. These two reasons I think are valid. To maintain an inbred colony, a good deal of technical competence is needed; and divergence does occur.

These difficulties have interested the Laboratory Animals Centre for some years, and we have suggested that if primary colonies of various strains are kept in one place and constantly controlled, genetically and from the points of view of health and specific responses, they are capable of producing a relatively small number of animals, which can be used for limited sub-cultivation to produce the large numbers required for experimental purposes. We have suggested that foundation stock from such primary colonies may be sub-cultivated elsewhere for up to about three generations, and that brother $\times$ sister mating for those three generations can be ignored. To avoid going beyond the three generations which can reasonably be regarded as a useful limit of sub-cultivation, the first generation should be distinguished by putting a green label on the box, the second generation by a yellow label, and the third by a red label. Animals from a red label box should never be used for breeding. We call this "traffic light sub-cultivation", and in practice it works well. It relieves the sub-cultivator of the need to study inbreeding techniques, and avoids tedious and troublesome quality control. The feedback of information about specific responses of mice sub-cultivated in this way would add greatly to our knowledge of inbred strains.

INTRODUCTORY PAPERS

PRACTICAL ASPECTS OF STRAIN VARIATION IN RELATION TO PHARMACOLOGICAL TESTING

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THE control of variation in animal experimentation particularly in the field of bioassay and pharmacological screening is perhaps one of the principal problems confronting the industrial pharmacologist. The application of statistical procedures and design to problems of toxicity and bioassay by Trevan (1927) and Gaddum (1933) pointed the way to refinements in experimental method thereby improving the accuracy of the tests. Further advances in bioassay procedure resulted from the classical research of Fisher who used statistical methods to determine precisely the variation due to different factors within the experiment and then by appropriate design, considering each factor, limited the variation to a minimum. With a view to reducing variation still further a number of industrial laboratories concentrated on the production of inbred lines in the hope that more constant and uniform responses would be obtained. This largely became established practice although little experimental evidence was brought forward to support the belief. Mather (1946) subsequently observed that F_1 hybrids gave more uniform responses and were generally more vigorous than either of the parent inbred strains. These observations were largely overlooked until McLaren and Michie (1954) and Biggers and Claringbold (1954) independently re-discovered this phenomenon. Biggers, McLaren and Michie (1961) feel in general that the F_1 hybrid is the most satisfactory laboratory animal. Chai (1960) has put forward an opposing viewpoint stating that "For the assay of a given substance, the choice of assay animal—either inbred or an F_1 hybrid—cannot be made in advance; it has to be based on existing data or determined experimentally". Brown (1961a, b) has lent additional experimental evidence to support this.

Environmental conditions, however, also play a vital part in biological assay. Chance has carried out extensive investigations into the effects of altering the environmental conditions. These investigations have been admirably summarised by Russell and Burch (1959).

Finally in this respect mention must be made of the work of the Laboratory Animals Centre under the direction of Dr. Lane-Petter and of the Animal Technicians Association who have done so much to improve the general quality and health of the laboratory animals. Without a healthy robust animal all experimental work involving animals, whether pharmacological or bacteriological, can be rendered virtually worthless.

Differences in the physiological response of various strains of mice have been clearly recognised. Elizabeth Russell (1955) in an excellent review of the "Significance of Physiological Pattern of Animal Strains in

Biological Research", has drawn attention to the considerable differences that occur between mice to disease susceptibility, nature of disease produced by a given pathogen, survival time of infected individuals; capacity for antibody production, cold tolerance, reaction to specific toxins; sensitivity to and content of various hormones and reaction to endocrine extirpation; differences in normal blood-cell levels, life-expectancy and pathological pattern. The pharmacologist has been perhaps rather slower to give consideration to strain variation and it is the purpose of this paper to draw attention to the considerable variations which may exist and to indicate how, by correct selection of strain the quality of pharmacological assays may be improved.

Siegmund, Cadmus and Lu (1957) have developed a screening technique for mild analgesic drugs which has gained considerable acceptance among industrial pharmacologists. In this test phenylquinone is injected intraperitoneally to mice and a typical response is produced which can be inhibited by analgesic drugs. Phenylquinone produces a characteristically biphasic response in a number of strains of mice—intermittent contraction of the abdomen, rubbing the abdomen on the floor of the cage and stretching of the hind limbs. Unless an adequate degree of this writhing response is produced, the test gives erratic and non-reproducible results (Hendershot and Forsaith, 1959). Similarly in our own laboratory we were unable to get satisfactory responses and we therefore decided to examine as many strains as possible in order to select the one which gave best writhing response. We obtained eight strains through the courtesy of Dr. Lane-Petter of the Laboratory Animals Centre, Carshalton, and three other strains from commercial sources all of which were examined for their writhing response.

Methods

Random samples of 6 male mice, 5 to 7 weeks old, from each strain were injected intraperitoneally with 2 mg./kg. phenylquinone and the individual reaction of each mouse was recorded for a period of 30 min., noting the cumulative number of writhes at 2 min. intervals. Two mice from each strain were tested on each of 3 days, the strains being randomised throughout the test days. From these results the frequency of writhing during each 2 min. period was computed. The phenylquinone was injected as an 0·2 mg./ml. solution in 5 per cent ethanol and was maintained at 37°, the solution being just below saturation at this temperature. In all tests the technician was unaware of the strain being used. In tests where aspirin was administered it was given orally 50 min. before the phenylquinone.

Strains used:

Pure inbred strains		Random-bred albino strains (nominal designation)
CBA	A2G	
C57BL/6	C57L	ALB1
DBA/2	C_3H	ALB2
C57Br/cd	CE	ALB3

Results

The frequency of writhing in eleven strains of mice is illustrated in Fig. 1, and the total number of writhes per mouse in a 30 min. period following administration of the phenylquinone is shown in Table I. The mice can be divided into three groups: those which show a large number of writhes

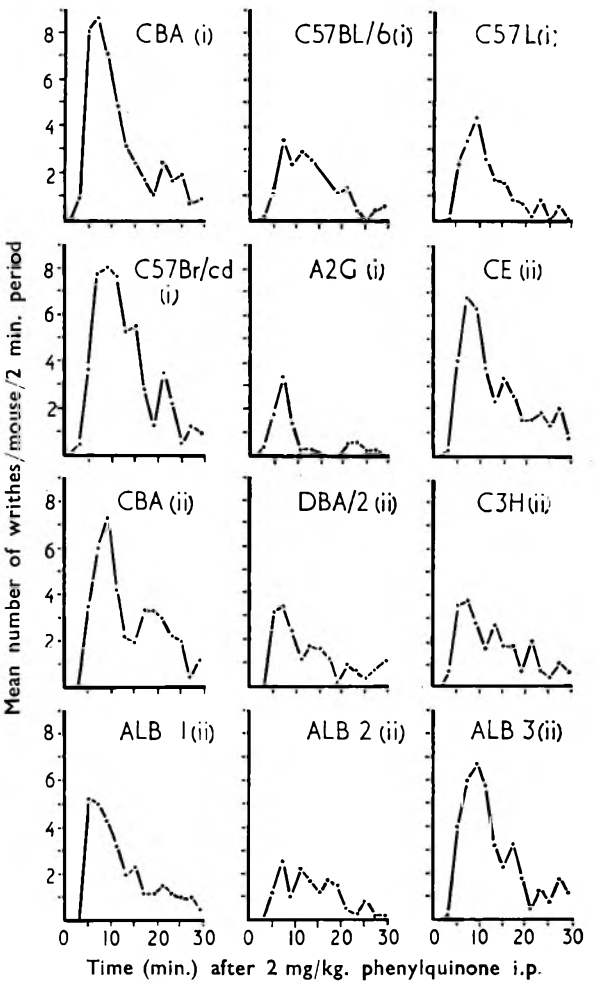

Fig. 1. Frequency of writhing of 11 strains of mice.

CBA, C57Br/cd, CE and ALB3; those which are intermediate in responses C57BL/6, C57L, C_3H and ALB1, and those which give poor responses A2G, ALB2 and DBA/2. The characteristics of the writhes also differ between those groups. In the strain which give a large number of writhes, the syndrome is quite distinct but in the others the phases are less clearly seen, and in the poorly responding animals a writhe may only be detected as a slight contraction of the abdomen or stretch of the limbs.

TABLE I

NUMBER OF WRITHES PER MOUSE IN 30 MIN. FOR EIGHT INBRED AND THREE COMMERCIAL MOUSE STRAINS FOLLOWING INTRAPERITONEAL ADMINISTRATION OF 2 MG./KG. PHENYLQUINONE

Experiment	Strain	Weight range (g.)	Writhes per mouse						Mean
			No. 1	No. 2	No. 3	No. 4	No. 5	No. 6	
(i)	CBA	14–17	38	60	16	90	41	38	47
	C57BL/6	12–15	3	16	49	19	8	22	19
	C57L	21–25	22	21	32	20	1	30	21
	C57Br/cd	15–18	47	17	53	65	49	75	51
	A2G	15–18	8	16	9	9	0	10	9
(ii)	CE	14–19	36	39	0	49	75	34	39
	CE*	13–15	43	40	34	25	33		35
	CBA	21–24	24	54	34	28	65	39	41
	DBA/2	8–14	7	1	20	36	43	14	20
	C_3H	20–23	12	27	55	20	10	21	24
	ALB1	20–23	40	62	5	25	13	32	29
	ALB2	17–19	17	12	7	18	16	19	15
	ALB3	15–19	39	18	33	77	43	24	39

* Litter mates.

Individual variation in the number of writhes within groups is also high even in mice which respond well but this does not detract from using the phenylquinone test to give a graded response with analgesic drugs. Records of the writhing frequencies of batches of five mice from one strain (ALB3) show that 15 mice will give a mean number of writhes of 129 $\pm$ 11·0 S.E.M. For a more accurate test the use of litter mates would obviously reduce this error still further (Table II) but this would generally be impracticable in a screening programme.

TABLE II

MEAN NUMBER OF WRITHES AND A COMPARISON OF THE COEFFICIENTS OF VARIABILITY AT 20 MIN. AND 30 MIN. IN RESPONSE TO INTRAPERITONEAL ADMINISTRATION OF 2 MG./KG. PHENYLQUINONE, IN DIFFERENT MOUSE STRAINS

Experiment	Strain	20 min.		30 min.	
		Mean no. of writhes	Coefficient of variation	Mean no. of writhes	Coefficient of variation
(i)	CBA	39·0	51	47·2	53
	C57BL/6	16·3	85	19·5	83
	C57L	18·7	58	21·0	55
	C57Br/cd	42·7	42	51·0	39
	A2G	7·3	54	8·7	59
(ii)	CE	31·7	62	38·8	63
	CE*	28·8	22	35·0	20
	CBA	31·8	38	40·7	39
	DBA/2	15·8	79	20·2	81
	C_3H	19·3	65	24·2	68
	ALB1	24·3	73	29·5	69
	ALB2	12·8	34	14·8	31
	ALB3	33·3	51	39·0	54

* Litter mates.

The phase of most frequent writhing is complete in 20 min. although the mice continue to writhe for some time afterwards but at a much reduced rate. No significant difference was found between the estimates of the variability of the number of writhes in 20 min. and 30 min. periods, therefore it is unnecessary to count for more than 20 min.

In order to verify that mice obtained from one source would still retain their writhing characteristics even when reared in a different laboratory, breeding pairs of CBA and C57BL/6 were obtained from the Laboratory Animals Centre. The first strain gave a good writhing response and the second an intermediate response. We raised sufficient numbers to repeat the tests, the results of which are shown in Fig. 2. The curves of the second test overlap precisely the initial curves obtained with both strains.

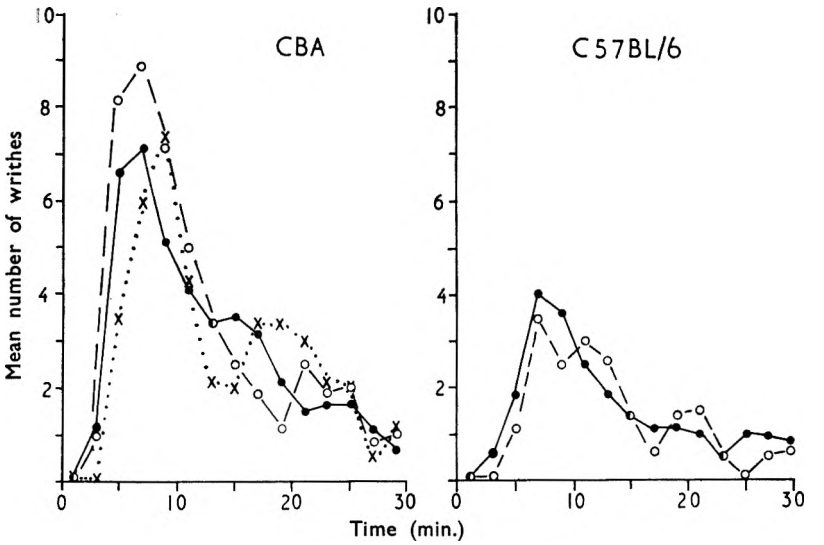

FIG. 2. Frequency of writhing of two strains of mice ○ – – ○ March, 1961 (6 mice); × – – × April, 1961 (6 mice); ●—● November, 1961 (30 mice).

Finally having shown that a mouse strain writhes consistently and then having selected a strain which gives an adequate degree of writhing, it is also necessary to confirm that the animals continue to give the same response to the analgesic drugs (Fig. 3). The dose response line obtained

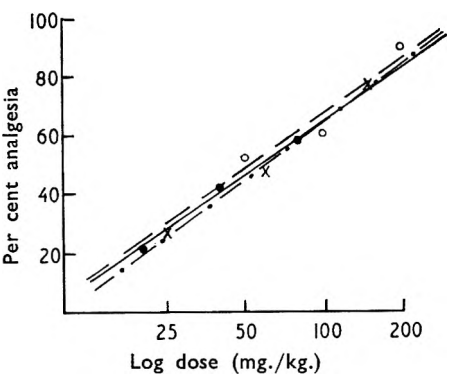

FIG. 3. Single day analgesic assays of aspirin, 15 mice/dose. ●—● 9.12.1960; ○ – – ○ 6.12.1961; × – – × 11.12.1961.

on 9/12/60 for the mean writhing response to graded doses of aspirin for the 20 min. period following phenylquinone was compared with the line obtained exactly a year later (6/12/61) using the same strain of mouse. The two lines were found to be in very close alignment. A further dose response line prepared 5 days after the last test (11/12/61) gave a slope which was also virtually identical to the other two. The ED50 values for each test were respectively 58, 54 and 60 mg./kg. Mice which responded poorly to the phenylquinone failed to give a linear response to aspirin.

Discussion

By consideration of the genetical, physiological and environmental background of the strain of animal used in assay and screening procedures the quality of a test can be considerably improved with a corresponding reduction in error variance. The sensitivity and accuracy of tests can be enhanced further by the selection of the strain of animal which gives the best pharmacological response. It is not uncommon that many pharmacological testing procedures are acceptable to one laboratory and not to another. The reason for these discrepancies can largely be found in the strains of animal used in the different laboratories. The results with phenylquinone writhing response indicate that there is a large variation between mouse strains, probably much larger than hitherto suspected, and that some strains give such poor responses that it is impossible to get a quantitative response to mild analgesic drugs. The expediency of using a selected strain which gives a high degree of writhing in the phenylquinone test is demonstrated by the consistency in the dose response lines obtained with aspirin and in the comparatively low doses of mild analgesic drugs required to achieve a pronounced and consistent reduction in the frequency of writhing. Additional support to this is provided by Dr. P. F. D'Arcy who tells us that, by using a similar strain of mouse and employing identical assay procedure, he has obtained ED50 values, mean 52 mg./kg. for aspirin. These figures which were obtained independently agree remarkably with our own.

Whilst it is not generally practicable to screen a wide selection of strains before performing every pharmacological test, this would be advantageous when establishing a particular long term programme for the screen or assay of a particular series of drugs. In any pharmacology laboratory, it would be useful to have available two to three strains of mouse of widely differing characteristics (possibly one or two home strains supplemented by commerical stocks), so that should any test appear not to be satisfactory, although other workers have found it to be so, the test could be performed in different strains. Certainly we would advocate that coloured mice be more widely used in pharmacology as well as albino mice instead of the present almost exclusive use of the latter.

With some species, the choice of strain is naturally more limited than with the mouse and it would not be practicable, let alone economical, to screen a variety of strains. Nevertheless, strain variation should be borne in mind as of practical importance to an experiment rather than simply theoretical, particularly when wide discrepancies of results appear between

work performed in different laboratories. It should not, however, become the scapegoat for all unsatisfactory results.

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STRAIN VARIATION IN MICE

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Everyone reading these papers already knows that there are a number of uniform strains of mice maintained in Great Britain. Of these the Laboratory Animals Centre maintains, according to the Catalogue of Uniform Strains, nine inbred and one random bred strain and in practice has one or two more strains that have been acquired recently. It has been my task for the past 3 years to investigate the differential characteristics of some of these strains of mouse.

Preliminary work on the response of mice from a number of our strains to histamine acid phosphate, after sensitising with pertussis vaccine and to insulin, after 24 hr. deprivation of food, gave the results shown in Fig. 1.

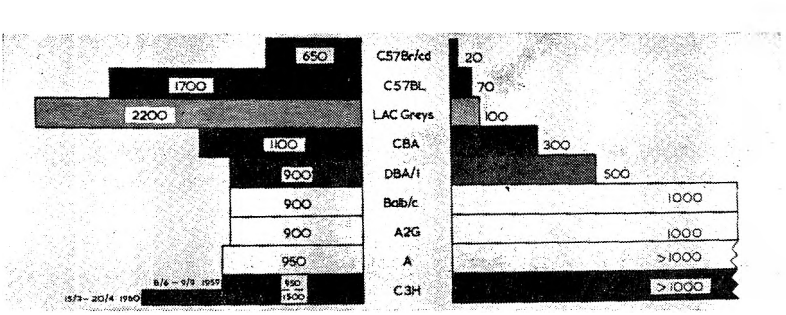

Fig. 1. Comparative sensitivities of some mouse strains for insulin and histamine acid phosphate.

In this work I used approximately equal numbers, usually between 20 and 30, of each sex of mouse, from 6 to 8 weeks of age. I estimated the approximate LD50 and ED50 of each strain by probit plots, and or Karber's method, and the histograms show the average obtained for two or three tests. It was not determined whether the difference between the ED50 of the two samples of C_3H mouse was significant. Two further points of interest should be noted from the figure. The first is that the C57Br/cd mice are the most sensitive for both responses, and the second that the three white strains of mouse resemble one another in each response, possibly due to their derivation.

The lack of relation between the order of magnitude of these quantal responses in any one strain of mouse has been the stimulus for more extensive work. This has been done using strains of mouse and their F_1 hybrids in a study of quantal response to insulin and of quantitative response to pentobarbitone sodium. Experimental conditions were arranged to be the same for all mice used on any one day. Not more than 5 or less than 4 mice were allowed to react to insulin in one jar, and not more than 5 mice were housed in one box between tests of response to

pentobarbitone sodium. The animal room was maintained at a temperature of 70° ± 2° F. The work on both these responses has defined the necessity for using mice of known weight, sex and age when investigating differential characteristics.

In considering weight of mouse in the quantal response to insulin, significant, or closely approaching significant correlations were found of the combined results for ED50, and of the mean slopes with mean strain weight, so that a rough assessment of the reaction of a strain to this response could be made from mean strain weight. In the response to pentobarbitone sodium, for one strain of mouse, the correlation of sleeping time with weights of mouse was such, that it seemed reasonable to adjust the mean sleeping times for body weights by co-variance analysis for all strains used.

TABLE I

COMPARISON BETWEEN THREE HYBRID STRAINS OF MICE FOR DIFFERENCE IN PATTERN OF RESPONSE AND IN ED50

P = probability D = difference

Strain	Pattern of response $BrA2F_1$ > $A2CF_1$ and $CA2F_1$		Mean ED50
	P	D	
$BrA2F_1$			885
$A2CF_1$	0·02	+	1,190
$CA2F_1$	0·001	+++	1,100

$BrA2F_1$ = C57Br/cd (F) × A2G (M).
$A2CF_1$ = A2G (F) × CBA (M).
$CA2F_1$ = CBA (F) × A2G (M).

Sex effect in the response to insulin is apparent from the significant qualitative difference in the pattern of response within certain strains between males and females. Pattern of response between strains is also significantly different, and I postulated for the LAC grey mice a relation between sensitivity and pattern of response. This is now confirmed for the F_1 strains in Table I and between the sexes of the F_1 strains in Table II.

TABLE II

COMPARISON BETWEEN THE PATTERN OF RESPONSE AND THE MEAN ED50 FOR MALES AND FEMALES IN FOUR HYBRID STRAINS

P = probability D = difference

Strain	Pattern of Response M < F		Mean ED50	
	P	D	M	F
$A2BrF_1$	0·2	—	999	946
$BrA2F_1$	0·7	—	882	877
$A2CF_1$	0·1–0·001	+++	1,230	973
$CA2F_1$	<0·001	+++	1,142	1,014

$A2BrF_1$ = A2G (F) × C57Br/cd (M). $A2CF_1$ = A2G (F) × CBA (M).
$BrA2F_1$ = C57Br/cd (F) × A2G (M). $CA2F_1$ = CBA (F) × A2G (M).

An interesting negative confirmation is given in Table III. It is not proven that an unequal balance of sex in the mice used for this response

would affect the results of an assay, but it is significant that for all the F_1 strains in Table IV the male mice have the greater slope of regression between dose and response. The hypothetical strain of mouse having male and female slopes equal to the average slopes for the F_1 strains and the variance of the difference calculated from the F_1 strains variance, has a significant difference between male and female slopes. For the response to pentobarbitone sodium a sex difference develops with age, showing that for such reactions a knowledge of both the age and sex of the animals is important.

TABLE III

COMPARISON BETWEEN THE PATTERN OF RESPONSE AND THE MEAN ED50 OF THE HYBRID A2DB/1F_1, AND ITS PARENTS

P = probability D = difference

Strain	Pattern of response A2DB/1F_1 = A2G and DBA/1		Mean ED50
	P	D	
A2DB/1F_1			890
A2G	0·05–0·1	—	885
DBA/1	0·05–0·1	—	875

TABLE IV

SLOPES OF THE RESPONSE TO INSULIN FOR THE MALE AND FEMALE MICE OF FOUR F_1 HYBRID STRAINS

Strain	Mean slope M	Variance M	Mean slope F	Variance F
A2BrF_1	1·597	0·789	1·105	0·267
BrA2F_1	3·455	0·975	1·996	0·140
A2CF_1	2·871	0·342	1·784	0·586
CA2F_1	1·453	0·310	0·545	0·318
Average	2·344		1·358	

Hypothetical strain of mouse comprising four hybrid strains.
Significant difference M slope > F slope 0·02 > P > 0·01.

Having settled that animals of known weight, sex and age must be used, it is possible to view general strain differences. The strain difference in sensitivity to insulin has already been seen in Fig. 1. The strain difference in the precision of this response is shown in Table V. It is important that no significant within strain difference in parallelism was found, but between strain differences were apparent and significant when within strain variance was low. Of the hybrid strains the crosses between the A2G and CBA strains were interesting in that each resembles its maternal parent more closely. That between DBA/1 and A2G strains is interesting in that it resembles neither parent. A correlation of ED50 and slope of regression for this response that has been previously published brings out clearly the genetic relation between the strains of this species (Fig. 2). With the quantitative response to pentobarbitone sodium it was necessary

to consider not only the weights, sex and age of the mice but also the homogeneity of the variance of their response. The heteroscedasticity of the groups was such that significant difference was defined at a level of

TABLE V

SUMMARY OF THE COMPARISON OF THE PARALLELISM OF THE REGRESSION BETWEEN THE CONVULSIVE RESPONSE AND THE DOSE OF INSULIN BY MEANS OF THE χ^2 summation for FIRST TEST ONLY

P = probability D = difference

Strains	No. of tests	Mean slopes	Within strain comparison		Between LAC grey mice and other strains	
			P	D	P	D
Inbred						
A2G	6	1·929	0·5	—	0·01–0·001	+++
A2G	2	1·962	0·1–0·2	—	0·1–0·2	—
CBA	3	0·905	0·1–0·2	—	0·2–0·3	—
DBA/1	2	2·842	ca 0·5	—	0·02–0·05	+
Hybrid						
$A2CF_1$	3	1·815	0·2–0·3	—	0·1–0·2	—
$CA2F_1$	3	0·985	0·8–0·9	—	*ca* 0·99	—
$BrA2F_1$	3	1·825	0·99	—	*ca* 0·05	+
$A2BrF_1$	2	1·625	0·2–0·3	—	0·7–0·8	—
$A2DB/1F_1$	2	1·283	*ca* 0·8	—	0·99	—

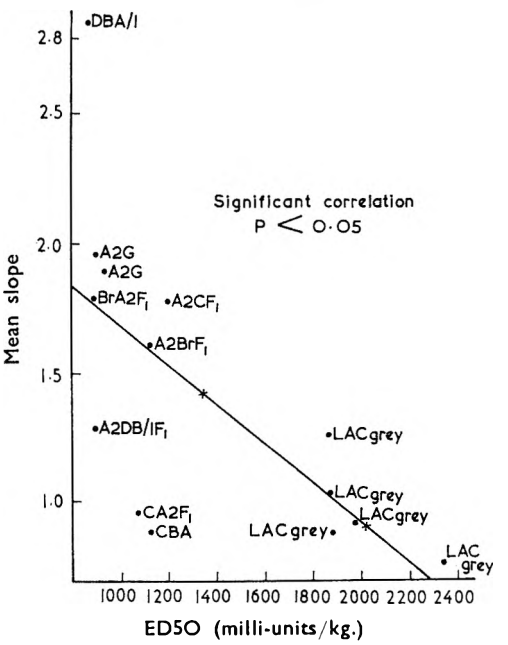

FIG. 2. Correlation of ED50 and mean slope of the regression in some strains of mouse.

probability 0·001. Fig. 3 shows the differences obtained in sensitivity and precision for some inbred and hybrid strains as compared with the

A2G strain. There is some indication that for this response both sensitivity and precision in parent strains are reflected in these characteristics of the hybrid strains.

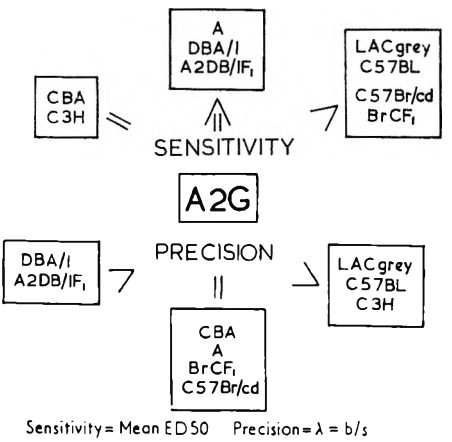

FIG. 3. Comparison of the sensitivity and precision of the response to pentobarbitone sodium in the A2G strain with that in other uniform strains of mouse.

In conclusion it may be said that uniform strains of mice vary significantly in their quantal and quantitative responses. Both the order of sensitivity of these responses and their precision must be determined empirically for each strain, in mice with known weight, and of similar age and sex.

THE CONDITIONING OF EXPERIMENTAL ANIMALS

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The conditioning of small laboratory animals for experiments, based upon the abolition of a conditioned reflex, is standard practice in the screening or evaluation of tranquillising drugs. However, it is less appreciated that other tests may benefit from the use of pre-conditioned animals. In this communication four experimental procedures are discussed in which conditioning of the animals brought about a marked improvement in the experimental results. The four procedures are: (1) Test for pyrogens (British Pharmacopoeia 1958). (2) Collection of urine in a urino-faecal separator (Brittain, 1959). (3) Studies on the acute toxicity of amphetamine in crowded and non-crowded conditions (D'Arcy and Spurling, 1961). (4) The assessment of purgative activity (D'Arcy, Grimshaw and Fairbairn, 1960).

Test for Pyrogens

The test is made as described in the British Pharmacopoeia. White Himalayan rabbits of either sex and of body weight 2·0–2·5 kg. are used (Allen & Hanburys breeding colony). The rabbits are restrained in stocks and a record is made of rectal temperature before, and at specific intervals after, the intravenous injection (marginal ear vein) of the test solution. Experience from testing over some 15 years has shown that the correct performance of the test depends on conditioning the animals to become accustomed to the various procedures involved in the test, before they are used routinely.

During the conditioning or training period which is virtually a "dummy" pyrogen test the rabbits are handled by the operators, allowed to become used to the testing laboratory and the restraining cages and also to experience the insertion and retention of the rectal thermocouple. The animals also receive an injection of a known non-pyrogenic sample of normal saline and thus indirectly become accustomed to the technique used by the operators to engorge the marginal ear vein. It is normal practice to introduce the rabbits into routine use after two or three training periods.

Table I illustrates the importance of using pre-conditioned rabbits in the pyrogen test; in this experiment, 12 rabbits which had already been conditioned to the pyrogen test procedure and 12 rabbits that had not been conditioned were used; the rabbits were similar in all other respects. The rabbits were subjected to a pyrogen test using a known non-pyrogenic solution of normal saline. Table I lists the maximum fluctuation in rectal temperature for each rabbit, that is the greatest difference between rectal temperatures before injection 30 min. after each animal was secured in its stock, and any subsequent temperature recorded after the injection of the test solution. It is evident that the mean fluctuation in temperature

for the non-conditioned group of rabbits is significantly larger ($P < 0.01$) than that of the conditioned animals.

TABLE I

THE EFFECT OF CONDITIONING RABBITS ON THE MAXIMUM FLUCTUATION IN RECTAL TEMPERATURE (+ or −°F) DURING A PYROGEN TEST*

NON-CONDITIONED		CONDITIONED	
Rabbit	Maximum fluctuation in rectal temperature (+ or −° F)	Rabbit	Maximum fluctuation in rectal temperature (+ or −° F)
1	2·6	1	0·7
2	2·1	2	0·6
3	1·4	3	0·1
4	0·8	4	0·2
5	0·3	5	0·3
6	0·4	6	0·2
7	0·6	7	0·4
8	0·3	8	0·4
9	1·3	9	0·6
10	0·8	10	0·4
11	2·2	11	0·7
12	0·9	12	0·4
Mean	1·14	Mean	0·42
S.E.	±0·22	S.E.	±0·06

Significance of difference between mean values $P = <0.01$.
* Samples of non-pyrogenic saline injected.

A large fluctuation in rectal temperature, especially if negative, may easily obscure a pyrogenic reaction, or, if positive, may give a false pyrogenic result. During a follow up of the experiments cited in Table I it was observed that a group of non-conditioned rabbits failed to show pyrogenicity in a test solution, which was classified pyrogenic when tested in conditioned rabbits.

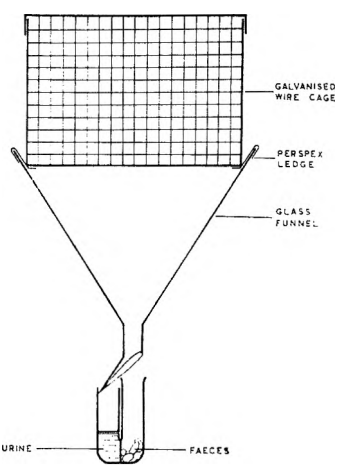

FIG. 1. A simple urino-faecal separator for use in metabolism experiments.

Collection of Urine in a Urino-faecal Separator

The collection of urine free from contact with faecal material is essential in many metabolic studies on laboratory animals. A simple urino-faecal

separator designed for rat experiments by Brittain (1959) has been found suitable (Fig. 1).

In routine tests a group of 5 male rats (Tuck strain, 100–120 g. body weight) is placed in the metabolism cage; food and water are withheld during the test. Urine flows to the tip of the glass funnel and collects in the left limb of the receiver; faecal pellets drop directly down the funnel into the right limb.

It is necessary to condition rats to the apparatus; rats without previous experience of the restraint of the separator, excrete very soft to semi-liquid faeces which either block the neck of the funnel or alternatively wash down with the urine into the receiver. Rats are normally conditioned by dosing them orally with tap water (5 ml./kg.) and placing them in the apparatus for about 6–8 hr. on two consecutive days, resting them for a day and then using them for experiment on the fourth day. Conditioning rats in this manner has given good experimental results.

The Acute Toxicity of Amphetamine in Crowded and Non-crowded Conditions

Chance (1946) showed that amphetamine is far more toxic to mice kept under crowded conditions than to mice housed singly. Whilst studying the importance of increased adrenocortical secretion in crowding toxicity (D'Arcy and Spurling, 1961), difficulty was experienced in establishing a constant difference between amphetamine toxicity in crowded and non-crowded mice.

The mice used for these experiments (male mice, Tuck strain, 20–25 g. body weight) were delivered from the dealer in boxes of 100, 7 days before the test. Between delivery and test, the mice were kept in cages of 50 in a laboratory at 68°–70°F.

The absence of a constant difference between the acute toxicity of amphetamine in crowded and in non-crowded mice in consecutive experiments was thought to be due to previous crowded conditioning of the mice, either during delivery from the dealer or during housing while awaiting test. Therefore on arrival, 7 days before the test, mice were kept in groups of 5 until the day of the test. This conditioning, or rather abolition of a conditioning produced by previous crowding, was successful and it was possible to demonstrate that increased adrenocortical secretion was a factor in causing crowded toxicity to amphetamine (D'Arcy and Spurling, 1961).

Assessment of Purgative Activity

A simple test for assaying purgative substances using mice has been developed by D'Arcy, Grimshaw and Fairbairn (1960), and is based upon a modification of a method described by Lou (1949).

Groups of 10 male mice (Tuck strain, initial body weight 18–20 g.) are dosed orally with a test or standard preparation; they are housed in pairs on a wire mesh grid in a small compartment of a large perspex cage, which is placed over a sheet of blotting paper. At specific time intervals (3, 6 and 24 hr.) after dosing the number of wet or unformed faeces is counted

for each pair of mice, and the total is related to the dose of purgative administered. Unformed faeces stain the blotting paper and are thus easily distinguished.

Conditioning of the mice before routine testing greatly improves both the precision and accuracy of the assay. This conditioning involves subjecting the mice, at weekly intervals, to the assay procedure in the containers in the testing laboratory for the normal duration of the assay for 2–3 weeks after which time the mice are used for routine assays.

TABLE II

THE EFFECT OF CONDITIONING MICE ON THE PRECISION AND ACCURACY OF A PURGATIVE ASSAY*

Week of conditioning period	Known relative potency	Estimated relative potency	95 per cent fiducial limits per cent	λ (s/b)
2	0·80	0·70	73–137	0·13
3	0·75	0·88	78–128	0·11
4	0·80	0·77	82–122	0·09

* A sample of senna extract was assayed against known dilutions of itself; all assays were performed using a 2 × 2 design with 5 pairs of mice per dose, i.e. 40 mice per assay.

Table II shows the effect of the conditioning procedure on the precision and accuracy of a purgative assay. In the experiment a sample of senna extract was assayed against a known dilution of itself on the same group of mice but at different times during their conditioning period. As conditioning progressed the estimated potency approached the actual potency, the fiducial limits narrowed and the index of precision (λ) became smaller.

TABLE III

THE EFFECT OF CONDITIONING MICE ON THE PRECISION AND ACCURACY OF A PURGATIVE ASSAY*

Week of conditioning period	Known relative potency	Estimated relative potency	95 per cent fiducial limits per cent	λ (s/b)
0	3·98†	2·26	Invalid‡	—
1	3·98	4·43	62–161	0·21
2	3·98	5·30	61–164	0·19
3	3·98	3·23	Invalid‡	—
5	3·98	4·09	76–131	0·12
7	3·98	3·77	74–136	0·14
9	3·98	4·26	65–153	0·19
11	3·98	4·97	68–146	0·20

* A sample of sennoside A was assayed against a standard senna extract; all assays were performed using a 2 × design with 5 pairs of mice per dose, i.e. 40 mice per assay.
† Mean of 10 assays using conditioned mice; 95 per cent fiducial limits 95–105 per cent.
‡ Dose-response lines of test and standard preparations deviated significantly from parallelism.

In a further series of experiments (Table III) a group of mice were used for 11 consecutive weeks in an assay relating the potency of sennoside A to that of a standard senna extract. It is again evident that, with conditioning, estimated potency approached known potency, and that the fiducial limits narrowed and the index of precision (λ) decreased.

However, the results also showed that too long a conditioning period had detrimental effects; a period of 5 weeks would seem to be the optimal period for using the mice. After this the fiducial limits widened and the index of precision (λ) increased.

Conclusions

In the four experimental techniques described, the value of conditioning the animals before experimentation has been established. It may well be that there are many other accepted procedures that could also benefit from using conditioned animals. The results obtained in the purgative assays indicate that, in specific instances, too rigorous a conditioning procedure can be detrimental to the results of the experiment. In addition, as shown in the amphetamine experiments, it may be necessary to abolish a pre-conditioned state in the animal, a state that may inadvertently be produced by normal handling, transport or housing.

References

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DISCUSSION

CHAIRMAN. I would like to clear up the question of terms. An "inbred strain" has been defined, certainly in relation to mice, as the result of not less than 20 generations of continuous brother × sister mating, or in exceptional circumstances of parent × offspring mating, to produce the maximum degree of homozygosity.

"Haphazard breeding" is defined as breeding simply by selection of suitable animals. "Random bred" can be used to mean haphazard breeding in that sense, but recently it has taken on a more precise meaning. The progeny from a colony maintained at a constant size, when mated, are deliberately randomised in order to achieve a constant relationship. This might be better described as "randomised bred".

"Healthy white mice drawn from uniform stock" is a meaningless phrase, because what is meant by healthy? The pathogen burden of conventional colonies is not uniformly distributed throughout the colonies. There is, therefore, an unpredictable variation in the pathogen burden between individuals, and this pathogen burden may, even without overt disease, vitiate certain types of experiment. Consequently there have been developed colonies which are called specific pathogen-free—SPF; animals that have been derived by caesarian section, thus breaking the chain of infection from mother to offspring in all infections which are not placentally transmitted. SPF animals are not the same as germ-free, because they live in a more or less conventional environment. In this country, I.C.I. at Alderley Park have developed a big colony of this kind, which is proving extremely useful.

Much trouble is entailed in achieving SPF conditions for animals, but the difficulties are often exaggerated.

DR. G. F. SOMERS. There are problems which arise out of careful selection and inbreeding of animals. When a manufacturer has assessed a new and potentially valuable drug he then has to investigate its possible toxicological properties. This is done with strains of animals which are as alike as possible, while with man, breeding is very haphazard and there is considerable variation between individuals. Therefore it is not surprising that when a drug is subsequently given to man we find that certain sections of the population are sensitive to toxic effects which have not been observed in the animals.

Also when a drug is stated to have an LD50 of so many mg./kg. this applies to that drug tested on a particular group of animals in a particular laboratory, and it is not surprising if the results are not always reproducible elsewhere. Dietary factors also play a part.

Are we misguiding ourselves by using mice for toxicological studies which are all alike, and thus failing to observe idiosyncracies, and do we need a standard strain of mice to which to refer our toxicity results?

Lastly, the official books give standard tests for freedom from undue toxicity, and they say, for example, "give 1,000 units intravenously to 5 mice and none shall die". The response may well depend on the strain of mice used, and when this occurs it may give rise to controversies between manufacturers and their customers.

DR. F. J. C. ROE. Dr. Somers has pointed out that random-bred animals are, in some circumstances at least, very useful. I agree. Basically there are two different ways in which experimental animals are used: firstly, in the assay of drugs for a kind of activity which they are known to have, and secondly, in screening a series of drugs to see whether they possess particular types of pharmacological activity. For the former purpose, it is advantageous to have as standard an animal as possible, and one that is sensitive to the drug in question and can respond in a quantitatively assessable fashion. In this instance an inbred strain, or a first generation hybrid between two inbred strains, is clearly the animal of choice. But for screening, truly random-bred animals may have their advantages. By using a suitably sized group of such animals one may hope to cover the extremes of susceptibility and resistance to all kinds of drug action. In theory it is possible to cover the same range by having in each test group, mice from several inbred strains. This would be a far more complicated procedure, and there would be no guarantee that both ends of the range of susceptibility were covered.

At the Chester Beatty Research Institute we are concerned with screening procedures of two kinds. Firstly, we screen drugs for anti-tumour activity. The use of transplantable tumours in animals for screening potential cancer chemotherapeutic agents is described in detail in the first report from the Cancer Chemotherapy National Service Centre. (See *Cancer Chemotherapy Reports*, 1959, **1**, 42–104.)

The second type of screening involves the testing of chemical and other agents for carcinogenic action. For this purpose, outbred stock animals are used. In any one group of test mice, for example, all shades from jet black to snow-white may occur. I have not been at the Institute long enough to know whether this is among the best ways of testing for carcinogenic activity, but certainly many carcinogens have been discovered by this means.

Tables I and II overleaf (see also Roe, F. J. C., Rowson, K. E. K. and Salaman, M. H., 1961. *Brit. J. Cancer*, **15**, 515) show the result of injecting 30 μg. of 9,10-dimethyl-1,2-benzanthracene in aqueous gelatine into newborn mice of two inbred strains, CBA and "101". The two strains were chosen in the light of Salaman's previous observation that adult mice of the "101" strain were highly sensitive, and those of the CBA strain only very weakly sensitive, to the carcinogenic effect of dimethylbenzanthracene plus croton oil applied to the skin (Salaman, M. H., 1956, *Rep. Brit. Emp. Cancer Campgn.*, **34**, 194). The results of the present test on the whole confirm Salaman's view of the relative sensitivity and resistance of the two strains to carcinogenic activity. But it is interesting that the difference in sensitivity to malignant lymphoma was expressed not so much in terms of the overall incidence (20 per cent in "101"s as against 15·4 per cent in CBA's) but more in terms of the length of the tumour induction period (average of 16·5 weeks in "101"s against an average of 26 weeks in CBAs). Again the incidence of lung tumours in the two strains at one year was similar but the largest tumours present in the "101" mice were significantly larger than those in the CBA mice.

TABLE I

CARCINOGENIC EFFECT OF 30 μG. OF 9,10-DIMETHYL-1,2-BENZANTHRACENE ADMINISTERED TO MICE SUBCUTANEOUSLY IN AQUEOUS GELATINE SUSPENSION DURING THE FIRST 24 HR. OF LIFE: COMPARISON OF EFFECTS IN "101"-STRAIN AND CBA-STRAIN MICE

Treatment	Mice injected	Deaths before weaning at 1 month	Deaths between 1 month and 1 year	Deaths from malignant lymphoma	Average induction time of malignant lymphoma	Deaths from other types of malignant disease	Deaths from non-cancerous conditions	Cause of death unknown (because of decomposition)
Dimethylbenzanthracene in gelatine	44	5	18	6	26 weeks	2	3	7
Gelatine only	44	17	1	0	—	0	1	0
None	49	10	0	0	—	0	0	0
Dimethylbenzanthracene in gelatine	57	12	19	9	16·5 weeks	2	3	5
None	47	3	4	0	—	0	1	3

TABLE II

INCIDENCE OF TUMOURS IN CBA AND "101"-STRAIN MICE INJECTED WITH 30 μG. 9,10-DIMETHYL-1,2-BENZANTHRACENE WHEN NEWBORN AND KILLED AT ONE YEAR

Treatment	Survivors at 1 year	Average number of lung adenomas per mouse	Average diameter of largest adenoma (mm.)	Average number of papillomas of the forestomach per mouse	Number of mice with miscellaneous tumours*
Dimethylbenzanthracene in gelatine	21	12·2	1·8†	0·1	6
Gelatine only	26	0	—	0	1
None	39	0·16	1·2	0	2
Dimethylbenzanthracene in gelatine	26	13·1	3·4†	3·2	9
None	40	0·25	1·3	0	1

† This difference was significant: $P < 0.01$.
* Including tumours of liver, kidney, ovary and haemangiomata of various sites.

If, in the experiment described, either strain of mouse had been used by itself as a screen for the carcinogenic activity of dimethylbenzanthracene, a clear positive result would have been scored. Moreover, experience leads one to the view that, although mouse strains differ widely in their sensitivity to carcinogenic agents, these differences are not usually absolute. Sometimes apparent resistance may be overcome by lengthening the exposure time to, or by giving a higher dose of, the test carcinogen.

Dr. Lane-Petter has clearly and, for the most part, rightly recommended the wider use of mice of inbred strains. But I doubt whether the use of pure line mice will materially increase until such animals are obtainable commercially. The regular supply of pure-line breeding nuclei from the Laboratory Animals Centre under the so-called "traffic light system", is a splendid service; but it is of little value to laboratories who have no facilities for breeding the animals they need. Surely it is for the users of experimental animals to agree what is needed, and then make sure that existing, or new, commercial breeding establishments provide the type and quality of animal required.

There is little point in attempting to obtain genetically pure animals, if the desirability of uniformity in environmental factors is not also considered. In particular, our knowledge of animal diets is inadequate. Diets such as 41B for mice have been developed haphazardly, from the results of comparisons of various unlikely concoctions of inconstant ingredients such as Sussex ground oats, fishmeal or cod liver oil. We are a long way from knowing what an ideal mouse diet is, and even further, it seems, from getting a reliable supply of such a diet. For instance, what do we know about the presence or concentration of oestrogens or antibiotics in animal diets? Who has measured the content of individual vitamins after cubing or after storage, and compared it with the theoretical content based on the levels present in the ingredients of the diet?

Unfortunately no individual firm or laboratory by itself has the resources to solve such problems. There is surely an opportunity at meetings such as this to draw up schemes for tackling them on a co-operative basis.

Bedding also deserves consideration. For a very long time we have been supplied with sawdust or wood shavings in old and often dirty sacks. At last, as a result of a concerted effort, bedding can be obtained in hygienic paper bags.

The main bar to the wider use of inbred lines of animals is the impossibility of obtaining them commercially. Possibly the only way of obtaining animals, food, and bedding of high standard, is for the users to agree minimum standards, undertake joint research particularly on animal diets, and then, unanimously, demand higher standards from commercial suppliers.

DR. M. R. A. CHANCE. It was reported some time ago by Gruneberg (*Nature*, 1954, **173**, 674), that the genetic propensity of particular strains of mice to possess an increased number of vertebrae in the spine is dependent on their eating the right food. This means that the right food

must be available in the environment, and the mice must eat it, before a genetic propensity can develop.

With regard to physiological variables, we have found a maternal effect of considerable proportions in the variance of sleeping time of a CBA/CE cross to quinalbarbitone sodium, confirmed by the results of Dr. Annie Brown. Each of these pure lines are more sensitive to changes of number of mice in the group than is the first cross, whichever way round it is made. Thus the first crosses show a low variance whether kept singly, two or eight in a cage, whereas the pure lines respond to isolation and to crowding with eight in a cage with a very high variance. Low variance of the pure lines is only possible if they are kept in pairs, when the variance is of the same order as that obtained with a first cross. This is an interesting interrelationship of the environmental and genetic factors controlling variance. (See Mackintosh, J. H., 1962, *Nature*, Lond., in the press).

The word "training" is being used to define not only specific procedures for obtaining a particular response from an animal, but also procedures for familiarising animals with a given situation. Familiarisation of the animals with the cage is the essential feature for producing dry faeces.

I wish to point out, however, that it is being too readily assumed that the effect of small numbers is to allow habituation to cage mates, just as the habituation to a new cage takes place. Evidence will be presented elsewhere on this point.

Now consider the effect of numbers on variation. The fact that isolated animals of inbred strains are much more variable than paired animals, and similarly that large numbers of animals, that is to say, eight per cage, are much more variable than paired animals, suggests that in both isolated and crowded conditions there is some element of distortion in the behaviour of the animals. This is of a different kind in the two situations, for the isolated mice are deprived of social stimulus, whereas crowded mice are receiving a different kind of social stimulus from those caged in small numbers. Hence we may expect that a different pattern of behavioural responses underlies what appears as an increase in variance in both conditions when one physiological or pharmacological response alone is being examined, rather than a whole pattern of motor outflows which constitutes the behaviour.

I would like to ask the industrial pharmacologists whether in routine assay procedure they could control variance and increase precision in bioassays by using two animals per cage.

Evidence has been brought forward at this meeting that keeping animals in fives, which might be described as the upper limit in numbers for a small group for small rodents, reduces the variance in subsequent assay procedures, compared with keeping them in larger numbers. It has been found that in the gonadotrophin assay, the quinalbarbitone anaesthesia test, in the insulin assay and in the gastric acid secretion, that two animals per cage is the one condition which produces minimal variance.

Keeping mice or rats in smaller numbers per cage must mean increasing the amount of space required for preparing animals for assay and keeping

them during the assay, provided the same number of animals are employed, but the reduction in variance means that a proportionate reduction in numbers used for the assay is achieved. If, for this reason it is worth reducing the numbers to five per cage, would it be worth still further reducing the numbers, and keeping the animals in pairs? Our experience suggests that a material reduction in variance would occur by keeping animals in pairs rather in fives, and it then might become possible to keep these pairs in much smaller cages, hence bringing about a reduction in the total caging area required.

DR. E. M. GLASER. There seems to be a need for more precise definition of the terms "learning", "conditioning" and "habituation". Learning is a qualitative change of responses, or the development of new responses which were not previously available. In conditioning, the response is constant, but the stimulus changes. In habituation, there is no qualitative change of stimulus or of response, only a gradual quantitative diminution of the response if the stimulus is repeated, though this diminution may lead to an absence of the response.

In the experiments described by Dr. D'Arcy there was a repeated uniform stimulus, for example, the rabbit being put in the cage, and the response to this stimulus was struggling which caused a rise of temperature. If the rabbit was put into the cage repeatedly, the response gradually diminished or disappeared, and this was presumably due to habituation. The same applies to the metabolic cage where fear or other factors may have caused diarrhoea, which gradually disappeared when the animal became habituated to the experimental situation.

With the purgative experiments it seemed that the habituation to the experimental conditions disappeared again after 5 weeks. I suspect that the animals were becoming habituated to the drug. We have found that the rate of habituation to different stimuli applied at the same time is not necessarily the same. So the animals might have become habituated quickly to the cage and lost their diarrhoea, and then become habituated to the drug.

The physiological basis of this problem has been recently considered (Glaser, E. M. and Griffin, J. P. 1962. *J. Physiol.*, **160**, 429).

DR. P. F. D'ARCY. It does appear to be habituation to the drug after 5 weeks, because Brittain and I have since shown that if the period of testing is extended so that the mice are subjected to the purgative assay procedure every 2 weeks rather than every week, good precision can be maintained for much longer than 5 weeks.

DR. ANNE MCLAREN. About the term "sensitivity" which Dr. Annie Brown used as meaning LD50. This term is sometimes used (by Chai, for instance) in a different sense, namely, to mean the slope of the dose response line. This might be a possible source of confusion.

Mr. D. Brown, in talking about the work that I did with Dr. Biggers and Dr. Michie, stated that we asserted that F_1 hybrid animals were "the most satisfactory laboratory animals". That is not so. What we did conclude was that F_1 animals had been shown, in most cases where they had been

tested, to be more uniform than the parental inbred strains; also that they possessed greater viability than inbred animals, and that for these two reasons they were at least worth consideration when one was looking for the best strain of animals for a particular assay.

Where random bred animals have been compared with inbred strains, the theoretical expectation that they prove more variable has not always been substantiated, and I think that a good fertile random bred strain is worthy of consideration for a particular test, especially for a laboratory where the LAC's excellent traffic light system for inbred strains is not available.

For screening procedures, where one requires to mimic the responses of a variable human population, I think it is more desirable to use a number of different inbred strains, all of which have had their responses for a number of properties tested, than a single random-bred strain. The point is that one is then dealing with *controlled* variability; the population is genetically variable but the ways in which it varies are known and the situation is repeatable.

On Dr. D'Arcy's work, I do not quite see why habituation should send the variance shooting up in the way he has stated, and I wondered whether all that purging wasn't just messing up the insides of the mice.

MR. D. BROWN. The variation can be equally large within an inbred strain as within a random bred strain. Inbreeding does not necessarily lead to increased or decreased accuracy.

Some time ago we did insulin dose responses and got a slope which agrees fairly well with Dr. Annie Brown's. The slope was about 2, and Mr. K. L. Smith, in published work on his insulin assays, had a slope of approximately 5. Could Mr. Smith account for these differences? Are the differences due to the fact that he has an inbred line, or does he select mice specifically from a random bred colony?

MR. K. L. SMITH. I think Dr. Annie Brown is wrong to use slopes to indicate strain differences since slopes only indicate uniformity within the group of mice being used. The more uniform the mice, the steeper the slope; the less uniform, the flatter the slope. So if there are different slopes in different strains of mice, this is really indicative of different variability.

In the commercial assay the level of sensitivity is not important. We find in our routine assays that we have to make large changes of dose in our colony, but this does not affect the slope of the response line, just its position.

I do not know what strain of mice we use. We buy them from a dealer, and they are perfectly satisfactory. I think the high slopes we obtain in our assays of insulin are related to our method of handling and feeding the mice and in the way they are deprived of food in preparation for the test, rather than to the strain.

DR. ANNIE BROWN. I was quite aware that the slopes of the insulin curves I obtained were much less steep than those obtained in commercial laboratories. I think this may be the conditions that I used. These were,

however, very stable. My mice were always kept and bred in the same room at the same temperature, and they always reacted at the same temperature and had the same number of mice in a jar when they reacted. And I think the curves, particularly the ED50 against the weight of mouse which was plotted in the published paper, show that whenever I did my tests the slope for the LAC grey mice, tested with every strain of mouse, was always in the same part of the graph. And similarly on repeating the A2G mice tests on different occasions the slope came on the same part of the graph. The conditions I used are not such that they can be used in a commercial laboratory, because I used six strains of mice, which I generally tested in one day, and I purposely reversed the strains from time to time. I don't say that the conditions were the best, but I still think that slope and strain have a very definite connection.

MR. K. L. SMITH. Nevertheless the slope is an indication of uniformity within the strain. Do your results mean that your inbreeding hasn't really produced a uniform strain?

DR. Annie BROWN. I should think that probably is what it does mean, and I don't think inbreeding does produce a uniform strain. I am not unreservedly in favour of inbred strains.

In answer to Dr. McLaren, LD50 and ED50 always mean sensitivity, but precision can be defined as either the slope for qualitative or lambda for quantitative responses. This is where confusion arises.

The number of mice affects the variance tremendously. When we did our A2G sleeping times to pentobarbitone sodium we used 20 males and 20 females each time. The tests were repeated eight or nine times during my work, and the homogeneity of variance was calculated for each test. For some tests χ^2 gave a probability of less than 0·001, and for others a probability of 0·9. This was certainly not a result of the way the mice were treated, because they were all treated similarly.

DR. L. GOLBERG. It is probably a common experience to have trouble of one kind or another with diet. One of the basic difficulties in this country arises from the use of Diet 41. This diet was devised by Bruce and Parkes (*J. Hyg.*, 1949, **47**, 209) under conditions of war-time and post-war shortages of various ingredients. Some of the defects of Diet 41 were soon recognised, as for instance its marginal content of vitamin E (Bruce, H. M., 1950, *J. Hyg.*, **48**, 171). Bearing in mind the fact that manufacturers of animal diets inevitably vary the ingredients to some extent according to market availability, the allowance of essential nutrients must provide an adequate margin of safety. Such a margin is not present in Diet 41.

Since the advent of the 41B modification, we have found on occasion frank vitamin B_6 deficiency in our animals which could be cured with pyridoxine. When we approached the manufacturers for an analysis of the diet for these important marginal nutrients we could never get satisfaction. There is no batch to batch analysis. Values for vitamin contents are calculated from tables, an unreliable procedure. There should be some attempt by manufacturers to standardise the ingredients and one can

envisage the time when the diet, like an AR chemical, will have the vital ingredients listed on the label.

It is also desirable that the whole question of the composition of laboratory animal diets was examined afresh. I have in mind a methodical study like that carried out by Brock and Wilk (*Arzneimitt.-Forsch.*, 1961, **11**, 1071; 1962, **12**, 64) which serves to emphasise that the time has come for British workers to reconsider their own animal diets.

DR. M. R. A. CHANCE. We had a major crisis in our rat colony about two years ago, which was the result of dietary deficiencies. The crisis occurred after a characteristic English summer which had very little sun, and in which English wheat was used as the source of protein. There was a serious drop in the breeding rate and a considerable eating of young. The manufacturers were able to give us precise details of deficiencies in that diet, but no authoritative step had been taken to alter its composition. We need to have proper control of the diet, and this presumably means that some control organisation will have to do the testing, with the samples being submitted to it by manufacturers.

About batch to batch variability; we get immense variations from time to time, and in our recommendations for different sizes of cages for controlling variance we have made allowance for this in the designs of this test. Even this, however, is not adequate, because the strains vary through the day. Comparisons should be made all at the same time on any one day, and then the data can be subjected to a variance analysis to eliminate this factor.

Bonnycastle's method of analgesic assay (*J. Pharmacol.*, 1950, **100**, 141–145) which was reported about 10 years ago, was one of the best examples of how training can make for uniformity of response. It shows also how the variance comes about. He identified three or four separate responses of the rat to a painful stimulus, and showed that the individuals composing a population may react in a variety of ways to the same stimulus. Throughout the same population, some rats will crouch, others will squeak, and yet others will remove their tail from the source of the painful stimulus. This variation in type of and not merely in extent of response is a fundamental of all biological populations, and probably a reason why we behave differently to the same stimulus ourselves; simply because this is one way in which the total population will survive in a situation which is novel. We must, therefore, expect individuals of the same species of animal to give a variety of responses to the same stimulus, and one way of overcoming that variation is by transferring all the responses to whichever one you wish to use, by training.

PROF. A. N. WORDEN. Laboratory animal diets are made in this country usually in machinery, by people and on premises that are used for the production of compound animal feedingstuffs for domestic animals and poultry. As such all the raw materials and production and formulation and other troubles inherent to that industry at the price it operates are also inherent in laboratory animal diets, and the costs are similar. For a diet of a

pharmaceutical standard of control the cost would be something like double.

DR. G. FEUER. At the Institute of Psychiatry, the Maudsley Hospital, by selective breeding, two strains of albino rats have been developed from a common parental strain which are different in emotional behaviour. One shows apparently fearful and anxious behaviour and the other strain is courageous.

With Dr. P. L. Broadhurst, we have found that during subsequent generations, using the same diet and experimental conditions, some of the endocrine organs in both strains were significantly different. Differences in size and activity in their thyroids and pituitary and some differences in adrenals and ovaries were found. We think that the diet had nothing to do with this selection, but at the behaviour test, certain slight stimuli which we used caused differences in the animals' behaviour which were either related to their endocrine organs or maybe the differences in endocrine activity resulted in the variations in the psychological reaction of the animals.

We found conditioning was against our experimental set-up. In our system the rats were assayed in exactly the same circumstances. By using different thyroid drugs, or thyrotropic hormones, stimulating their thyroid or pituitary we wanted to change the psychological reaction, and found that these rats became used to the behaviour test, and when it was repeated, we did not obtain very good responses.

MR. W. R. BUCKETT. While Mr. Brown is pursuing the right path in finding a strain of mice with the maximal number of writhes due to phenylquinone, it is a recognised fact that it is an antagonistic reaction, and the mice themselves would vary in sensitivity both to the agonist and the antagonists tested. Hence for the screening machine necessary in industry I think we need not one specific strain but a good mixed strain so that we can detect untoward reactions.

MR. D. M. BROWN. It is impossible to rear a standard mouse which would satisfy all pharmacological tests. Screening procedures should not be restricted to one test alone; for the evaluation of any activity a number of different types of tests should be employed, and where possible, using the strain of animal which is best suited for the purpose.

MR. W. R. BUCKETT. When can you rely on one strain of mouse?

MR. D. M. BROWN. You can rely on a strain of mouse once you have proved it is the best strain for the purpose, and so far we have found by random breeding of a particular strain, that the strain does not lose its characteristics.

MR. J. M. HARRIS. I would like to mention some results which Dr. G. B. West and I have obtained with dextran in rats. On injection it usually produces a marked oedema of the paws, and this has been used as a screening test for anti-inflammatory, antihistamine and antiserotonin substances.

DISCUSSION

We set out to establish a dose-response curve to dextran but found that this was almost impossible because the animals we were using at that time were random-bred "Wistar" rats obtained from the A.R.C. colony at Compton. On average 23 per cent of these animals failed to show any reaction at all and over the last 2 years the percentage of "non-reactors" has varied from 11 to 43 per cent. When we used other strains of rat, for example, Sprague Dawley, Hooded Lister or August rats, all the animals reacted to the dextran. Then we tested rats from other "Wistar" colonies and found that about 17 per cent of rats from S.K.F. were "non-reactors" (this colony having been started with A.R.C. animals about 2 years ago) yet rats from the Wellcome colony were all "reactors".

We suggest that a genetic factor is involved here, non-reactivity being the recessive characteristic. By mating two reactors, we have obtained either a litter that were all reactors or a litter with about one non-reactor out of four if both parents were heterozygous reactors. When we mated a heterozygous reactor with a non-reactor, we obtained a litter with two non-reactors out of four, and when we mated two non-reactors we always obtained all non-reactors. These non-reactors breed true, and we have now bred five generations with more than 100 animals of this type.

Thus it seems that when this reaction is used in a screening test then the animals must be tested first to be sure that they react to dextran or else there is a danger that the results will contain false negatives.

DR. G. W. CAMBRIDGE. Have you ever found animals in which there was consistent 100 per cent writhing responses, since there was a paper recently published which claims that this can be done and any deviation from 100 per cent on the test is significant, that is, it could be used as a quantal assay. I have never been able to obtain writhing responses in 100 per cent of the animals.

MR. D. BROWN. No.

MR. B. O. HUGHES. Mr. W. R. Buckett inferred that Mr. Brown advocated the use of one strain of mouse for all tests on analgesic drugs, because this strain was found to be the most satisfactory for the phenylquinone writhing test. This was not what Mr. Brown had meant. We too found a different strain more effective in the tail clip test to the one which we used in the writhing test.

MR. K. L. SMITH. We used Wistar strain rats for Vitamin D assay successfully over a number of years, when suddenly they refused to develop rickets. We changed to a black and white hooded rat, which has proved satisfactory, but since then our Wistar rats have given rickets again.

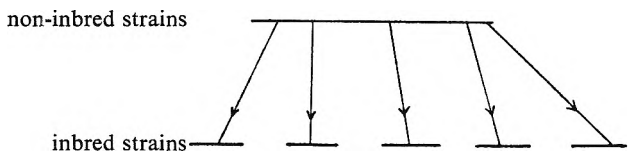
DR. F. J. C. ROE. In attempting to produce a uniform animal, the influence of litter size on rate of growth should not be forgotten. For absolute uniformity of animal, uniform litter size would be necessary. However, even this would not be sufficient, for within any one litter animals would still differ both physically and psychologically depending on their initial success in the fight for the best nipple.

Summary

CHAIRMAN. In trying to define or describe laboratory animals, and what we do to them, it is clear that we have very few precise or defined terms. The term "Wistar rat", for instance, for many years has been given to any rat with a white coat. So much so that the term, like the term "Swiss" as applied to mice, has little if any meaning, and should be abandoned.

Toxicity tests require a wide spectrum animal, or animals of several species.

Now, a non-inbred group of mice will have a spectrum of variation that may be represented by the long upper line in the figure. From it may be developed a number of non-inbred strains.



Inbred strains, whose within-strain variation is much smaller, are represented by the short lower lines in the figure. But the maximum between-strain variation of the inbred derivatives is much greater than that of the parent non-inbred strain. It would seem that a judicious mixture of inbred strains will, therefore, be better for, say, toxicity tests than a single non-inbred.

Reference has been made to the sources of laboratory animals, especially mice. The best of the commercial breeders in this country are producing as good an animal as they are inherently capable of doing. But if you want inbred strains, you have either got to breed them yourself or get them from a breeding station which is under laboratory rather than commercial control.

The diet situation in this country is deplorable. Some experiments that we have been doing at Carshalton for the last 12 months have made some interesting comparisons in mice fed on five different commercially-produced so-called complete diets. Three of them were reputedly Diet 41B (Bruce, H. M. and Parkes, A. S. 1956. *J. Anim. Tech. Ass.*, 7, No. 3, p. 54), and these included both the best and the worst.

If a better diet is wanted it will cost something like twice the present prices, but as the cost of the experiment for which the animal is used is anything from 10 to 25 times the cost of the animal, doubling the price of the diet, if it is accompanied by an improvement in the work for which it is used, is obviously wise economy.

The case for standardising bedding is not nearly so urgent.

The "general purpose mouse" is a myth. No chemist expects to have one reagent on his laboratory shelf, and the evidence that has been produced today indicates that the differences between different strains kept under different conditions or treated in different ways rules out the concept of a general purpose animal.

On usefulness of inbred strains compared with random or F_1 hybrids, I think it has been made clear that all three groups of animals, together with the controlled environments in which they are kept, have their usefulness.

It would be foolish to say that you must use inbred strains, or F_1 hybrids, or random breds. They all have their place. I hope that the fact that it is practical to have representatives of any of those three groups will stimulate people to match their animals to the work for which they are going to be used.

RESEARCH PAPERS

MONOMOLECULAR LAYERS OF SYNTHETIC PHOSPHATIDES*

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In studying the functions of phosphatides in biological membranes, investigations have been made into monomolecular layers of synthetic phosphatides. Force-area curves of monolayers of phosphatidic acid, phosphatidylcholine, phosphatidylethanolamine and phosphatidylserine, all containing identical fatty acids, showed small differences, obviously to be attributed to differences in size and charge of the end groups. The shifts of the force-area curves within one class of phosphatides were more pronounced and are brought about by variations of the apolar moiety. Shortening of the chain length and particularly unsaturation of the fatty acid constituents greatly expanded the films of the L-α-lecithins, thereby increasing the closest stable packing attainable. Force-area curves of structurally isomeric mixed-acid L-α-phosphatides, carrying dissimilar fatty acids in different positions, were identical. Mixed films consisting of phosphatides and cholesterol in molar equivalents—at proportions also found in red cell membranes—revealed a condensing effect of cholesterol on the film of phosphatides containing certain unsaturated fatty acid constituents.

In theories formulating the structure of biomembranes, bimolecular layers of phosphatides and other lipids are presumed to be associated on both sides through their polar groups with layers of proteins, thus forming the framework of the biological interfaces (Booij and Bungenberg de Jong, 1956; Stein and Danielli, 1956). Electron microscopic studies on natural objects as well as on artificially produced systems for instance myelins, formed from isolated phosphatides, appear to sustain the idea that a bimolecular phosphatide layer represents the backbone of the numerous cell membranes and boundaries inside the cells (compare Robertson, 1959; Stoeckenius, 1959, 1960; Stoeckenius, Schulman and Prince, 1960; Engström and Finean, 1960). Although the numerous biomembranes may be constructed according to a general pattern (a "unit membrane"), it is likely that differences exist in their fine structure, accounting for the apparent variations in properties between various membranes. Perhaps some of these variations may be brought about by differences in the chemical structure of the membranous lipid constituents.

Studies from this laboratory, made on the chemical composition of

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lipids of a number of animal tissues, membranes and membranous fragments, have already supplied information on how far variations in the lipid moiety of the membrane play an essential part in the properties of membranes. We found from results of comparative fatty acid analyses made on lipid fractions of a number of mammalian tissues that neutral lipids of the species studied possessed animal specificity; the fatty acid patterns differed from animal to animal, but resembled each other for different tissues within one species. On the other hand, the group of phosphatides revealed some tissue specificity, inasmuch as their fatty acid patterns differed for various tissues within one animal, but showed some degree of similarity in several homologous tissues (e.g. lung and brain) of different species of mammals (Veerkamp, Mulder and van Deenen, 1962). Furthermore, analyses on phosphatides extracted from whole tissues, may supply information about the fatty acid composition of lipids from intracellular membranes, for example mitochondrial membrane fragments. These results suggested that, beside the proportions of various lipid groups, a specific composition of the apolar part of the phosphatides may also play an essential part in the function of phosphatides in membranes. This view was supported by investigations on lipids from red cell membranes from different animal species. These lipids appeared to differ significantly. In the sheep, ox, pig, man, rabbit, rat, the lecithin percentage of the membranous phosphatides increases from about 1 to 58 per cent. A considerable shift in the sphingomyelin content compensates this effect, thereby giving a nearly equal content of choline-containing phosphatides in the red cells of the species studied (de Gier and van Deenen, 1961). Moreover, the fatty acid patterns of red cell lipids were found to be highly specific for each animal species and to vary between the red cells of different mammals. After alkaline saponification of the lipids from red cell ghosts, a decrease of arachidonic acid and palmitic acid was observed in the sequence rat, man, rabbit, pig, ox, sheep, whereas in that order the oleic acid content was found to be significantly increased with these groups of lipids (Kögl, de Gier, Mulder and van Deenen, 1960; de Gier, Mulder and van Deenen, 1961).

A comparison of these lipid characteristics with data on the permeability properties of the red cells from these animals, as determined by other investigators (Jacobs, Glassman and Parpart, 1935, 1950; Höber and Orskow, 1933), surprisingly showed that the shift observed in the lipid composition varies in the same sequence of animals as does the permeability behaviour when determined by osmotic means. This coincidence suggested that lipid composition and permeability properties of biomembranes may be closely related. One possible approach to elucidate such a relation, in progress now in our laboratory, involves the induction of changes in the chemical composition of membranous lipids by dietary means and the study of consequent changes in the permeability characteristics of the membranes.

Another approach will be by the perhaps devious but necessary route of making artificial membrane models composed of defined phosphatides and to search for differences in structure and properties of these

models in connection with the chemical composition of their lipid constituents.

During introductory attempts in this direction it became desirable to study mono-molecular layers of defined phosphatides composed of different, but known, fatty acids.

Studies on Monolayers of Phosphatides

Although it is unlikely that a phosphatide film at the air/water interface bears a very close resemblance to a cell membrane, studies on such monolayers certainly have improved the insight into the structure of biomembranes. This was demonstrated as early as 1925 by Gorter and Grendel, who deduced, from the spreading of lipids from red cell ghosts, that these bio-interfaces contain sufficient lipid material to constitute a bimolecular layer covering the red cell surface.

Leathes (1923, 1925) found that, when lecithin was spread on the surface of water, an expanded film was formed, while hydrolecithin gave a more condensed film. The possibilities offered by studying the action of phospholipases on monolayers of phosphatides was recognised by Hughes (1935). Recently, Dawson and Bangham (1959; compare also Bangham and Dawson 1960) developed this method in an attractive way, using labelled substrates. Beside studies on the complex formation of phosphatides with cholesterol, glycerides and fatty acids (Leathes, 1925; Dervichian and Pilet, 1944; Dervichian and Joly, 1946; Guastalla, 1949; Croes and Ruyssen, 1950; de Bernard, 1958) the interaction of monolayers of phosphatides with proteins was also investigated (Eley and Hedge, 1956, 1957; Schulman, 1957; Payens, 1960). These studies appear to be very important to the understanding of the binding between different types of lipids and between lipids and proteins, combinations which probably exist in membranes. The results of the studies on the interaction between proteins and phosphatides, however, have been interpreted in different ways.

As a consequence of Rideal's (1939) valuable suggestions, various attempts have been made to obtain information about the possible action of biologically active compounds on biomembranes by studying their effects on monolayers of phosphatides, for example sulphonamides (Veldstra and Havinga, 1947), local anaesthetics (Skou, 1961; Shanes, 1960), antibiotics (Few and Schulman, 1953), chemotherapeutic agents inhibiting neoplasms (Hirt and Berchtold, 1961), plant growth hormones (Veldstra and Havinga, 1948). Although many interesting results were obtained it is not possible, at the present, to draw definite conclusions about the mechanism of action of these substances in the living cell from the results of studies on phosphatide films at the air/water interface or other artificial systems. Evaluation of membrane models, which in composition and structure more closely resemble living membranes, probably will furnish further knowledge about the mechanism of drug action in the future. Encouraging possibilities arise from the work of Saunders (1953), who succeeded in studying stable films of phosphatides formed between two aqueous liquids, bearing a close resemblance to

boundaries separating a living cell from a liquid environment. Furthermore the possibilities offered by electron microscopy of artificial boundary systems (compare Trurnit and Schidlovsky, 1960), may give fresh information on the ultra structure of these synthetic membrane systems in comparison with biomembranes.

Most results in this field have been obtained by using isolated lecithin specimens of biological origin, whereas only few studies were made on reliable synthetic compounds (Anderson and Pethica, 1955; Eley and Hedge, 1957). Because of the differences in the lipid composition of biomembranes, presumably related to differences in membrane properties, it appeared desirable to carry out future experiments with well-defined phosphatides.

Materials and Methods

Synthetic Phosphatides

To verify the effects of the chain length and the presence of double bonds of the fatty acid constituents on the interfacial behaviour of phosphatides, a method for the synthesis of a series of L-α-lecithins in a simple way was developed (Kögl, de Haas and van Deenen, 1960). By this method, involving a reacylation of L-α-glyceryl-phosphorylcholine prepared from egg lecithin, defined L-α-lecithins were obtained, which are composed of two identical fatty acids per molecule (I). The fatty acid chain length varied from C_2 to C_{24}, while also compounds composed of an unsaturated acyl chain are included (Table I).

TABLE I

SYNTHETIC L-α-LECITHINS CARRYING TWO IDENTICAL FATTY ACIDS (Formula I)

L-α-Lecithins	R	Abbreviations†
L-α-(Diacetyl)lecithin	CH_3—	$2^c/2^c$-L-α-PC
L-α-(Dibutyryl)lecithin	CH_3—CH_2—CH_2—	$4^c/4^c$-L-α-PC
L-α-(Diheptanoyl)lecithin	CH_3—$(CH_2)_5$—	$7^c/7^c$-L-α-PC
L-α-(Didecanoyl)lecithin	CH_3—$(CH_2)_8$—	$10^c/10^c$-L-α-PC
DL-α-(Ditetradecanoyl)lecithin*	CH_3—$(CH_2)_{12}$—	$14^c/14^c$-DL-α-PC
L-α-(Di-pentadecanoyl)lecithin	CH_3—$(CH_2)_{13}$—	$15^c/15^c$-L-α-PC
L-α-(Di-stearoyl)lecithin	CH_3—$(CH_2)_{16}$—	$18^c/18^c$-L-α-PC
L-α-(Di-tetracosanoyl)lecithin	CH_3—$(CH_2)_{22}$—	$24^c/24^c$-L-α-PC
L-α-(Di-undecenoyl)lecithin	CH_2=CH—$(CH_2)_8$—	$11^1/11^1$-L-α-PC
L-α-(Di-oleoyl)lecithin	CH_3—$(CH_2)_7$—CH=CH—$(CH_2)_7$—	$18^1/18^1$-L-α-PC

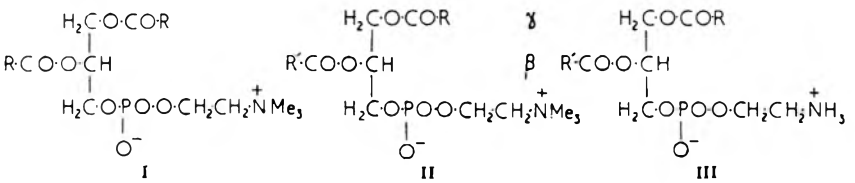
* This lecithin was synthesised by an unpublished procedure involving a preparation of dimyristoyl-DL-α-glycerylphosphoryl(*NN*-dimethyl)ethanolamine according to de Haas and van Deenen (1961a). The latter compound was converted into the corresponding lecithin by quaternisation with methyliodide.

† The various groups of phosphatides are abbreviated in the usual way, as follows: PC, phosphatidylcholine (lecithin); PE, phosphatidylethanolamine; PS, phosphatidylserine; PA, phosphatidic acid.

The fatty acid constituents are indicated by means of the number of carbon atoms and double bonds; e.g. 18^1 stands for oleic acid. The abbreviation of the fatty acid in γ-position of the phosphatide molecule preceeds that of the β fatty acid.

Most naturally occurring lecithins are known to be composed of both saturated and unsaturated fatty acid consituents. From the recent proof of the β-specificity of the mono fatty acid-releasing phospholipase present in snake venom (Tattrie, 1959; Hanahan, Brockerhoff and Barron, 1960; de Haas, Mulder, and van Deenen, 1960; de Haas and van Deenen, 1961), it is now concluded that saturated fatty acids are preferentially

located in the γ-ester position, whereas the unsaturated ones are predominantly esterified at the β-position. The biological significance of this asymmetrical distribution of the fatty acids in this most abundant type of phosphatides is not yet elucidated. The synthesis of mixed-acid lecithins (II) was first achieved by de Haas and van Deenen (1960; 1961a),



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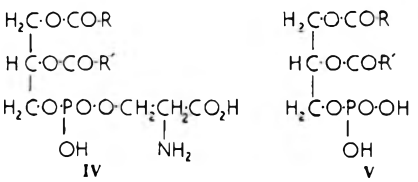

IV V

according to a fully synthetic method being suitable for the preparation of compounds having the naturally occurring L-α-configuration, as well as for D isomers (Table II). After elucidating the β-specificity of snake venom phospholipase with these compounds, a less elaborate partial synthesis of mixed-acid L-α-lecithins was developed, implying the specific mode of action of this enzyme (de Haas and van Deenen, 1960a). The synthetic mixed-acid lecithins contained either two saturated fatty acids of unequal chain length or one saturated and one unsaturated fatty acid in different positions (Table II).

TABLE II

SYNTHETIC MIXED-ACID LECITHINS (Formula II)

Compounds	Fatty acid position		Abbreviations*
	R	R'	
(γ-Stearoyl-β-lauroyl)-L-α-lecithin	Stearic	Lauric	18°/12°-L-α-PC
(γ-Stearoyl-β-lauroyl)-D-α-lecithin	Stearic	Lauric	18°/12°-D-α-PC
(γ-Stearoyl-β-lauroyl)-DL-α-lecithin	Stearic	Lauric	18°/12°-DL-α-PC
(γ-Lauroyl-β-stearoy)-DL-α-lecithin	Lauric	Stearic	12°/18°-DL-α-PC
(γ-Oleoyl-β-stearoyl)-L-α-lecithin	Oleic	Stearic	18¹/18°-L-α-PC
(γ-Stearoyl-β-oleoyl)-L-α-lecithin	Stearic	Oleic	18°/18¹-L-α-PC
(γ-Stearoyl-β-oleoyl)-D-α-lecithin	Stearic	Oleic	18°/18¹-D-α-PC

* For explanation see Table I.

In addition, mixed-acid L-α-phosphatidylethanolamines (III) (de Haas and van Deenen, 1961a; Daemen, de Haas and van Deenen, 1962), and a DL-α-phosphatidylserine (IV) (de Haas and van Deenen, 1961b) were prepared fully synthetically. Besides these mixed-acid cephalins, DL-α-(distearoyl)phosphatidylethanolamine, DL-α-(distearoyl)phosphatidyl

serine and DL-α-(distearoyl)phosphatidic acid (V) were also synthesised accordingly by comparable methods (Table III).

TABLE III

SYNTHETIC CEPHALINS AND PHOSPHATIDIC ACID (Formulae III, IV and V)

Compounds	Fatty acid position		Abbreviations*
	R	R′	
Formula III,			
DL-α-[Distearoyl]-phosphatidylethanolamine	Stearic	Stearic	$18^0/18^0$DL-α-PE
(γ-Stearoyl-β-oleoyl)-L-α-phosphatidylethanolamine	Stearic	Oleic	$18^0/18^1$-L-α-PE
(γ-Oleoyl-β-stearoyl)-L-α-phosphatidylethanolamine	Oleic	Stearic	$18^1/18^0$-L-α-PE
Formula IV			
DL-α-(Distearoyl)phosphatidylserine	Stearic	Stearic	$18^0/18^0$-DL-α-PS
(γ-Stearoyl-β-lauroyl)-DL-phosphatidylserine	Stearic	Lauric	$18^0/12^0$-DL-α-PS
Formula V			
DL-α-(Distearoyl)phosphatidic acid	Stearic	Stearic	$18^0/18^0$-DL-α-PA

* For explanation see Table I.

Spreading Methods

A conventional Langmuir-Adam trough assembly was used for surface pressure measurements. The phosphatides were spread from a 5×10^{-4} molar solution in highly purified chloroform on a phosphate buffer pH 7·4, ionic strength 0·14, at room temperature (21–24°). In a number of experiments a citrate-phosphate buffer pH 4·0, ionic strength 0·14, was used as substrate. The initial surface per molecule was about 200 Å^2, at which the pressure varied between 1 to 2·5 dynes/cm. The measurement of a force area-curve lasted for 1·5 to 2 hr., depending on the compressibility of the film and the closest stable packing attainable.

Force area curves are generally averages of at least three measurements.

RESULTS AND DISCUSSION

Force-area Curves of Monolayers of Several Types of Glycerol Phosphatides

Effects brought about in force-area characteristics of phosphatides by different polar end groups are demonstrated in Fig. 1. For comparison, films of phosphatides containing identical fatty acids were spread. Apparently the molecular areas occupied by the various types of phosphatides at the various pressures differ only to a limited extent. The closest stable packing for the distearoyl homologues of DL-α-phosphatidic acid and DL-α-phosphatidylethanolamine approaches at pH 7·4 near to 36 Å^2/molecule. Since this value is in keeping with the molecular dimensions of two fatty acid chains, it appears that the area per molecule of these groups of phosphatides is determined mainly by the apolar moiety of their molecules. The closest stable packing of L-α-(distearoyl)lecithin, however, was found to be about 39–40 Å^2/molecule, being in agreement with the data reported by Anderson and Pethica (1955). The differences in molecular area occupied in the films of L-α-lecithin and DL-α-phosphatidic acid and DL-α-phosphatidylethanolamine respectively, might be caused by differences in steric configuration. But it is not unlikely that the three methyl groups of the choline moiety or its charge may be involved

in determining the packing of the lecithin molecules. In fact, the force-area curves of more expanded films of mixed-acid L-α-lecithins and L-α-phosphatidylethanolamines, (Figs. 4 and 5), showed a comparable difference. With DL-phosphatidylserine, values observed for the closest stable packing attainable at pH 7·4 amounted to 39 Å².

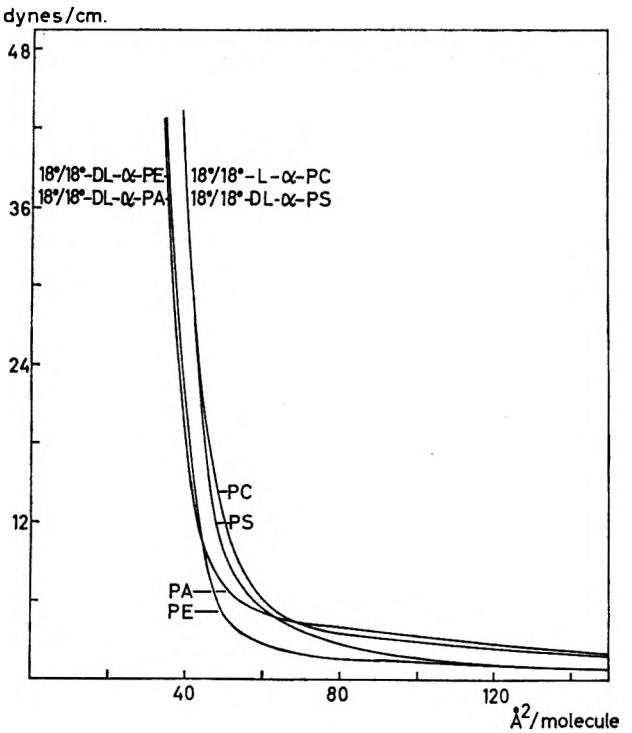

FIG. 1. Force-area characteristics of distearoyl homologues of lecithin (phosphatidylcholine; PC), phosphatidylethanolamine (PE), phosphatidic acid (PA) and phosphatidylserine (PS) on a phosphate buffer substrate of pH 7·4 (abbreviations are also indicated in Tables I–III).

Furthermore some differences were noted in the response of the various phosphatide films to changes in pH of the substrate medium. At pH 4·0 the films of phosphatidylethanolamine, phosphatidylserine and particularly of phosphatidic acid, showed a decreased area per molecule at low pressures if compared with the films spread at pH 7. The lecithin films were identical at both pH values. The closest stable packing of phosphatidyl ethanolamine and phosphatidic acid was not altered appreciably by this pH variation, while phosphatidylserine films revealed a slight decrease to 37 Å². However, (γ-stearoyl-β-lauroyl)-DL-phosphatidylserine, at pH 7·4, gave a more expanded film than the distearoyl homologue, and showed, when spread on an underlayer of pH 4·0, a significant phase transition at a pressure of about 24 dynes/cm., resulting

in the closest stable packing comparable to the value of the distearoyl compound (Fig. 2). In addition, Fig. 2 demonstrates that the force-area characteristics of (γ-stearoyl-β-lauroyl)-DL-lecithin did not respond to variations of pH 7·4 to 4·0. At pH 7·4, the differences between the film characteristics of the DL-α-lecithin and DL-α-phosphatidylserine, both having an identical fatty acid composition, appeared to be small.

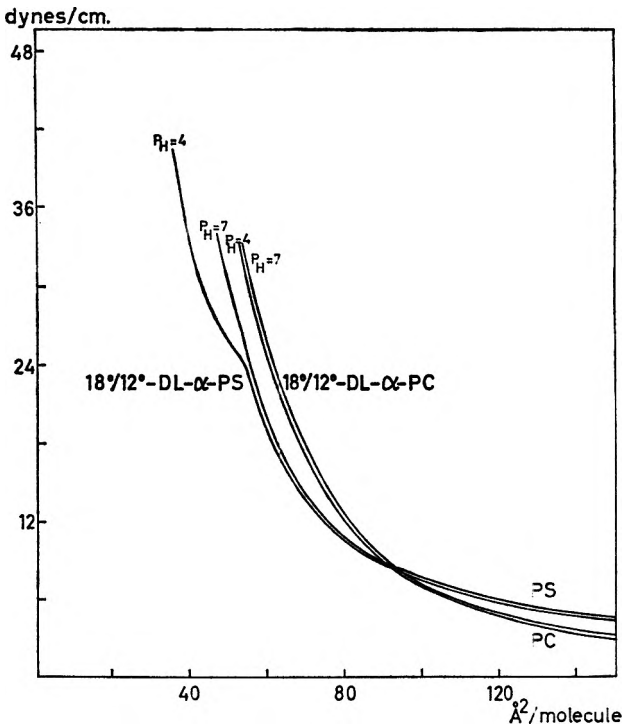

FIG. 2. Effect of pH on force-area characteristics of (γ-stearoyl-β-lauroyl)-DL-α-phosphatidylserine and (γ-stearoyl-β-lauroyl)-DL-α-phosphatidylcholine (Compare also Table II and III.)

Effect of Fatty Acid Chain Length on Force-area Characteristics of Lecithins

The pioneering studies of Adam (1930) on monolayers of fatty acids, alcohols and derivatives, clearly indicated that the force-area curves of amphiphatic molecules generally are highly dependent on the chain length of the apolar part, which determines the van der Waals' forces involved. In this respect, however, information about the behaviour of the films of the more complex phosphatide molecules is lacking. Representative characteristics of a number of synthetic lecithins, containing different fatty acids, are given in Fig. 3. The long-chain compounds, L-α-(ditetracosanoyl)lecithin and L-α-(distearoyl)lecithin, produce monolayers of the liquid condensed type. Shortening of the chain length of

the fatty acid constituent results in far more expanded films, like those of L-α-(dipentadecanoyl)lecithin and L-α-(didecanoyl)lecithin. Furthermore, the compressibility of both foregoing long-chain lecithins was about 45×10^{-4} cm./dyne, whereas the latter short-chain lecithins revealed a value of 90×10^{-4} cm./dyne (compare Harrap, 1954).

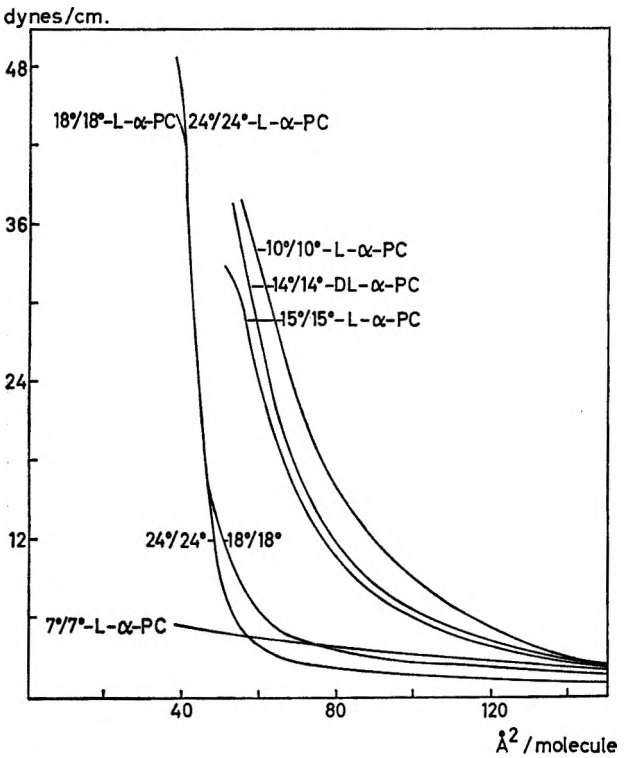

FIG. 3. Force-area characteristics of various saturated lecithins (phosphatidyl choline; PC) on a phosphate buffer substrate, pH 7·4. All compounds contain two equal fatty acid constituents per molecule. The nature of the fatty acid constituents is indicated by the number of carbon atoms (compare Table I).

As expected, the fairly water-soluble L-α-(diheptanoyl)lecithin and the highly water-soluble L-α-(dibutyryl)lecithin and the L-α-(diacetyl)lecithin failed to give a stable film.

Effect of Unsaturated Fatty Acid Constituents on Force-area Characteristics of Phosphatides

Naturally occurring phosphatides, for example from red cell membranes, often show great variation in their content of unsaturated fatty acids. Introduction of one mono-unsaturated fatty acid (oleic acid) in the lecithin molecule causes the monomolecular film of the phosphatide to be more expanded than the film of the corresponding saturated lecithin (Fig. 4), and changes the compressibility from 45×10^{-4} cm./dyne to 83×10^{-4}cm.

/dyne. Consequently, the presence of two oleic acid chains in the lecithin molecule effect a further shift of the force-area curve, increasing the expanded character of the lecithin film. The presence of oleic acid within the phosphatidylethanolamine molecule causes effects similar to those noted for the film characteristics of lecithin (compare Fig. 5). Apparently, the presence of unsaturated bonds in the acyl chain of the phosphatides greatly influences their interfacial properties. Taking into account the significant amounts of poly-unsaturated fatty acids, such as arachidonic acid, present in membranous phosphatides, it seems important to extend these studies to these types of compounds. The synthesis of phosphatides composed of highly unsaturated fatty acids is in progress in our laboratory.

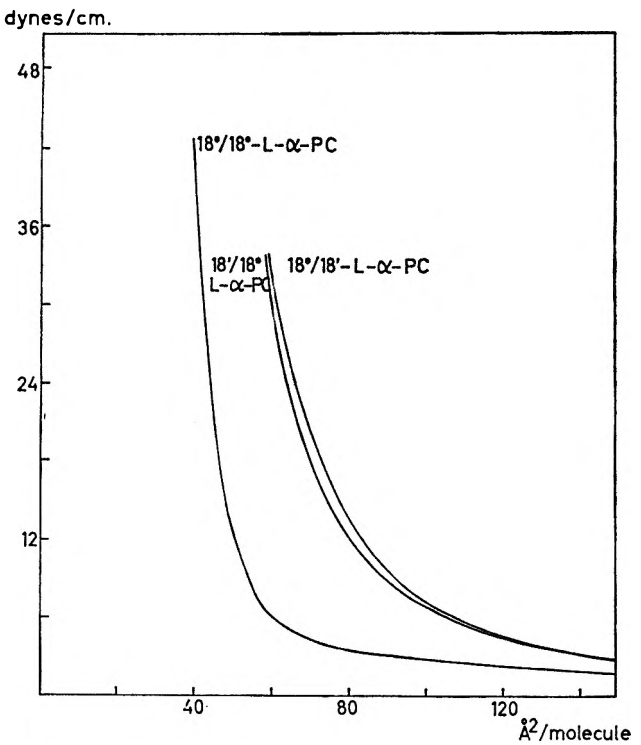

FIG. 4. Force-area characteristics of a saturated lecithin (PC) (stearic acid) and of two lecithins containing one mole of stearic acid and one mole of oleic acid in different positions (compare Table I and Table II).

Monolayers of Structurally Isomeric Mixed-acid Phosphatides

The significance of the asymmetrical distribution of fatty acids in naturally occurring lecithins viz. the preferential location of saturated acyl groups in the γ-position, and of the unsaturated ones in the β-position, has not yet been elucidated. Force-area curves of structurally isomeric mixed-acid phosphatides, having the two dissimilar fatty acids attached in different positions, appear to be identical.

Examples presented in Figs. 4 and 5 show that the curves of two mixed-acid L-α-lecithins and two L-α-phosphatidylethanolamines respectively,

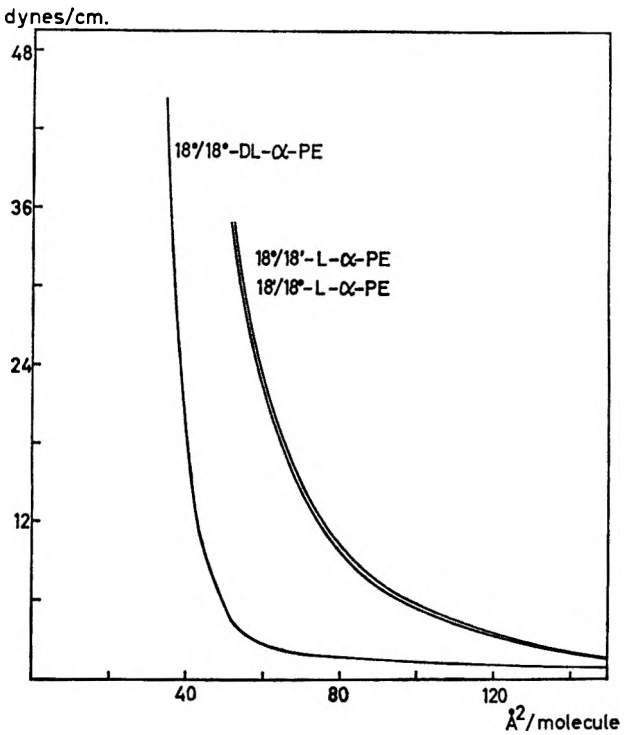

FIG. 5. Force-area characteristics of a saturated (stearic acid) phosphatidyl ethanolamine (PE) and of two phosphatidylethanolamines containing one mole of stearic acid and one mole of oleic acid in different positions (compare Table III).

composed of one oleic and one stearic acid chain, are equally situated for each group of phosphatides. This is true also when two saturated fatty acids of unequal chain length are located in different positions (Fig. 6). These findings do not preclude the asymmetrical distribution of fatty acids in the phosphatides from having a structural significance, since in membranes phosphatides are associated with other lipids and with proteins, forming complicated complexes. On the other hand the possibility exists that this asymmetrical fatty acid distribution is merely a result of the biosynthetic pathways of phosphatides. Generally, our knowledge of the fundamental background of the specific configuration in natural compounds for example L-amino acids, is rather restricted.

Furthermore, the curves of the expanded films of D- and L- isomers of lecithins composed with stearic and lauric acid, were found to be identical, while the DL compounds gave slightly decreased areas per molecule (Fig. 6).

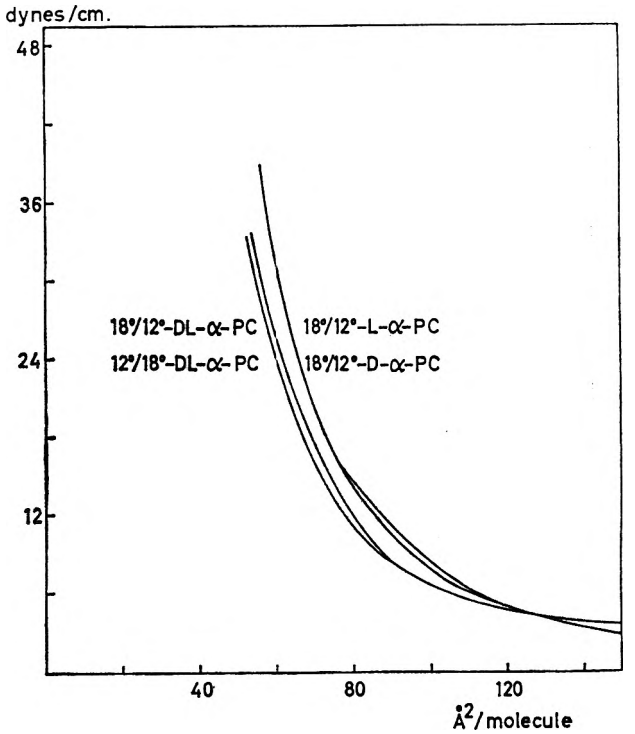

FIG 6. Force-area characteristics of D, L and DL isomers of α-lecithins (pH 7·4) The two DL compounds contain stearic acid and lauric acid in different molecular positions (compare Table II).

Mixed Films of Cholesterol and Synthetic Lecithins

Various investigations (de Bernard, 1958; Dervichian, 1958; Finean, 1953, 1961; Willmer, 1961) have emphasised the importance of cholesterol -phosphatide interaction because of the occurrence of such complexes in living tissues, such as myelin sheaths. Since our analyses of red cell membranes from different species of mammals (de Gier and van Deenen, 1961) demonstrated, that the molecular proportion of cholesterol to phosphatides is approximately 1 : 1, mixed films were prepared of cholesterol and various lecithins in this ratio. Fig. 7 indicates that even the already condensed film of L-α-(distearoyl)lecithins undergoes a small shift in the presence of cholesterol. Assuming that the molecular area of cholesterol, being about 35 Å² per molecule at 33 dynes/cm., is not changed in the mixed film, it can be derived from the mixed film area (37 Å² per molecule), that the molecular area of lecithin in the mixed film was 39 Å² per molecule at 33 dynes/cm., whereas L-α(distearoyl)lecithin, when spread alone, had a value of 41·5 Å² at this pressure. Presumably the molecular area of L-α-(distearoyl)lecithin in the mixed film with cholesterol is no longer attributed to the dimensions of the choline moiety, as was supposed to be likely in the single lecithin film.

The effect of cholesterol on the expanded film of a lecithin containing one oleic acid constituent, however, is quantitatively much more pronounced (Fig. 8). The presence of an equimolar amount of cholesterol resulted in a significantly more condensed film giving a decrease of the molecular area per lecithin molecule, when compared with the spreading characteristics of the lecithin alone. Taking into account the above presumption, the molecular area of (γ-stearoyl-β-oleoyl)-L-α-lecithins in the mixed film was calculated to be 49 Å^2 per molecule, while the lecithin alone had a value of 59 Å^2 at the same pressure of 33 dynes/cm. This

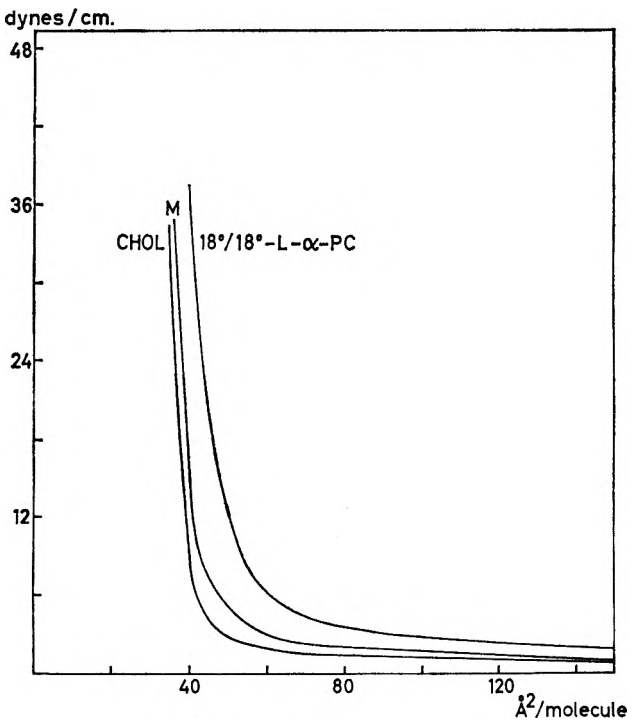

FIG. 7. Force-area characteristics of cholesterol (Chol), L-α-[distearoyl]lecithin (PC) and a mixed film (M) of both compounds in equimolar amounts.

effect was also met in experiments on mixed films of cholesterol with L-α-lecithins containing two unsaturated fatty acid chains. Apparently the presence of cholesterol in the monolayers of these lecithins causes a re-orientation of the unsaturated fatty acids, thereby partly abolishing the expanded character of lecithin films attributed to unsaturated fatty acid constituents. Endorsing previous reports on the importance of cholesterol-phosphatide complexes (Dervichian, 1958; Finean, 1953), the observed effects may be of interest for the understanding of the intermolecular arrangements of the phosphatide and the cholesterol at biological interfaces, e.g. red cell membranes.

Comments

Thus it can be concluded that the interfacial behaviour of various types of glycerol phosphatides is a function of the nature of the apolar moiety. Because of the significant differences brought about in the film characteristics of the monolayers of phosphatides by variation of the chain length and particularly by unsaturation of the fatty acid constituents, it may be imagined that the fatty acid composition of membranous phosphatides plays an important part in the fine-architecture and properties of biomembranes. Studies from our laboratory have demonstrated that the lipid characteristics of red cell membranes from various mammals differ greatly, and their differences could be tentatively related to variations in the permeability behaviour of the membranes. It is not yet advisable

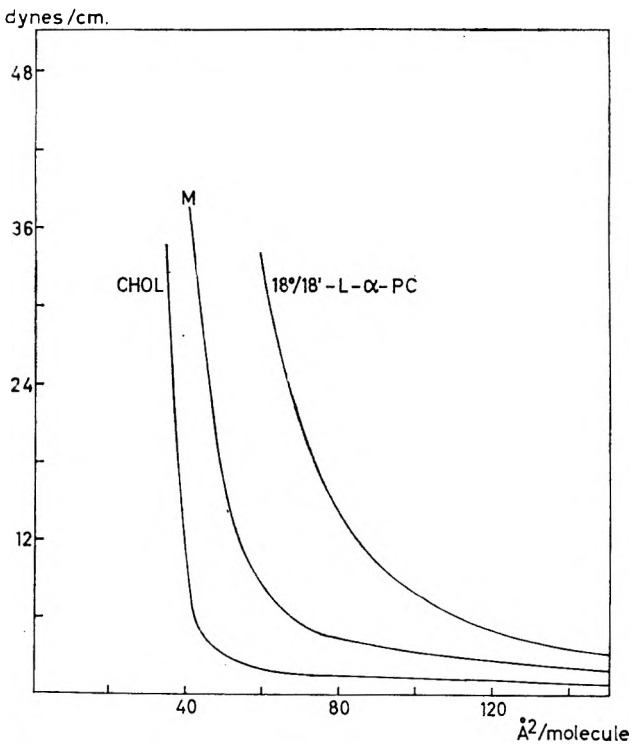

FIG. 8. Force-area characteristics of cholesterol (Chol), (γ-stearoyl-β-oleoyl)-L-α-lecithin (PC) and a mixed film (M)of both compounds in equimolar amounts.

to attempt an interpretation of this relationship between lipid composition and biophysical properties of membranes on the basis of the results presented on phosphatide monolayers. Apart from the fact that a comparison of data obtained at an air/water interface, with the complex framework of a cell membrane separating two aqueous phases, is always imperfect, the variations in the lipid composition of the red cell membranes

are complicated. As well as the variation in fatty acid composition, the sphingomyelin content and the amount of plasmalogen also differed greatly; data on monolayers of the latter phosphatides are not available at present. But the results obtained so far, prompt us to consider further attempts, to formulate the structure and the functional mechanism of biomembranes, and to study the influence of variations of fatty acid composition of the membranous constituents.

The better understanding of the relationship between monolayer properties and fatty acid composition in the phosphatides, will probably facilitate further studies on more complex systems in which phosphatides are combined with other lipids and proteins. In particular, extension of the experiments on mixed films of several types of phosphatides with cholesterol may supply further information about the precise orientation of these molecules. It should also help in elucidating the stabilising effect in biomembranes, such as in red blood cells, produced by the condensation of cholesterol with phosphatides. Furthermore, the fresh information obtained on monolayers of defined phosphatides, together with the new analytical values of the lipid composition of red cell membranes will stimulate the investigation of monolayers of lipids extracted from red cells of various animal species, to ascertain whether the earlier work of Gorter and Grendel (1925, 1926), leading to the bimolecular lipid theory, is consistent with present data.

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THE SOLUBILISING PROPERTIES OF LIQUORICE

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Conductivity and surface tension measurements have shown that micelle formation occurs in liquorice extracts. A method of determining solubilising power by vapour pressure measurements is described, and results quoted for benzene, chloroform, ethyl acetate, and hexane.

Liquorice has been used in pharmacy for many years, principally as a flavouring agent. It is sometimes used for its expectorant properties, and in recent years attention has been drawn to its anti-inflammatory action. Little, however, has been said of its value as an excipient. The production of a stable foam on agitating liquorice extracts is an indication of the existence of at least one amphipathic constituent. This property has been utilised in non-pharmaceutical fields, since liquorice extracts have been used as foam stabilisers (Housemann, 1929), and as dispersing agents for insoluble dyes (Marnon, 1954). A more useful indication of the surface-activity of liquorice is that liquorice extract B.P. will form an apparently homogeneous mixture with considerable quantities of materials which are insoluble in water, such as chloroform and volatile oils. On dilution of these mixtures with water a clear solution is produced. This behaviour is characteristic of solubilisation, and it was the purpose of this investigation to determine if solubilisation does in fact occur and, if so, how efficient a solubiliser liquorice is.

Experimental

Materials

Liquorice was of B.P. quality. The organic liquids were of analytical reagent quality. Each was redistilled before use, and its purity confirmed by boiling point and refractive index.

Preparation of Extracts

Two extracts were prepared, one according to the instructions of the British Pharmacopoeia. In the second extract water was substituted for ethanol. These will be termed "aqueous extract with ethanol" and "aqueous extract" respectively throughout this paper. 0·02 per cent phenyl mercuric nitrate was added to the aqueous extract to prevent fermentation.

Standardisation of Extracts

Since liquorice extracts vary in composition from batch to batch, the extracts were characterised by determining a range of physical and

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chemical properties. These are summarised in Table I. Physical constants were measured at 20°. Starches and gums were precipitated with ethanol and weighed, and glycyrrhizin determined gravimetrically, after precipitation, with sulphuric acid, from the solution remaining after removal of starches and gums. The experimental procedure described by Housemann (1922) was adopted, except that to completely precipitate the gums and starches it was found necessary to set the mixture aside overnight. Ethanol was determined by the British Pharmacopoeia method.

TABLE I

PHYSICAL AND CHEMICAL PROPERTIES OF LIQUORICE EXTRACTS

Property	Aqueous extract with ethanol	Aqueous extract
Density	1·135	1·154
n_D^{20}	1·416	1·403
pH	5·45	5·23
λ max.	262·5 mμ	262·5 mμ
Chemical composition (per cent):		
Ethanol (v/v)	17·3	—
Starches and gums (w/w)	13·53	12·37
Glycyrrhizin (w/w)	8·26	7·51

Determination of Critical Micelle Concentration

Conductimetric method. The conductances of dilutions of the aqueous extract were measured at 25° using a Cambridge Bridge. The specific conductance first increased with dilution, reaching a maximum at a concentration of 80·7 per cent of extract. A plot of specific conductance divided by the per cent concentration of extract in the dilution (a function of equivalent conductance), against the square root of the per cent concentration of the extract in the dilution is shown in Fig. 1, and gives a

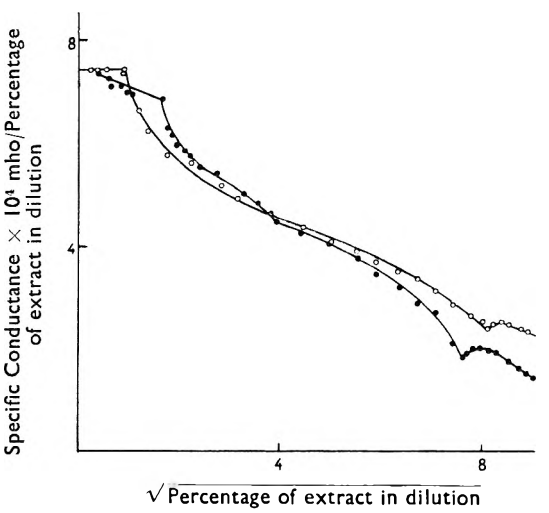

FIG. 1. Concentration-conductance curves for liquorice extracts. ○ Aqueous extract. ● Aqueous extract with ethanol.

similar inflection, the maximum this time occurring at the lower concentration of 71 per cent. Beyond this the curve continued in a manner characteristic of micelle forming systems, with a critical micelle concentration of 0·9 per cent. The aqueous extract with ethanol gave the same type of curve, with an inflection at 61 per cent, and critical micelle concentration at 2·7 per cent.

A similar curve was obtained with ammoniated glycyrrhizin, but there was no initial maximum in either specific or equivalent conductance. Measurements made with the aqueous extract to which ammoniated glycyrrhizin had been added, showed a maximum in the specific conductance-concentration curve, but this disappeared when equivalent conductances were plotted.

Surface tension method. Surface tensions were determined by measuring with a chainamatic balance the weight necessary to pull a platinum plate out of the surface of the extract. This method has been described fully by Harkins (1930). Measurements were made on aqueous dilutions of the extracts, and surface tension plotted against the per cent concentration of extract in the dilution: the graphs are shown in Fig. 2.

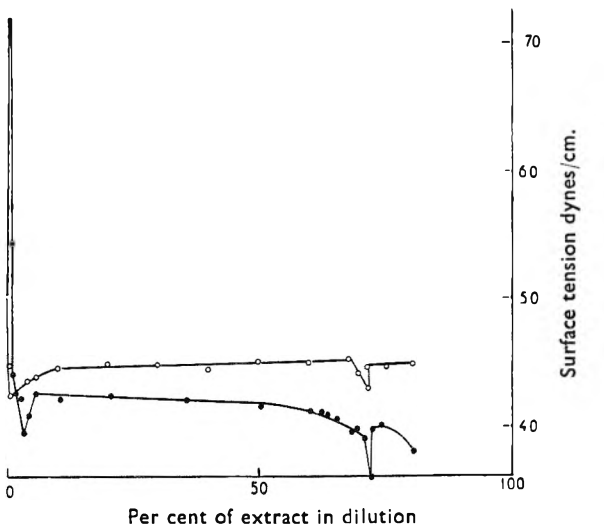

FIG. 2. Concentration-surface tension curves of liquorice extracts. ○ Aqueous extract. ●Aqueous extract with ethanol.

A minimum occurred in the curve for the aqueous extract at 71 per cent, corresponding to the inflection in the conductance curve, but the aqueous extract with ethanol gave a minimum value at 72 per cent, 11 per cent higher than the conductance maximum. Beyond these minima, McBain type III curves were obtained, with critical micelle concentrations at 1 per cent for the aqueous extract, and 3 per cent for the aqueous extract with ethanol. Ammoniated glycyrrhizin gave a similar curve, but without the initial minima shown by the extracts.

The Investigation of Solubilisation

Visual methods cannot be used as liquorice extracts are opaque, and a vapour pressure method similar to that described by McBain (1940, 1941) was therefore adopted. 3·5 g. of extract was weighed into a 100 ml. distilling flask, the side arm of which was fused to a mercury manometer. A rubber bung was used to seal the neck of the flask, and was pierced by a hypodermic needle, through which measured volumes of solubilisate were forced using an Agla micrometer syringe. The apparatus was immersed in a water bath at 25°, and the extract stirred with a magnetic stirrer. The solubilisate was added in increments, and after equilibrium was reached, the manometer level read with a cathetometer. A blank to each determination was carried out using water, or aqueous ethanol of the same strength as the aqueous extract with ethanol.

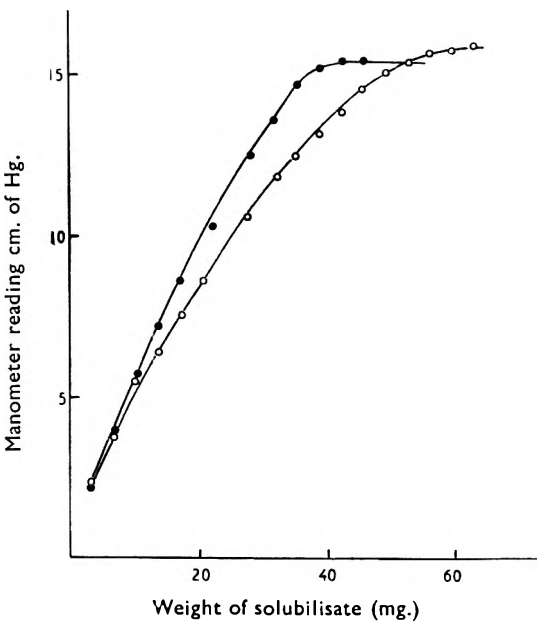

FIG. 3. Vapour pressures of chloroform in aqueous liquorice extract. ● Water. ○ Aqueous extract.

A graph of amount of solubilisate added against vapour pressure is shown in Fig. 3 and is typical of the results obtained. With the blank the vapour pressure rose sharply to a constant limiting value, while with the extract more solubilisate was required to attain the same value. The intersection of the two curves was taken as the limit of solubilisation. After intersection, the vapour pressure curve of the extract rose to a higher limiting value. McBain (1940) has predicted such behaviour when solubilisation gives way to emulsification. Fig. 3 represents the solubilisation of chloroform by the aqueous extract. Other solubilisates examined

were benzene, ethyl acetate, and hexane. The quantities solubilised are shown in Table II.

TABLE II

SOLUBILISATION BY LIQUORICE EXTRACTS

Solubilisate	Percentage (w/v) solubilised			
	Aqueous extract	Aqueous extract with ethanol	Sodium oleate	
			Vapour pressure	Visual
Chloroform	4·7	6·5	4·2	3·9
Benzene	1·4	1·9	1·9	1·2
Hexane	1·8	2·2	2·4	1·4
Ethyl acetate	0	0	1·0	1·0

Results are corrected for solubility.

As a check on the method, the solubilising power of a 2·76 per cent solution of sodium oleate was examined and compared with results obtained by the visual method. These are given in Table II.

Addition of ammoniated glycyrrhizin to the extracts increased the quantity of insoluble liquid solubilised.

DISCUSSION

The object of this work has been twofold, firstly to determine if the process by which liquorice extracts form clear solutions with materials which are insoluble in water is one of solubilisation, and secondly to investigate the extract's potential in the formulation of mixtures containing insoluble liquids. The first object has been fulfilled by showing that micelles occur in liquorice extracts, and that three liquids which are insoluble in water, namely benzene, chloroform and hexane, are solubilised. Both the conductance and surface tension curves are typical of micelle forming systems, and since the same curves have been produced with solutions of ammoniated glycyrrhizin, glycyrrhizin is a micellating constituent of the extracts. For the second requirement, the quantity of chloroform which can be solubilised by liquorice extract B.P. is given, while the results for benzene and hexane suggest that a volatile oil would be solubilised, probably to the extent of 1 to 2 per cent.

The vapour pressure method described is simpler and more rapid than that used by McBain (1940, 1941) but has the disadvantages that it is probably less accurate, and is limited to those solubilisates having a higher vapour pressure than water. It was for this reason that the number of solubilisates examined was small, and results were not quoted for substances, such as paraldehyde, and volatile oils, which are frequently formulated with liquorice extracts. The aqueous extract with ethanol solubilised more of the three solubilisates examined than the aqueous extract, indicating that ethanol enhances the solubilising power. This is in contrast to the conductance and surface tension results which show that ethanol increases rather than decreases the critical micelle concentration.

The reasons for the initial inflections in the conductance and surface

tension curves is not clear, but the fact that they occur at the same concentration on both aqueous extract curves, and are accompanied by a fall in surface tension indicates that they represent an interfacial change, and could be the critical micelle concentration of another constituent, possibly gum. The effect of ethanol on this point on the conductance curves could be taken as support for the observed effect of ethanol on solubilisation. However, since this did not occur with the surface tensions, and would be contrary to observations on other systems (Ward, 1940; Corrin, 1946; Ralston, 1948) it was assumed to be due to some other phenomenon, probably a change in dielectric of the solvent on dilution with water.

Although examination of the extract makes the results more interesting pharmaceutically, it does present a system which is difficult to work with, and which contains at least two amphipathic substances, namely glycyrrhizin and gum. For this reason work subsequent to this paper is being carried out on the constituents of liquorice.

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HYPOGLYCAEMIC AGENTS. PART II

By D. F. Hayman, V. Petrow, O. Stephenson and A. J. Thomas

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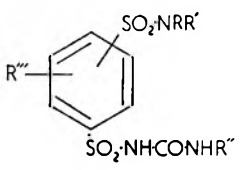
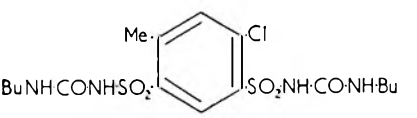
Various derivatives of 1-benzenesulphonyl-3-butyl(or cyclohexyl)ureas substituted in the aryl nucleus are described.

Some aromatic 1,3- and 1,4-disulphonamides containing one unsubstituted sulphamoyl residue were described by Petrow, Stephenson and Wild (1960). Several of them have now been converted into the corresponding sulphonylureas (cf. I, where R = H or alkyl and R′ = alkyl, or NRR′ = heterocyclic nucleus; R″ = butyl or cyclohexyl and R‴ = H, Me or Cl) by reaction with the appropriate isocyanate in aqueous acetone in the presence of one equivalent of sodium hydroxide. Additionally the bissulphonylurea (II) was obtained by reaction of 5-chlorotoluene 2,4-disulphonamide (Boggiano and others, 1960) with butyl isocyanate. A few related 1,4-bissulphonylureas were also prepared.

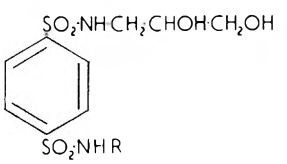


I



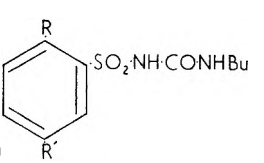
II

Most of the foregoing compounds were insoluble in water. A more soluble type was obtained by the reaction of *p*-(2,3-dihydroxypropyl-sulphamoylbenzenesulphonamide (III a, R = H) with butyl isocyanate. The product so formed failed to crystallise and was assumed to be the *O*-carbamate ester. It was readily converted into the required product (IIIb, R = CONHBu) by hydrolysis with boiling aqueous sodium hydroxide.



III

IV



V

Finally, ethyl 2-methyl-5-sulphamoylbenzoate, ethyl 4-chloro-3-sulphamoylbenzoate and methyl 4-methyl-3-sulphamoylphenyl sulphone (Jackman, Petrow, Stephenson and Wild, 1962) were condensed with butyl isocyanate to yield compounds IV, Va(R = Cl; R′ = CO_2Et)Vb(R = Me; R′ = SO_2Me) respectively.

TABLE I

$R = -SO_2.NH.CO.NH.C_4H_9$

$R' = -SO_2.NH.CO.NH.C_6H_{11}$

Substituent at position					Fomula	m.pt ° C.	Found					Required				
1	2	3	4	5			C	H	Cl	N	S	C	H	Cl	N	S
R		SO_2NMe_2			$C_{13}H_{21}N_3O_5S_2$	138–140	42·9	5·9		11·6	17·4	43·0	5·8		11·6	17·6
R′		SO_2NMe_2			$C_{15}H_{23}N_3O_5S_2$	166–168	46·9	6·2		10·6	16·1	46·3	6·0		10·8	16·5
R′			NH_2		$C_{15}H_{19}N_3O_3S$	179–180	52·6	6·1		14·5	11·0	52·5	6·4		14·1	10·8
R′			NHAc		$C_{17}H_{21}N_3O_4S$	206–207	53·1	6·2		12·4	9·2	53·1	6·2		12·4	9·5
R			SO_2NHMe		$C_{12}H_{19}N_3O_5S_2$	189–191	41·3	5·6		12·5	18·4	41·3	5·5		12·0	18·4
R′			SO_2NHMe		$C_{14}H_{21}N_3O_5S_2$	199–200	44·7	5·6		11·4	17·0	44·8	5·6		11·2	17·1
R			SO_2NHEt		$C_{13}H_{21}N_3O_5S_2$	177–179	43·1	5·7		11·6	17·5	43·0	5·8		11·6	17·6
R			SO_2NMe_2		$C_{13}H_{21}N_3O_5S_2$	168–170	43·0	5·9		11·7	17·6	43·0	5·8		11·6	17·6
R′			SO_2NMe_2		$C_{15}H_{23}N_3O_5S_2$	196–197	46·5	5·9		10·8	16·0	46·3	6·0		10·8	16·5
R			SO_2NHPh		$C_{17}H_{21}N_3O_5S_2$	186–188	50·0	5·3		10·1	15·2	49·6	5·2		10·2	15·6
R			SO_2N 		$C_{16}H_{29}N_3O_5S_2$	146–147	48·0	5·9		10·6	16·0	47·9	5·8		10·5	16·0
R			SO_2N 		$C_{16}H_{25}N_3O_5S_2$	165–167	47·4	5·9		10·6	16·0	47·6	6·3		10·4	15·9
R			SO_2N 		$C_{15}H_{23}N_3O_6S_2$	177–179	44·1	5·9		10·5	15·9	44·4	5·7		10·4	15·8
R			$SO_2NH.CH_2.CH(OH)$	CH_2OH	$C_{14}H_{23}N_3O_7S_2$	161–162	40·8	5·3		9·9	16·1	41·1	5·7		10·3	15·7
R			CO_2Et		$C_{14}H_{20}N_2O_5S$	141–142	50·9	5·9		8·5	10·2	51·2	6·1		8·5	9·8
R			R		$C_{16}H_{36}N_4O_6S_2$	218–220 (d)	44·3	6·1		12·9	14·8	44·2	6·0		12·9	14·8
R′			R′		$C_{20}H_{32}N_4O_6S_2$	212–214 (d)	49·1	6·2		11·5	13·5	49·4	6·2		11·5	13·2
Me	SO_2NHMe		R		$C_{13}H_{21}N_3O_5S_2$	150–152	43·6	5·9		11·5	17·7	43·0	5·8		11·6	17·6
Me	SO_2NMe_2		R		$C_{14}H_{23}N_3O_5S_2$	156–158	44·6	6·1		10·9	17·0	44·5	6·1		11·1	17·0
Me	R		SO_2Me		$C_{13}H_{20}N_2O_5S_2$	158–159	44·7	5·5		8·2	18·7	44·8	5·8		8·0	18·4
Me	R′		SO_2Me		$C_{15}H_{22}N_2O_5S_2$	191–192	48·3	6·0		7·4	17·1	48·1	5·9		7·5	17·1
Me	R			SO_2NMe_2	$C_{14}H_{23}N_3O_5S_2$	154–155	44·4	6·1		11·1	16·9	44·5	6·1		11·1	17·0
Me	SO_2NMe_2			R	$C_{14}H_{23}N_3O_5S_2$	151–152	44·6	6·1		11·2	17·2	44·5	6·1		11·1	17·0
Me	CO_2Et		R		$C_{15}H_{22}N_2O_5S$	111–112	52·5	6·2		7·8	9·1	52·6	6·5		8·2	9·4
Cl	SO_2NHMe		R		$C_{12}H_{18}ClN_3O_5S_2$	164–166	37·7	4·8	9·2	11·1	16·9	37·5	4·7	9·2	10·9	16·7
Cl	SO_2NHMe		R′		$C_{14}H_{20}ClN_3O_5S_2$	180–183	41·6	5·2	8·9	10·3	15·2	41·0	4·9	8·7	10·3	15·6
Cl	SO_2NHEt		R		$C_{13}H_{20}ClN_3O_5S_2$	153–155	39·3	5·2	9·1	10·7	16·2	39·2	5·1	8·9	10·6	16·1
Cl	R′		SO_2NHMe		$C_{14}H_{20}ClN_3O_5S_2$	168–170	41·3	5·0	9·1	10·6	15·0	41·0	4·9	8·7	10·3	15·6
Cl	SO_2NMe_2		R		$C_{13}H_{20}ClN_3O_5S_2$*	160–162	39·5	5·0	9·2	10·6	16·3	39·2	5·1	8·9	10·6	16·1
Cl	R		R		$C_{16}H_{28}ClN_4O_5S_2$	176–178	40·9	5·6	7·8	12·1	13·5	41·0	5·4	7·6	12·0	13·7
Cl	R		CO_2Et		$C_{14}H_{19}ClN_2O_5S$	170–171	46·5	5·1	8·9	7·7	9·9	46·3	5·3	8·8	7·7	9·8
Cl	R		SO_2NHMe	Me	$C_{13}H_{20}ClN_3O_5S_2$	168–170	38·8	4·8	9·0	10·6	16·5	39·2	5·1	8·9	10·6	16·1
Cl	R′		SO_2NHMe	Me	$C_{15}H_{22}ClN_3O_5S_2$	174–176	43·0	5·6	8·3	10·0	15·4	42·5	5·2	8·4	9·9	15·1
Cl	R		R	Me	$C_{17}H_{27}ClN_4O_5S$†	214 (d)	42·4	5·5	7·5	11·6		42·3	5·6	7·3	11·6	

* Prepared by Mr. A. M. Wild. † Prepared by Dr. G. B. Jackman.

The foregoing sulphonylureas were tested for oral hypoglycaemic activity by Dr. A. David and his colleagues but proved to be without interest.

Table I summarises analytical data for those compounds not described in detail in the Experimental Section.

EXPERIMENTAL

The intermediates employed below are described by Petrow, Stephenson and Wild (1960) and Jackman, Petrow, Stephenson and Wild (1962).

p-*Sulphamoylbenzenesulphonethylamide.* Sulphamoylbenzene-*p*-sulphonyl chloride (17 g.) was added in portions with stirring to 5 per cent aqueous ethylamine (240 ml.) previously cooled to 0°. After 2 hr., excess of ethylamine was boiled off and the residual liquid acidified with dilute hydrochloric acid. The *product* was collected and washed with cold water. It had m.p. 166–168° after crystallisation from water. Found: C, 36·4; H, 4·4; N, 10·8. $C_8H_{12}N_2O_4S_2$ requires C, 36·4; H, 4·6; N, 10·6 per cent.

p-(2,3-*dihydroxypropylsulphamoyl*)*benzenesulphonamide* was prepared by reaction of sulphamoylbenzene-*p*-sulphonyl chloride with 3-aminopropane-1,2-diol in aqueous solution at below 5°. It had m.p. 141–143° after crystallisation from a small amount of water. Found: C, 34·9; H, 4·2; N, 8·7; S, 20·3. $C_9H_{14}N_2O_6S_2$ requires C, 34·8; H, 4·5; N, 9·0; S, 20·7 per cent.

p-*Sulphamoylbenzenesulphonanilide*, had m.p. 230–231° after crystallisation from aqueous ethanol. Found: C, 46·4; H, 3·8; N, 9·0; S, 20·4. $C_{12}H_{12}N_2O_4S_2$ requires C, 46·1; H, 3·9; N, 9·0; S, 20·5 per cent.

N-(p-*Sulphamoylbenzenesulphonyl*)*piperidine.* A solution of sulphamoylbenzene-*p*-sulphonyl chloride (15·35 g.) in chloroform (60 ml.) was added with stirring to a solution of piperidine (15·3 g.) in water (100 ml.). After 30 min. excess of chloroform and piperidine were evaporated at reduced pressure. The residue was dissolved in water and acidified with hydrochloric acid to yield the product (15·3 g.), m.p. 220–221° (from aqueous ethanol). Found: C, 43·5; H, 5·1; N, 9·2; S, 21·0. $C_{11}H_{16}N_2O_4S_2$ requires C, 43·4; H, 5·3; N, 9·2; S, 21·1 per cent.

1,2,3,4-*Tetrahydro*-N-(p-*sulphamoylbenzenesulphonyl*)*pyridine* was prepared as above. It was obtained in 90 per cent yield, m.p. 181–182° (from aqueous ethanol). Found: C, 43·7; H, 4·6; N, 9·0; S, 21·1. $C_{11}H_{14}N_2O_4S_2$ requires C, 43·7; H, 4·7; N, 9·3; S, 21·2 per cent.

N-(p-*Sulphamoylbenzenesulphonyl*)*morpholine* had m.p. 191–192° (from aqueous ethanol). Found: C, 39·6; H, 4·7; N, 9·2; S, 20·6. $C_{10}H_{14}N_2O_5S_2$ requires C, 39·2; H, 4·6; N, 9·2; S, 20·9 per cent.

2-*Chloro*-5-*sulphamoylbenzenesulphonethylamide* was prepared by reaction of 2-chloro-5-sulphamoylbenzenesulphonyl chloride with 5 per cent aqueous ethylamine at below 10°. It had m.p. 144–145° (from water). Found: C, 32·5; H, 3·8; Cl, 11·5; N, 9·3; S, 21·5. $C_8H_{11}ClN_2O_4S_2$ requires C, 32·2; H, 3·7; Cl, 11·9 per cent; N, 9·4; S, 21·5.

2-*Chloro*-5-*sulphamoylbenzenesulphondimethylamide* had m.p. 146–147° (from aqueous ethanol). Found: C 32·6; H, 3·8; Cl, 11·7. N, 9·3; S, 21·5. $C_8H_{11}N_2O_4S_2Cl$ requires C, 32·2; H, 3·7; Cl, 11·9; N, 9·4; S, 21·5 per cent.

5-*Nitrotoluene*-2-*sulphondimethylamide.* A solution of 5-nitrotoluene-2-sulphonyl chloride in chloroform (100 ml.) was added over 20 min. with stirring to 25 per cent aqueous dimethylamine at room temperature. After 30 min., excess of amine and chloroform were boiled off and the residue was cooled and acidified with dilute hydrochloric acid. The *product* was collected and washed with cold water. It had m.p. 103–104° (from 95 per cent ethanol). Found: C, 44·3; H, 5·1; N, 11·2; S, 12·8. $C_9H_{12}N_2O_4S$ requires C, 44·3; H, 5·0; N, 11·5; S, 13·1 per cent.

5-*Aminotoluene*-2-*sulphondimethylamide.* A mixture of the foregoing nitro-compound (34·6 g.), iron powder (28·6 g.), acetic acid (1·7 ml.), water (220 ml.) and octanol (1 ml.) was heated under reflux with stirring for 6 hr. Ethanol (200 ml.) was then added and the mixture boiled with charcoal and filtered. The filtrate was cooled and saturated with hydrogen chloride to yield the product as the *hydrochloride* (28 g.), m.p. 172–174° (from ethanol-ether). Found: C, 43·4; H, 5·9; Cl, 14·0; N, 11·1; S, 12·7. $C_9H_{15}ClN_2O_2S$ requires C, 43·1; H, 6·0; Cl, 14·2; N, 11·2; S, 12·8 per cent.

5-*Chlorosulphonyltoluene*-2-*sulphondimethylamide.* A solution of the foregoing hydrochloride (28 g.) in 24 per cent hydrochloric acid was diazotised at 0° by the addition of sodium nitrite (8·36 g.) in water (20 ml.). The resultant solution was added with stirring to a saturated solution of sulphur dioxide in acetic acid (350 ml.) containing cuprous chloride dihydrate (7·7 g.) at 20–25°. After 30 min. the mixture was diluted with ice-water and the sulphonyl chloride collected and washed with cold water. It had m.p. 109–111° after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°). Found: C, 36·6; H, 3·9; Cl, 12·0; N, 4·6; S, 21·5. $C_9H_{12}ClNO_4S_2$ requires C, 36·3; H, 4·1; Cl, 11·9; N, 4·7; S, 21·5 per cent.

5-*Sulphamoyltoluene*-2-*sulphondimethylamide.* A solution of the foregoing sulphonyl chloride (29·8 g., moist material) in chloroform (150 ml.) was added with stirring to ammonium hydroxide solution (300 ml., d = 0·880) at below 10°. After 1 hr. excess of ammonia and chloroform were boiled off, and the cooled residue was acidified with hydrochloric acid. The *product* (22·6 g.) was collected and washed with cold water. It had m.p. 149–151° (from ethanol). Found: C, 39·2; H, 4·8; N, 10·0; S, 23·3. $C_9H_{14}N_2O_4S_2$ requires C, 38·8; H, 5·1; N, 10·1; S, 23·0 per cent.

4-*Chlorosulphonyl*-2-*methylbenzoic acid.* A mixture of *o*-toluic acid (68 g.) and chlorosulphonic acid (200 ml.) was heated at 125° for 2½ hr. when it was cooled and poured on to ice. The *product* (101·5 g.) was collected and washed with ice-water. It had m.p. 154–155° after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°). Found: C, 40·8; H, 2·7; Cl, 15·1; S, 13·4. $C_8H_7ClO_4S$ requires C, 41·0; H, 3·0; Cl, 15·1; S, 13·7 per cent.

Ethyl 2-methyl-5-sulphamoyl benzoate, obtained by esterification of the carboxylic acid, had m.p. 146–147° (from aqueous ethanol). Found: C, 49·6; H, 5·0; N, 6·0; S, 13·0. $C_{10}H_{13}NO_4S$ requires C, 49·4; H, 5·4; N, 5·8; S, 13·2 per cent.

1-*Cyclohexyl*-3-[p-(p-*toluenesulphonamido*)*benzenesulphonyl*]*urea*. Toluene *p*-sulphonyl chloride (0·69 g.) was added in portions with cooling to a solution of 1-(*p*-aminobenzenesulphonyl)-3-cyclohexylurea (1·07 g.) in pyridine (1·5 g.). After 1 hr. the mixture was heated on the steam-bath for 30 min., cooled, diluted with water and acidified with hydrochloric acid. The *product* (1·1 g.) was collected and washed with water. It had m.p. 195° after crystallisation from aqueous ethanol. Found: C, 53·0; H, 5·4; N, 9·3; S, 14·0. $C_{20}H_{25}N_3O_5S_2$ requires C, 53·2; H, 5·6; N, 9·3; S, 14·2 per cent.

1-*Chloro*-2,4-*di*(*ethylcarbamoylsulphamoyl*)*benzene* (by Dr. G. B. Jackman). A solution of 4-chlorobenzene-1,3-disulphonamide (13·5 g.) in acetone (60 ml.) was treated with a solution of sodium hydroxide (4·0 g.) in water (40 ml.). The mixture was cooled below 10° and ethyl isocyanate (8·2 g.) added dropwise with stirring. After 1 hr. at room temperature the mixture was acidified to Congo red with concentrated hydrochloric acid and the solids collected and washed with water. Extraction with boiling ethyl acetate yielded the insoluble *product* (16 g.), m.p. 203° (decomp.). Found: C, 35·0; H, 3·9; Cl, 8·3; N, 14·0. $C_{12}H_{17}ClN_4O_6S_2$ requires C, 34·9; H, 4·2; Cl, 8·6; N, 13·6 per cent.

1-*Butyl*-3-[p-(2,3-*dihydroxypropylsulphamoyl*)*benzenesulphonyl*]*urea*. A solution of *p*-(2,3-dihydroxypropylsulphamoyl)benzenesulphonamide (12·4 g.) in acetone (100 ml.) was treated with a solution of sodium hydroxide (1·6 g.) in the minimum of water. The mixture was cooled to 0°, stirred and treated with butyl isocyanate (13·1 g.) added dropwise. After 30 min. the mixture was heated at 60° for 6 hr. when it was cooled, poured on to ice and acidified to pH 5 by the addition of hydrochloric acid. The resultant oil was extracted with ethyl acetate and the extract dried with anhydrous sodium sulphate. Removal of the solvent left an oil which failed to crystallise. The oil was heated at reflux temperature with 4N sodium hydroxide (100 ml.) for 30 min. when the solution was treated with charcoal and filtered. The filtrate was cooled and acidified to pH 5 with hydrochloric acid when the solid product (9 g.) separated. It had m.p. 161–162° after crystallisation from water.

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FLOCCULATION AND CONDUCTIVITY OF PHOSPHATIDE SOLS

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Results obtained in flocculation and conductivity studies with sols of lecithin and lysolecithin and solutions of calcium and potassium chlorides are reported. Calcium chloride interacts with lecithin, the extent of interaction depending upon the method of preparation of the lecithin sol. Potassium chloride does not interact with lecithin. These results may explain why potassium ions can be transported across a cell membrane whilst calcium ions cannot.

Danielli and Stein (1956) have summarised the information that exists about the nature of a red cell membrane. It contains roughly equal weights of lipid molecules including phosphatides and of complex protein molecules. The lipid content in a membrane is just sufficient to constitute a layer two molecules in thickness and the molecules are thought to be arranged in the form of a bimolecular leaflet, with the non-polar parts directed inwards and with the polar groups on the exterior. Proteins are adsorbed on both surfaces of the leaflet.

Why certain salts diffuse through a cell membrane whilst other salts do not is still a mystery. It is possible that phosphatides play a part in the functioning of a membrane by interacting with certain salts and preventing their passage across the membrane, whilst other salts which do not interact with phosphatides may be those which are transferred across it. The interactions between the phosphatides lecithin and lysolecithin, and calcium and potassium chloride solutions have been studied by means of flocculation and conductivity experiments.

Experimental

Apparatus

The conductivity measurements were made using a Pye conductance bridge (Cat. No. 11700). A Cambridge conductivity cell (Ref. No. 43932/2), having a cell constant of 0·1 and bright platinum electrodes, was used. Constant results could not be obtained with a cell the electrodes of which had been covered with platinum black owing to the difficulty of removing impurities from the surface of the electrodes.

Materials

The methods for preparing lecithin and lysolecithin have been described previously (Saunders, 1957). The substances had the following characteristics:

	Lecithin	Lysolecithin
Nitrogen	1·83 per cent	2·73 per cent
Phosphorus	3·92 per cent	5·86 per cent
Specific rotation	$[\alpha]_D^{16°} + 7·17$ (5 per cent w/v solution in ethanol)	$[\alpha]_D^{17·5°} + 1·60$ (5 per cent w/v solution in ethanol)
Iodine number	74·3	3·0
Mean molecular weight (from N and P contents)	778	521

Preparation of Sols

Lysolecithin sol. Lysolecithin was dissolved in water with warming. The sol was passed down a column containing mixed ion-exchange resins (Amberlite IR-120(H) and IRA-400(OH)). The resins were then washed with small successive quantities of distilled water and the sol was finally made up to volume.

Lecithin sol. Lecithin was dissolved in 5 ml. of ether. About 20 ml. of water was added with intermittent shaking. The ether was removed by displacement with nitrogen which in turn was removed under reduced pressure. The solution was then passed down a column containing mixed resins and finally made up to volume.

Mixed sols. Method A. Sols of lecithin and lysolecithin prepared by the above methods were mixed in the required proportions. *Method B.* Weighed quantities of lecithin and lysolecithin were dissolved in ethanol. The ethanol was removed under vacuum leaving a film of intimately mixed phosphatides. Water was added and the flask shaken for 5 hr. at 55°. The sol was then passed down a column containing mixed resins and finally made up to volume.

All sols were aged for 24 hr. before being used, since viscosity studies have shown that changes occur in the sols with time (Thomas and Saunders, 1958).

Flocculation Studies

1 ml. of a sol containing 0·5 g. of phosphatide in 100 ml., prepared by method A, was placed in a small sample tube and 0·2 ml. of a salt solution was added by means of an Agla micrometer syringe. After stirring the sols, the tubes were placed in an incubator at 25° (±1°).

TABLE I

EFFECT OF CALCIUM CHLORIDE ON THE STABILITY OF LECITHIN SOLS

Concentration of $CaCl_2$ in moles/litre ($\times 10^5$)	After standing for 24 hr.		After standing for 72 hr.	
	0·42 per cent lecithin	0·21 per cent lecithin	0·42 per cent lecithin	0·21 per cent lecithin
1·25	−	−	−	−
3·00	−	−	−	−
6·00	+	−	+	+
125	+	−	+	+
300	−	−	−	−
600	−	−	−	−

\+ = Flocculation.
− = No flocculation.

Effect of total phosphatide concentration on flocculation produced by salt solutions. Two series of experiments were made, one with a 0·42 per cent lecithin sol, and the other with a 0·21 per cent sol. To them was added 0·2 ml. of various concentrations of calcium chloride and potassium chloride solutions, with the results given in Tables I and II.

The results given in Tables I and II show that with a sol containing 0·42 per cent (w/v) of lecithin flocculation occurs much more rapidly

than with a sol containing 0·21 per cent. However, after allowing the mixtures to stand for three days identical results were obtained with both sols. Calcium chloride concentrations from 6×10^{-5} moles per litre to $1{\cdot}25 \times 10^{-3}$, caused flocculation of the sols. With potassium chloride concentrations above 3×10^{-3} moles per litre caused flocculation.

TABLE II

EFFECT OF POTASSIUM CHLORIDE ON THE STABILITY OF LECITHIN SOLS

Concentration of potassium chloride in moles/litre ($\times 10^3$)	After standing for 24 hr.		After standing for 72 hr.	
	0·42 per cent lecithin	0·21 per cent lecithin	0·42 per cent lecithin	0·21 per cent lecithin
1·25	−	−	−	−
3·00	+	−	+	+
6·00	+	−	+	+
8·33	+	−	+	+

Effect of lysolecithin on the flocculation of lecithin sols by salt solutions. The effect of the presence of varying amounts of lysolecithin on the stability of lecithin sols towards various concentrations of calcium chloride and potassium chloride was studied. The rate of flocculation was slower with the mixed sols than with lecithin sols, and the mixtures were allowed to stand until constant results were obtained. The results obtained with calcium chloride are given in Table III. With a sol having a weight

TABLE III

EFFECT OF CALCIUM CHLORIDE ON THE STABILITY OF MIXED PHOSPHATIDE SOLS

(Total phosphatide concentration in each mixture = 0·42 per cent (w/v).

Calcium chloride in moles/litre ($\times 10^3$)	Weight fraction of lecithin				
	1·0	0·9	0·8	0·7	0·6
1·25	−	−	−	−	−
3·00	−	−	−	−	−
6·00	+	−	−	−	−
8·33	++	+	+	−	−
10·0	++++	+++	++	+	−
12·5	++++	+++	++	+	−
30·0	++++	+++	++	−	−
60·0	+++	++	+	−	−
83·3	+++	+	−	−	−
125·0	++	−	−	−	−
300·0	−	−	−	−	−
600·0	−	−	−	−	−

++++ = Large precipitate.
+ = Small precipitate.
− = No precipitate.

fraction of lecithin of 0·9, concentrations of potassium chloride of $1{\cdot}25 \times 10^{-2}$M and above were required to cause flocculation. When the weight fraction of lecithin was 0·8, concentrations of $8{\cdot}33 \times 10^{-2}$M and above were required to cause flocculation, and when the weight fraction was 0·7 or less no flocculation occurred with any concentrations below $1{\cdot}25 \times 10^{-1}$M.

Conductivity Studies

Effect of concentration of phosphatide sol. Two per cent sols of lecithin and lysolecithin were prepared by the methods described previously and their conductivities measured. These sols were then diluted with successive quantities of conductivity water and the conductivities of the diluted sols were measured. That due to the phosphatide was calculated by subtracting the conductivity of the water from the specific conductivity of the sol. The results obtained are given in Table IV.

TABLE IV

THE VARIATION OF SPECIFIC AND EQUIVALENT CONDUCTIVITIES OF LECITHIN AND LYSOLECITHIN SOLS WITH CONCENTRATION

Concentration in per cent (w/v)	Lysolecithin		Lecithin	
	Specific conductivity mhos cm.$^{-1}$ ($\times 10^5$)	Equivalent conductivity	Specific conductivity mhos cm.$^{-1}$ ($\times 10^6$)	Equivalent conductivity
2·0	1·472	0·383	0·580	0·225
1·8	1·352	0·391	0·528	0·228
1·6	1·213	0·395	0·478	0·232
1·4	1·096	0·407	0·430	0·239
1·2	0·952	0·413	0·372	0·241
1·0	0·806	0·419	0·312	0·242
0·8	0·687	0·447	0·254	0·247
0·6	0·540	0·468	0·194	0·251
0·4	0·435	0·566	0·155	0·301
0·3	0·340	0·590	0·142	0·368
0·2	0·232	0·604	0·097	0·377
0·1	0·142	0·739	0·080	0·622

Effect of phosphatide sols on the conductivities of salt solutions. The specific conductivities of calcium chloride and potassium chloride in the presence of phosphatides were compared with the specific conductivities of the salt solutions of the same concentration in water.

The reproducibility of the specific conductivities obtained on adding certain volumes of calcium chloride or potassium chloride solutions to water were within ± 1 per cent. Therefore changes in the specific conductivity less than this were not considered to be significant.

In each experiment 20 ml. of the phosphatide sol was placed in the conductivity cell which was then placed in a thermostat bath at 25° for 15 min. to attain constant temperature. The specific conductivity of the sol did not vary with time once temperature equilibrium had been attained. Small volumes (1 ml.) of salt solutions were added to the sol successively by means of a pipette.

The specific conductivity of the salt in water was calculated by subtracting the specific conductivity of the water from that of the salt solution. The specific conductivity of salt in the presence of a phosphatide sol was calculated by subtracting the specific conductivity of the phosphatide sol (after making a small correction to allow for the small dilution of the sol by the salt solution), from the specific conductivity of the sol containing salt.

The effects of varying the proportions of lecithin to lysolecithin in the sols on the conductivity of calcium chloride are given in Tables V and VI.

TABLE V

EFFECT OF PHOSPHATIDE SOLS (FREPARED BY METHOD A) ON THE SPECIFIC CONDUCTIVITIES OF CALCIUM CHLORIDE SOLUTIONS

Molar concentration of $CaCl_2$ ($\times 10^5$)	Specific conductivity due to $CaCl_2$ mhos $cm.^{-1}$ ($\times 10^{-5}$)	Weight fraction of lecithin in the mixtures							
		0	0·3	0·5	0·6	0·7	0·8	0·9	1·0
		Change per cent in specific conductivity of $CaCl_2$							
4·762	1·313	−0·53	+0·76	+0·30	−2·13	−2·22	−2·83	−3·12	−4·95
9·091	2·498	−0·88	+0·48	+0·28	−2·80	−3·12	−3·60	−3·96	−5·08
13·04	3·567	−0·73	+0·36	−0·39	−2·45	−2·38	−3·28	−3·28	−4·85
20·00	5·407	+0·64	+0·61	+0·11	−1·42	−1·98	−1·98	−2·68	−4·34
57·69	15·33	−0·26	−0·19	−0·20	−1·63	−1·74	−1·89	−1·83	−2·74
92·59	24·28	−0·04	−0·37	+0·33	−1·06	−1·12	−1·32	−1·28	−1·73
125·0	32·42	−0·55	−0·74	−0·77	−0·74	−0·24	−1·06	−1·17	−1·28
155·2	39·81	−0·15	−0·05	−0·70	−0·78	−0·18	−0·60	−0·48	−0·68
183·3	47·07	−0·44	−0·28	−0·06	−0·87	−0·25	−0·60	−0·17	−0·79
500·0	123·0	−0·27	+0·49	−0·32	−0·37	−0·65	−0·66	−0·24	−0·18

TABLE VI

PERCENTAGE CHANGES IN THE SPECIFIC CONDUCTIVITIES OF CALCIUM CHLORIDE SOLUTIONS IN THE PRESENCE OF PHOSPHATIDE SOLS (PREPARED BY METHOD B)

Concentration of $CaCl_2$ in moles/litre ($\times 10^5$)	Weight fraction of lecithin in mixture					
	0·3	0·5	0·6	0·8*	0·9*	1·0*
4·762	−7·10	−11·33	−13·14	−9·92	−11·63	−12·01
9·091	−6·00	−9·66	−11·37	−10·8	−10·97	−9·98
13·04	−6·61	−9·16	−11·18	−10·98	−10·90	−10·07
20·00	−3·72	−6·69	−7·99	−9·84	−8·18	−6·88
57·69	−2·19	−3·73	−4·96	−6·26	−5·86	−5·08
92·59	−1·10	−2·57	−3·47	−4·94	−4·57	−3·47
125·0	−1·94	−2·88	−3·73	−4·29	−4·15	−4·03
155·2	−1·52	−2·56	−3·02	−3·59	−3·63	−3·27
183·3	−1·32	−1·78	−2·82	−3·61	−4·03	−3·24
500·0	−0·93	−1·49	−1·72	−1·30	−1·24	−1·32

* Sols were opaque.

The effects of lecithin and lysolecithin on the conductivities of potassium chloride solutions are given in Table VII.

TABLE VII

PERCENTAGE CHANGES IN THE SPECIFIC CONDUCTIVITIES OF POTASSIUM CHLORIDE SOLUTIONS IN LECITHIN OR LYSOLECITHIN SOLS (0·5 PER CENT W/V)

Concentration of KCl in moles/litre ($\times 10^3$)	Lecithin	Lysolecithin
0·4762	+0·29	−0·73
0·9091	−0·82	−0·97
5·217	−0·73	−0·80
9·167	−0·23	−0·46
48·80	+0·86	+0·54
85·38	+0·10	0

DISCUSSION

From the results it can be seen that calcium chloride is much more effective in causing flocculation of lecithin sols than is potassium chloride. Flocculation starts at 6×10^{-5}M with calcium chloride, whilst with potassium chloride a concentration of 3×10^{-3}M is required. These results are in agreement with earlier studies which showed that the higher

the valency of the ions in the added salt the more effective was the coagulating effect on lecithin sols (DeJong and Teunissen, 1935; Remesov, 1930; Saunders, 1957).

Malquori (1932), Rona and Deutsche (1926) and Saunders and Elworthy (unpublished), have reported that divalent metal chlorides give two precipitation zones, with a region of peptisation between them. The results given in Table III show one region of precipitation and the beginning of the region of peptisation. If the effect of much higher concentrations of calcium chloride had been studied another region of precipitation would have been expected.

Conductivity studies indicate that some interaction occurs between lecithin and calcium chloride. It is possible that the calcium ions interact with the negatively charged phosphate group of the lecithin molecules, forming either an insoluble complex or cross-links between lecithin molecules in different micelles causing aggregation of the micelles to form a precipitate. Also it is possible that some calcium ions are adsorbed on to the micelles and neutralise their small negative charge and so cause them to aggregate. At higher concentrations of calcium chloride more calcium ions are probably adsorbed and the charge on the micelles may be reversed and consequently no precipitation occurs. This is supported by the fact that at higher concentrations of calcium chloride, conductivity studies indicate that the extent of interaction between lecithin and calcium chloride is increased. The narrowing of the range of concentration of calcium chloride over which flocculation occurs on increasing the proportion of lysolecithin in the sol, may be because lysolecithin solubilises or protects the "complex" formed between lecithin and calcium chloride and thus prevents its precipitation.

The precipitating action of potassium chloride is probably a salting out effect. The results given in Table VII show that there is no significant interaction between potassium chloride and lecithin or lysolecithin. The relatively high concentrations required to cause precipitations probably remove the water of hydration around the particles, thus facilitating their aggregation. Lecithin sols are protected by lysolecithin against the precipitating action of potassium chloride, probably because the hydration layer is much more difficult to remove from lysolecithin than from lecithin.

Lysolecithin was much more effective in protecting lecithin sols against potassium chloride than it was against calcium chloride. This is further evidence that the mechanisms of precipitation are different for the two salts. The physical appearance of the precipitates also differed. The precipitates caused by calcium chloride were bulky, whilst those caused by potassium chloride were much less so and more granular.

The very low conductivities exhibited by lecithin and lysolecithin sols indicate that the phosphatides exist as large, slightly negatively charged micelles in aqueous solution. The equivalent conductivity of lysolecithin sols is higher than that for lecithin sols and thus suggests that the size of the micelles is smaller in lysolecithin sols. This is in agreement with diffusion and light-scattering studies (Robinson and Saunders, 1959, 1960; Saunders and Thomas, 1958; Thomas and Saunders, 1959).

From Table V it can be seen that lysolecithin sols do not have a significant effect upon the conductivity of calcium chloride solutions. This finding is in agreement with the fact that calcium chloride does not have any effect upon the viscosity or diffusion rate of lysolecithin sols (Saunders and Thomas, 1958).

The effect of mixed sols on the conductivity of calcium chloride has been found to be dependent upon the method of preparation. The sols prepared by method A were clear if the weight fraction of lecithin present was 0·6 or below, and translucent if the weight fraction of lecithin was 0·7 or above. Only sols having a weight fraction of lecithin of 0·6 or above caused a significant fall in the conductivity of calcium chloride solutions, although an interaction probably occurred with sols having a weight fraction below 0·6, but the interaction was not large enough to cause a significant fall in the conductivity.

TABLE VIII

TABLE SHOWING THE MOLAR RATIO OF LECITHIN/$CaCl_2$ REACTED ($\times 10^{-2}$) FOR SOLS PREPARED BY METHOD A

Molar concentration of $CaCl_2$ ($\times 10^5$)	Weight fraction of lecithin				
	0·6	0·7	0·8	0·9	1·0
4·762	38·0	42·6	38·2	38·9	27·3
9·091	15·2	15·9	15·7	16·1	13·9
13·04	12·1	14·5	12·0	13·5	10·2
20·00	13·6	11·4	13·0	10·8	7·40
57·69	4·10	4·48	4·72	5·48	4·07
92·59	3·93	4·34	4·21	4·88	4·01
125·0	—	—	3·88	3·95	4·02

TABLE IX

TABLE SHOWING THE MOLAR RATIO OF LECITHIN/$CaCl_2$ REACTED ($\times 10^{-2}$) FOR SOLS PREPARED BY METHOD B

Molar concentration of $CaCl_2$ ($\times 10^5$)	Weight fraction of lecithin					
	0·3	0·5	0·6	0·8*	0·9*	1·0*
4·762	5·70	5·96	6·16	10·9	10·4	11·2
9·091	3·53	3·66	3·73	5·24	5·80	7·09
13·04	2·24	2·69	2·64	3·59	4·07	4·89
20·00	2·59	2·40	2·41	2·61	3·54	4·67
57·69	1·53	1·49	1·35	1·42	1·71	2·19
92·59	1·89	1·35	1·20	1·12	1·37	2·00
125·0	0·795	0·893	0·827	0·959	1·11	1·28
155·2	0·815	0·810	0·822	0·923	1·03	1·27
183·3	0·798	0·986	0·745	0·777	0·784	1·08
500·0	0·416	0·431	0·448	0·791	0·934	0·973

* Sols were opaque.

When the sols were prepared by method B, clear sols were again obtained when the weight fraction of lecithin present was 0·6 or below, whilst opaque sols were obtained with higher weight fractions of lecithin. Sols prepared by this method, contrary to expectations, caused larger changes in the conductivity of calcium chloride than did sols prepared by method A, and significant falls occurred with sols having a weight fraction of lecithin of less than 0·6.

The extent of interaction between the lecithin molecules and the calcium chloride can be estimated from the molar ratio obtained by dividing the number of moles of lecithin present by the number of moles of calcium chloride which have reacted with the lecithin. These ratios are given in Tables VIII and IX.

The results in Table VIII show that for a given concentration of calcium chloride the extent of interaction between the lecithin and calcium chloride is constant and the presence of lysolecithin does not appear to affect it. As the concentration of calcium chloride is increased, so the interaction is increased.

The results given in Table IX show that the interaction between lecithin and calcium chloride is far greater when the sols are prepared by method B. Even the sols having a weight fraction of lecithin of 0·7 or above, which were opaque and had very large particles present in them reacted to a greater extent with calcium chloride than did sols prepared by method A. With sols prepared by method B, the interaction seems to increase with increasing amounts of lysolecithin present. This is probably explained by the fact that the greater the amount of lysolecithin present in the sol, the greater will be the amount of lecithin which is completely solubilised, and consequently the greater the interaction between the lecithin and the calcium chloride. If all the lecithin had been completely solubilised, the extent of interaction would probably have been independent of the amount of lysolecithin present, since with clear sols having a weight fraction of lecithin of 0·6 or below the extent of interaction is independent of the lysolecithin content of the sols.

Calcium chloride interacts with lecithin, whilst potassium chloride does not. This may have a bearing on the different permeabilities of a cell membrane towards the two cations. The binding of calcium ions by lecithin may be the reason for the relative impermeability of a cell membrane to calcium ions, whilst the lack of such binding between potassium chloride and lecithin may be the reason for the permeability of a cell membrane to potassium ions.

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NEW APPARATUS

A MODIFIED PLETHYSMOGRAPHIC APPARATUS FOR RECORDING VOLUME CHANGES IN THE RAT PAW

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Oedema of the paws is one of the most easily recognised and characteristic outward signs of the anaphylactoid reaction in the rat, and many workers have used the degree of swelling as a measure of the severity of the reaction. Examples of such a measurement include the increase in weight of the paws (Halpern and Briot, 1950), the silhouette area of the paw recorded photographically (Bergel, Parkes and Wrigley, 1951), and the increase in water content of the paws (Rowley and Benditt, 1956). A

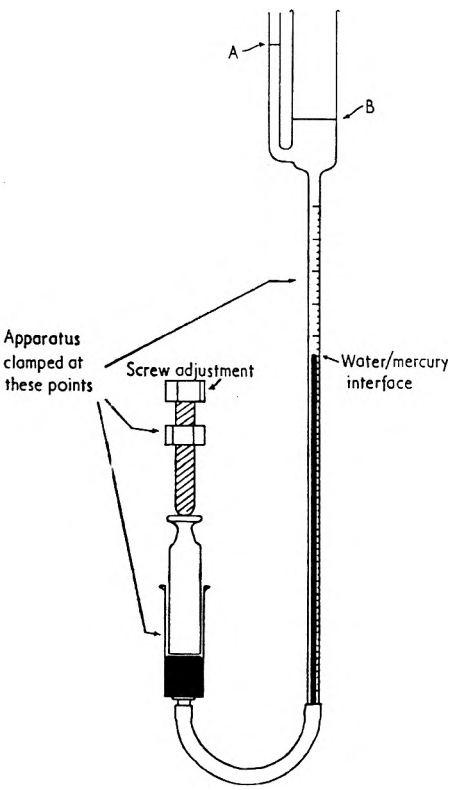

Fig. 1. The modified apparatus for measuring paw volume (see text for explanation).

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more rapid measurement of oedema in a rat paw may be made by a plethysmographic method (Wilhelmi and Domenjoz, 1951; Cerletti and Rothlin, 1955; Adamkiewicz, Rice and McColl, 1955). Buttle, D'Arcy, Howard and Kellett (1957) described a simple apparatus to measure paw volume, in which the paw of an anaesthetised animal was immersed to a pre-determined depth in water, the volume of water so displaced being the volume of that part of the paw immersed.

Two important modifications to this apparatus are now proposed: a side-tube has been added to the mouth of the apparatus, and mercury has replaced the carbon tetrachloride solution of sudan III in the lower part of the apparatus (see Fig. 1).

The modified apparatus consists of a 3 ml. microburette with a side-tube of approximately 4 mm. internal diameter fused to the reservoir above the stem. Thick-walled rubber tubing connects the base of the burette to a 5 ml. syringe. Mercury fills the syringe and lower part of the burette, whilst the upper part contains water to which a little surface-active agent (for example, Teepol) has been added. This ensures complete wetting of the paw during measurement. There are two marks on the upper part of the burette: A, in the side-tube, is approximately 1·5 cm. below the top of the burette, and B, in the burette reservoir, about 5 cm. below the top.

The animal is lightly anaesthetised with ether, and the hind-limb is shaved 24 hr. before the first measurement is made, care being taken to avoid injury to the skin. Before each measurement, the animal is re-anaesthetised with ether just sufficiently deeply to ensure that the limbs are flaccid. The level of the water meniscus is adjusted to mark A by means of the syringe and screw attachment, and the burette reading of the water: mercury interface recorded. The rat's paw is now placed into the mouth of the burette until the tip of the third toe coincides with mark B, thereby causing the water level to rise in the burette and side-tube. The syringe plunger is then withdrawn to bring the water meniscus back to mark A, the new reading of the water: mercury interface being recorded. It has been found in practice to be more accurate if, both before and after immersion of the paw, the syringe is first withdrawn a little too far and then the water level brought to the mark A from below. The difference between the two readings represents the volume of the immersed part of the limb.

The two modifications have been made to the original apparatus for the following reasons. With a side-tube fused on to the burette reservoir and the mark A transferred from the reservoir to this, the water meniscus remains clearly visible when the animal's paw is present in the burette reservoir, and this aids accurate adjustment of the water level. Mercury has a number of advantages over carbon tetrachloride. The carbon tetrachloride solution used in the original apparatus emulsified in the presence of the wetting agent, and made accurate measurement impossible. Also, when making a large number of readings, continuous evaporation of the carbon tetrachloride necessitated its frequent replacement.

Repeated measurements may be made on individual rats. However, we have found that prolonged anaesthesia with ether, urethane or the

barbiturates inhibits the anaphylactoid reaction to egg-white or dextran in rats. For this reason, severe ether intoxication must be avoided and measurements on individual rats should be made at not less than 10 to 15 min. intervals.

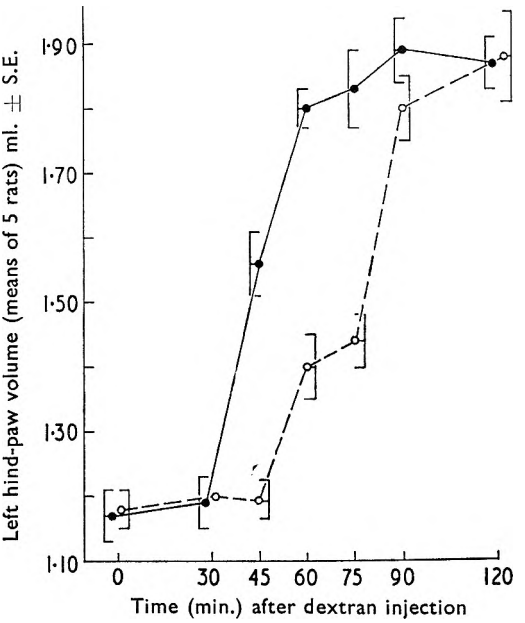

FIG. 2. Effect of intraperitoneal dextran (300 mg./kg.) on left hind-paw volume in untreated male rats (○- - -○), and rats pretreated with soluble insulin (20 units/kg.) 60 min. before challenge (●——●).

Fig. 2 shows a typical record of hind-paw volume changes after the intraperitoneal injection of dextran (300 mg./kg.) into untreated male rats and male rats pretreated with soluble insulin (20 units/kg.) 1 hr. before challenge.

Measurement of paw volume in these animals has revealed that insulin pretreatment hastens the onset of oedema, but does not affect the final degree of oedema after dextran injection.

This apparatus was first demonstrated at the Winter Meeting 1960, of the British Pharmacological Society.

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LETTER TO THE EDITOR

Absence of General Anaesthetic Properties in a Number of Terpenoid Hemisuccinates

SIR,—The demonstration of central depressant activity in a number of steroids (Seyle, 1942; Figdor and others, 1957 and refs. cited), one of which, sodium 21-hydroxypregnane-3,20-dione hemisuccinate (hydroxydione sodium) is employed clinically as a basal anaesthetic (e.g. Murphy, Guadagni and De Bon, 1955; Galley and Rooms, 1956) raises the question as to whether these compounds share a common mechanism of action with other general anaesthetics, many of which have been considered to act by a "physical" mechanism (Ferguson, 1939) in which favourable lipid solubility has long been considered a necessary prerequisite (see review by Butler, 1950). It has been further suggested (e.g. Warburg, 1921) that adsorption of the agent on or in the cell membranes produces the primary effects responsible for the anaesthesia and Mullins (1956) has presented a model of the membrane permitting visualisation of these changes. Stress is laid upon the importance of the molecular dimensions of the anaesthetic, an aspect which has also been emphasised by Wulf and Featherstone (1957). More recently, Pauling (1962) has divided general anaesthetics into two classes. Those capable of hydrogen bond formation, for example barbiturates and aliphatic alcohols, are regarded as specifically inhibiting the processes supplying energy for the maintenance of cerebral electrical activity, whilst those incapable of hydrogen bonding, for example the simple gaseous anaesthetics and the magnesium ion, are postulated to depress the electrical oscillations of the brain through hydrated microcrystal formation.

It seemed, therefore, to be of some interest to establish whether central depressant activity was present in alcohols possessing molecular weights between those of the simple aliphatic alcohols and those of the anaesthetically-active steroids. Since the water-soluble hydroxydione is known to suffer hydrolysis to the centrally-active lipid-soluble parent steroid (Figdor and others, 1957; Jakoby and Tomkins, 1956) we investigated several representative terpenoid hemisuccinates. Thus the hemisuccinates of menthol, borneol, citronellol, fenchol, farnesol, dehydroabietinol and podocarpinol were prepared by refluxing the alcohol with succinic anhydride in pyridine or quinoline and administered intravenously to mice in the form of their sodium salts, in doses of up to 500 mg./kg. These compounds, like the sodium salts of arachidyl, erucyl and dodecyl hemisuccinates, produced convulsions at or below this dose level and in no case was anaesthesia observed. Intraperitoneal injection into rats at doses up to 100 mg./kg. gave no effect. Hydroxydione itself is active in the mouse on intravenous injection at an AD50 of 21·5 mg./kg. (Figdor and others, 1957).

These results support the conclusions of Figdor and others (1957) that the steroidal general anaesthetics display a high degree of structural specificity as indicated by the marked reduction or loss of activity on hydroxylation, epoxidation or halogenation, or on introduction of nuclear unsaturation. Moreover, convulsant activity is present in certain analogues of hydroxydione and evidence has been advanced (Gordon and others, 1956) that the steroidal general anaesthetics act in a similar way to the barbiturates where it is well established that minor structural modifications can lead to convulsant activity (e.g. Swanson and Chen, 1939). It is tempting to view these facts in terms of Pauling's (1962) contention that anaesthetics capable of hydrogen bonding act by a specific mechanism and to regard the appearance of convulsant activity as a manifestation of retention of affinity for the receptors coupled with marked changes in intrinsic

activity (cf. Ariëns, 1954). If this picture is correct convulsant activity in these instances could result from a direct stimulant action, rather than from a preferential depressant action on the higher centres with functional release of automatic lower motor mechanisms as is generally considered to be the case in stage II, the excitement stage of general anaesthesia.

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