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 ($P < 0{\cdot}01$) 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 $P = < 0{\cdot}01$.
* 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.

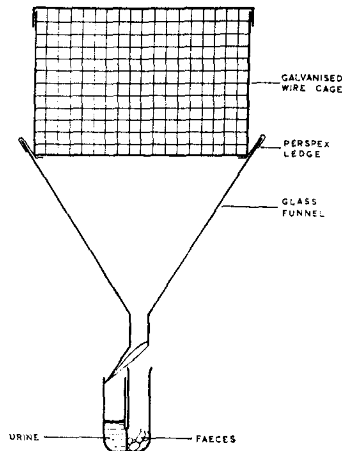

FIG. 1. A simple urino-faecal separator for use in metabolism experiments.

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.

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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 new-born 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: $P < 0{\cdot}01$.
* 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.

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.

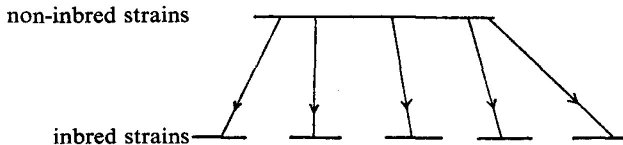
DR. F. J. C. ROE. In attempting to produce a uniform animal, the influence of litter size on rate of growth should not be forgotten. For absolute uniformity of animal, uniform litter size would be necessary. However, even this would not be sufficient, for within any one litter animals would still differ 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.