RESEARCH PAPERS

SOME PHARMACOLOGICAL PROPERTIES OF A NEW INTRAMUSCULAR IRON PREPARATION

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The pharmacological properties of a new intramuscular iron preparation containing 50 mg. of iron as an iron-sorbitol-citrate complex has been studied in animal experiments. The acute cardiovascular effects of intravenous injections, as well as toxicity in mice and rabbits, are reported. The preparation is stable *in vivo* and has no apparent antigenic properties.

Parenteral iron administration by the intramuscular route was made possible with the introduction of colloidal iron preparations which were well tolerated and produced no or minimal pain at the injection site. The intramuscular iron therapy has proved to be effective in the treatment of many iron-deficiency anemias. Disadvantages with this form of iron medication include pain and discolouration at the injection site (Karlefors and Nordén, 1958). The recent report of Haddow and Horning (1960) demonstrating sarcoma formation in animals after long-term administration of massive doses of iron-dextran has focused interest on the carcinogenic properties of intramuscular iron preparations. Though the exact cause of the sarcoma induction is not clear, inadequate absorption may be a factor of importance.

In the preparation of some new iron-sorbitol-citrate complexes in our laboratories (Lindvall and Anderson, 1961), special regard has been given to the absorption characteristics of these preparations. One such complex, Jectofer, is described by Lindvall and Andersson. In the present study some pharmacological properties of this complex will be presented and compared with those of an iron-dextran preparation.

Methods

The iron-sorbitol-citrate complex contained 50 mg. of iron per ml. and was used as sterile solutions with a pH of 7·5 $\pm$ 0·2. For comparison, an iron-dextran preparation Imferon, pH 5·8 containing 50 mg. iron per ml. or a preparation of saccharated oxide of iron, Intrafer, pH 10·9 containing 20 mg. of iron per ml. were used. The iron preparations will be referred to as iron-sorbitol, iron-dextran and saccharated oxide of iron respectively.

Acute Effects in Anaesthetised Cats and Rabbits

Cats (2–3·5 kg.) were anaesthetised with sodium pentobarbitone (35 mg./kg.) and rabbits (2–2·75 kg.) with urethane (1·2 g./kg.). Blood pressure, respiration (pneumotachogram) and heart rate were measured with conventional transducers and recorded on a Grass Model 5 Polygraph. In some instances peripheral vascular effects were studied by the

insertion of a constant volume perfusion pump (Sigmamotor, Inc., Middleport, N.Y.) in one femoral artery. Injections were made intravenously in one external jugular vein and intra-arterially, proximally to the pump.

Acute Toxicity

Male albino mice (17–24 g.) of an inbred strain were divided in three series of 160, 140 and 120 animals and injected by the intraperitoneal, intravenous and subcutaneous routes respectively. In each series the animals were further divided into groups of 20, each group receiving the same dose per unit weight. Injections were made with a 0·5 per cent saline dilution of iron-sorbitol. The ratio between successive doses was held constant at 1 : 2. The observation period was 7 days. The LD50 values were calculated by the method specified in the Scandinavian pharmacopoeia. Student's "*t*" test was used for testing the significance of the differences between the LD50 values.

Male albino rabbits (2–2·5 kg.) were divided into two series of 45 animals each and injected intravenously and intramuscularly. In addition, 20 male albino rats received intramuscular injections.

Subchronic Toxicity

Forty male albino rabbits, initial weight 1·1–2·5 kg., were kept in separate cages and given a diet of hay, oats, rabbit pellets, green vegetables and water *ad libitum*. They were observed for 7 days before treatment. The iron-sorbitol complex corresponding to 5 mg. Fe/kg. was given, in series I, to 10 animals intramuscularly (deep intragluteally) and to 10 animals intravenously, while 5 rabbits were kept as controls. In the 9 weeks of treatment the total dose corresponded to 215 mg. Fe/kg. In series II, 4 animals were injected intramuscularly and 4 intravenously with the same dose 5 days a week as in series I. Seven animals were kept as controls. In the 5 weeks of treatment of this series a total of 110 mg. Fe/ kg. was given. Blood samples of approximately 0·3 ml. were taken weekly from all animals, 10 ml. (by heart puncture) twice from each animal in series I, and once weekly in series II. Urine was collected, measured, and analysed for protein qualitatively by Esbach's reagent and quantitatively by the biuret method (Kingsley, 1941) involving measurement of the extinction at 540 mμ with a Beckman DU spectrophotometer. The amount of protein was calculated from a standard curve obtained by Kjeldahl analysis.

Haemoglobin concentration was determined according to King (1947), red cell counts by the method of Ellerman, hematocrit by conventional methods, iron in serum and iron binding capactity by procedures described by Lindvall and Andersson (1961) and serum proteins quantitatively by the biuret method as with urine. Paper electrophoresis was carried out on 10 μlitre aliquots of serum on 4 cm. paper strips (Whatman No. 1) in veronal buffer at pH 8·6 and a current of 0·5 mA/cm. for 16 hr. The strips were stained with bromphenol blue and scanned in a EEL densitometer.

All animals were killed 7 days after the last iron injection, the internal organs weighed and their iron content analysed. Histological specimens were fixed in ethanol, imbedded in paraffin and 10 μ sections were stained with hematoxylin-eosin, or for iron according to deVinal with the modification by Wöhler (1959) and counterstained with carmalum.

Antigenicity

Five guinea-pigs (209–243 g.) were injected intraperitoneally twice with 3 days interval with 0·35 ml. of a 1 : 50 saline dilution of iron-sorbitol (approx. 1·5 mg. Fe/kg.). Fourteen days later a challenging dose of about 2·5 mg Fe/kg. was given intracardially and observations made as recommended by the U.S. Pharmacopeia (1955). *In vitro* tests on isolated ilial segments from similarly treated guinea-pigs were also made. In

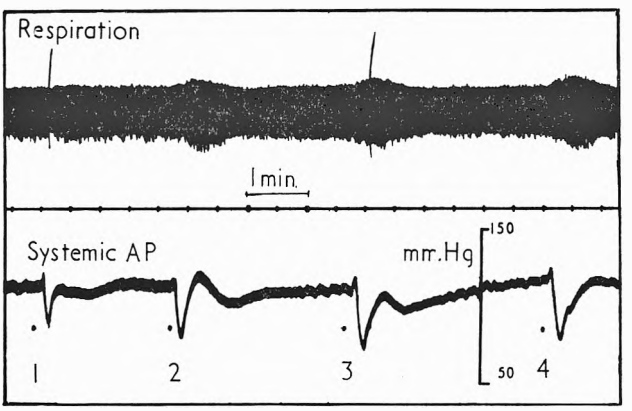

FIG. 1. Rabbit 2·65 kg. The hypotensive effect of increasing doses of intravenous iron-sorbitol. The dose at 1, 2, 3 and 4 corresponds to 2·8, 5·7, 11·3 and 22·6 mg. Fe/kg. respectively.

addition, antigenicity was studied by the agar gel-diffusion method (Ouchterlony, 1958) in 8 rabbits treated for 9 weeks with a total of 215 mg. Fe/kg. 1 ml. serum was tested against 1 ml. 0·5 or 0·1 per cent saline dilution of iron-sorbitol using the two-basin-plate technique.

RESULTS

Acute Effects in Anaesthetised Cats and Rabbits

Rapid intravenous injections in cats and rabbits of moderate doses (2–10 mg. Fe/kg. weight) of iron-sorbitol produced a fall in systemic blood pressure. The response was rapid, of short duration and, at the beginning of an experiment, often directly proportional to the dose injected (Fig. 1). The response of blood pressure and heart rate was qualitatively the same as that produced by acetylcholine (Fig. 2), and quantitatively, 3·5 mg. Fe/kg. as iron-sorbitol approximately corresponded to 0·1 mg. Fe/kg. as ferrous sulphate or 5·3 mg. Fe/kg. as iron-dextran. After repeated doses tachyphylaxis usually developed. The hypotensive effect was also greatly

influenced by the rate of injection. If the rate of injection was less than 1·5 mg. Fe/kg./min. the response was absent. The depressor effect of intravenous iron-sorbitol was not influenced by full doses of atropine or antihistamine drugs. Iron-sorbitol, like iron-dextran, antagonised the action of subsequently injected adrenaline. This antagonism lasted about

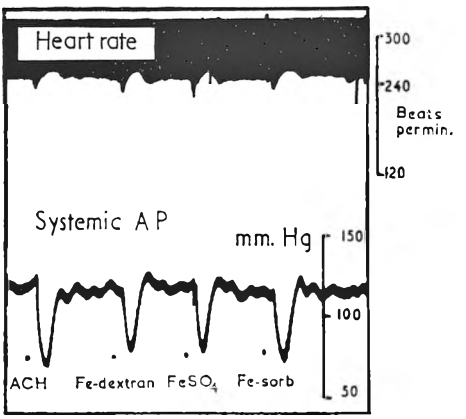

FIG. 2. Cat 2·85 kg. Comparison between the depressor effects of intravenous acetylcholine (ACH 1 μg.), iron(Fe)-dextran (5·3 mg. Fe/kg.), ferrous sulphate ($FeSO_4$, 0·1 mg. Fe/kg.) and iron(Fe)-sorbitol (5·3 mg. Fe/kg.).

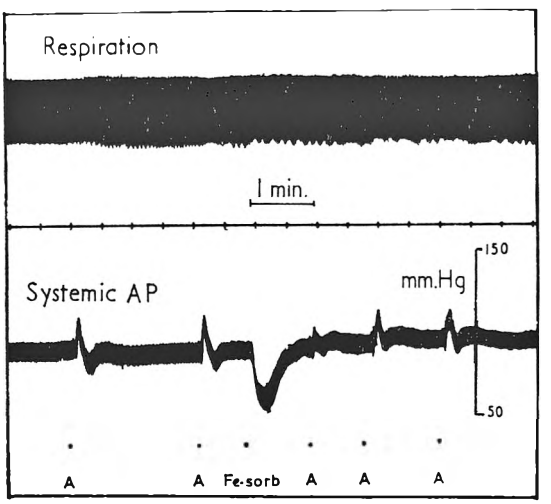

FIG. 3. Rabbit 2·75 kg. The adrenaline-antagonistic effect of i.v. iron-sorbitol (5·5 mg. Fe/kg.). At A, 4 μg. of adrenaline was administered intravenously.

two min. (Fig. 3). Recordings of the pressure in the femoral artery, perfused at constant rate, showed that local intra-arterial injection of iron-sorbitol (60 mg. Fe/kg.) was followed by only a slight fall in peripheral resistance. A similar result was obtained with iron-dextran. Fractionated doses of iron-sorbitol given by this route up to a total amount

of 100 mg. Fe/kg. did not elevate peripheral resistance as might have been expected if intravascular precipitation had taken place. On the other hand 1 mg. Fe/kg. given as ferric chloride, which precipitates when mixed with blood, and 30 mg. Fe/kg. as saccharated oxide of iron both caused an abrupt and long-lasting increase in peripheral resistance (Fig. 4).

With rapid intravenous injections of increasing doses of iron-sorbitol (3·5–350 mg. Fe/kg.) the magnitude of the fall in pressure was no longer related to the dose, but the duration of the pressure-drop progressively increased. When this occurred the duration of the acetylcholine response was also augmented. This would seem to indicate that large doses of the iron-sorbitol depressed the compensatory vasomotor mechanisms. However, considerable amounts of iron-sorbitol in fractionated doses,

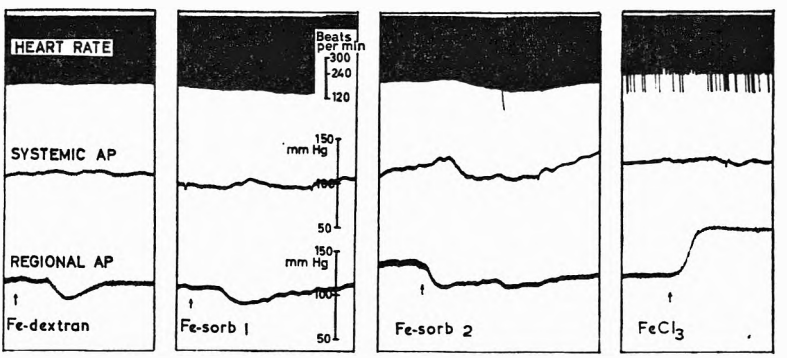

FIG. 4. Cat 2·4 kg. The effect on peripheral resistance of intra-arterial injections of iron(Fe)-dextran (4·5 mg. Fe/kg.), iron(Fe)-sorbitol (4·5 mg. Fe/kg. at 1 and 56·3 mg. Fe/kg. at 2) and ferric chloride ($FeCl_3$ 1 mg. Fe/kg.).

totalling up to 2 g. Fe/kg. in 3 hr., could be given in these acute experiments to the anaesthetised cats without significant changes in the resting blood-pressure level.

Respiration in rabbits was largely unaffected by intravenous iron-sorbitol in doses below 100 mg. Fe/kg., higher doses producing episodes of apnoea. Cats seemed less sensitive in this respect and doses of 170 mg. Fe/kg. given in rapid succession did not noticeably influence respiration. The normal E.E.G. pattern was not significantly influenced unless very high doses (1–2 g. Fe/kg.) were given, which produced a pronounced fall in blood pressure and imminent death.

Acute toxicity. In mice the intravenous LD50 was found to be 35·5 $\pm$ 1·33 (S.E.) mg. Fe/kg. weight. By the intraperitoneal and subcutaneous routes the average lethal doses were 50·25 $\pm$ 2·05 mg. Fe/kg. and 35·50 $\pm$ 1·08 mg. Fe/kg. respectively. In rabbits the intravenous and intramuscular LD50 values were 38·0 $\pm$ 5·3 and 38·0 $\pm$ 3·2 mg. Fe/kg. respectively. In rats the intramuscular LD50 was approximately 48 mg. Fe/kg.

Subchronic toxicity. The weight gain of the iron-sorbitol treated rabbits was similar to that of the control animals. The rabbits appeared healthy and had a normal appetite. The frequent injections seemed to

cause no discomfort. In series I (25 animals) given a total of 215 mg. Fe/kg., one rabbit in each of the intravenous, intramuscular and control groups died during the course of the experiment. The animal in the intravenous group died after 2 days, the other 2 animals died after 6 and 5 weeks respectively. All 3 animals had diarrhoea before death and except

TABLE I

THE EFFECT OF IRON-SORBITOL TREATMENT ON HAEMOGLOBIN CONCENTRATION (MG./100 ML.)

	Days of treatment	i.m. route	i.v. route	Control
Series I Total dose 215 mg. Fe/kg.	0	9·8 ± 0·1	9·6 ± 0·5	10·9 ± 0·5
	12	10·0 ± 0·2	10·2 ± 0·3	8·8 ± 0·5
	27	11·8 ± 0·3	10·9 ± 0·4	8·2 ± 1·1
	62	11·8 ± 0·3	11·4 ± 0·6	8·2 ± 0·3
Series II Total dose 110 mg. Fe/kg.	−2	9·9 ± 0·3	9·1 ± 0·2	10·4 ± 0·3
	8	10·3 ± 0·8	11·0 ± 0·4	10·4 ± 0·2
	14	8·6 ± 0·1	8·5 ± 0·2	9·4 ± 0·2
	22	9·3 ± 0·8	10·0 ± 0·7	10·1 ± 0·5
	28	11·7 ± 1·3	10·4 ± 0·4	10·0 ± 0·6
	36	10·7 ± 0·3	10·3 ± 0·3	11·0 ± 0·4

for signs of enteritis, postmortem examination was negative. In series II (15 rabbits) receiving a total of 110 mg. Fe/kg., 4 rabbits died as a result of the trauma of heart puncture.

Iron-sorbitol did not cause any obvious change in urine production nor did it produce proteinuria. During the 7 days' observation period before treatment small amounts (<50 mg./24 hr.) of protein were found in the urine of 4 rabbits on five occasions. During iron-sorbitol treatment the

TABLE II

THE EFFECT OF IRON-SORBITOL TREATMENT ON HEMATOCRIT (H) AND RED CELL COUNT (RBC) IN SERIES II, RECEIVING A TOTAL OF 110 MG. FE/KG.

Days of treatment	Administration					
	i.m.		i.v.		Control	
	H	RBC × 10^6	H	RBC × 10^6	H	RBC × 10^6
−2	29·0 ± 14·8	5·4 ± 0·1	30·0 ± 2·0	5·3 ± 0·1	34·0 ± 3·5	5·3 ± 0·1
8	30·3 ± 3·0	5·2 ± 0·2	28·3 ± 2·8	5·1 ± 0·1	32·0 ± 1·6	5·6 ± 0·2
14	31·2 ± 0·8	5·0 ± 0·1	31·3 ± 0·9	4·7 ± 0·1	31·0 ± 1·3	5·3 ± 0·3
22	35·5 ± 2·5	4·8 ± 0·5	37·3 ± 2·7	4·7 ± 0·1	36·2 ± 1·7	4·8 ± 0·2
28	42·3 ± 3·6	5·1 ± 0·6	40·0 ± 0·6	5·3 ± 0·1	36·2 ± 1·7	4·8 ± 0·1
36	40·5 ± 1·4	5·2 ± 0·3	38·7 ± 0·7	5·1 ± 0·1	41·2 ± 1·6	5·0 ± 0·3

urine from 3 different rabbits showed a positive protein test on five different occasions. The amount of protein excreted in these few instances was less than 80 mg. per 24 hr. By paper electrophoresis and chemical estimation no qualitative or quantitative changes in serum proteins were found in the treated animals.

The haematological effects of the iron-sorbitol treatment are summarised in Tables I and II. No consistent changes were found in either haemoglobin concentration, hematocrit value or red cell count. The intramuscular and intravenous groups did not differ significantly from each other or from the control group (Student's "*t*"-test).

In the rabbits of series I, the transferrin, when determined after a total of 215 mg. Fe/kg., was found to be saturated in 5 out of 6 animals. The mean value for iron in serum in these 5 animals was 290 ± 20 μg. per cent. The values for iron in serum and the unsaturated iron-binding capacity in series II showed much individual variation both before and during treatment and no consistent changes could be attributed to the iron-sorbitol administration.

At post-mortem examination no excess pleural or peritoneal fluid was observed. There was no discolouration of the internal organs except for the livers, which in some animals showed a deeper reddish-brown colour than those of the controls. In the intramuscularly treated rabbits the lymph nodes in the groin displayed a light-brown colour. At the sites of injection the muscles were markedly brown coloured and in 3 animals necrotic areas were observed. The organ : body weight ratios for liver, spleen and kidney of the treated rabbits did not differ significantly from

TABLE III

THE EFFECT OF IRON-SORBITOL TREATMENT ON IRON CONTENT (MG./G. WET WEIGHT) AND TISSUE/WEIGHT RATIO OF INTERNAL ORGANS. SERIES I.

	Administration					
	i.m.		i.v.		Control	
Tissue	Fe	weight ratio	Fe	weight ratio	Fe	weight ratio
Liver	0·59 ± 0·03	3·53 ± 0·16	0·54 ± 0·06	3·86 ± 0·21	0·06 ± 0·01	3·30 ± 0·27
Kidney ..	0·28 ± 0·03	0·58 ± 0·03	0·25 ± 0·03	0·61 ± 0·01	0·05 ± 0·01	0·67 ± 0·04
Spleen ..	0·29 ± 0·02	0·07 ± 0	0·32 ± 0·14	0·07 ± 0	0·05 ± 0·01	0·08 ± 0·01
Lungs ..	0·086 ± 0·014	0·47 ± 0·02	0·103 ± 0·011	0·51 ± 0·02	0·03	0·61 ± 0·06
Heart	0·03	0·35 ± 0·05	0·03	0·57 ± 0·04	0·04	0·30 ± 0·02
Inj. site ..	0·54 ± 0·05	—	—	—	0·02	—

those of the control animals (Table III). The greatest concentration of iron was found at the injection site in the muscle and in the liver (Table III). Iron values well above the control were also seen in spleen, kidney and lungs, while the iron level in the heart was not significantly changed.

The liver contained large quantities of stainable iron mainly localised to the portal areas. Conglomerates or iron granules were contained within the Kupffer cells and also in the sinusoidal lining cells. In the peripheri of the lobules, the parenchymal cells were filled with smaller iron granules. There were no signs of tissue damage. In the spleen, large iron deposits were observed in the capsule and in the fibrous trabeculae, sometimes to such an extent that the cell limits were difficult to distinguish. Aggregates of iron granules were observed in macrophages and apparently also extracellularly in the sinusoids of the red pulp. The sinusoidal lining substance displayed a diffuse blue colour. None or only small quantities had entered the lymphoid tissue which seemed to be normal in total amount. The general pattern for the distribution of iron in these organs did not differ essentially from that previously described for the iron-dextran and saccharated oxide of iron complexes (Nissim 1953, Pinninger 1956).

In the kidney the iron content was confined to the cortical substance. The epithelial cells of the proximal tubules contained fine iron granules

in the cytoplasm. Some of these cells showed disintegration of the cell membrane. This lesion is probably of the same nature as that observed by Golberg (1958) since the amount of damage seemed to be related to the time elapsed between autopsy and fixation of the tissue sample. In the heart and brain no significant amounts of iron and no pathological changes were observed.

The injection site showed large deposits of iron mainly in the connective tissue surrounding the muscle bundles. A large number of iron-filled macrophages were observed in the fatty tissue and occasionally interstitially in the muscle tissue.

Antigenicity. In none of the three different tests could any antigenic properties of iron-sorbitol be observed. There were no signs of anaphylaxis in the sensitised guinea-pigs when they were injected with the challenging dose intracardially. The isolated ilial strips from sensitised guinea-pigs did not respond to iron-sorbitol added to the bath. In the gel-diffusion experiments no reaction was observed when iron-sorbitol was tested against sera from the rabbits treated for 9 weeks with a total of 215 mg. Fe/kg.

Discussion

The acute toxicity of the iron-sorbitol is high when compared with the iron-dextran or the best preparations of saccharated oxide of iron (Martin and others 1955). This is presumably the consequence of the high diffusibility of the complex (Lindvall and Andersson 1961). Intravenous or intra-arterial injections to anaesthetised cats and rabbits are well tolerated and the effects are comparable to those of iron-dextran. After rapid i.v. injections to cats equi-effective depressor doses of iron-dextran, iron-sorbitol and ferrous sulphate showed a 60:40:1 relationship. The hypotensive effect of iron-sorbitol and iron-dextran seems to be due to the presence in small amounts of ferrous iron, which has been shown by Rajapurkar (1960) to exert an adrenergic blocking action. Chemical analysis by Dr. Lindvall revealed that both iron-dextran and iron-sorbitol contain ferrous iron and although the depressor effect is not directly proportional to the amount it shows the expected relationship.

The acute experiments on anaesthetised animals further demonstrated a good *in vivo* stability of the iron-sorbitol complex. When injected intra-arterially into the hind leg of the cat perfused with blood at a constant rate the peripheral resistance did not increase. Saccharated oxide of iron, and ferric chloride, in similar experiments augmented the peripheral resistance presumably as a result of intravascular precipitation. The absence of proteinurea in the subchronic experiments in rabbits also indicates that the iron-sorbitol complex is stable *in vivo* in the sense that it does not precipitate or cause precipitation of blood constituents. For the ultimate usefulness of iron-sorbitol the apparent lack of antigenic properties will also be of importance.

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MECHANISM OF ABSORPTION OF TWO INTRAMUSCULAR IRON PREPARATIONS

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The mechanism of absorption from an intramuscular depot of an iron-sorbitol-citrate and an iron-dextran complex has been studied in anaesthetised cats in which the lymph vessels were cannulated. The results are discussed in relation to the molecular size of the two iron complexes.

In a recent study from this laboratory (Lindvall and Andersson 1961) the properties of an iron-sorbitol-citrate complex for intramuscular injection were described. This complex was rapidly absorbed from the injection site in rabbits. In the following experiments the mechanism of absorption from the intramuscular injection site in cats was studied and the iron-sorbitol-citrate preparation compared with an iron-dextran complex.

Methods

The iron-sorbitol-citrate complex, Jectofer, and the iron-dextran complex, Imferon, both contained 50 mg. of elementary iron per ml. and the pH of the solutions were 7·5 and 5·8 respectively. They will be referred to as iron-sorbitol and iron-dextran.

Cats, 3·0–5·0 kg., were anaesthetised with pentobarbitone sodium (35 mg./kg.). After laparotomy the thoracic duct was cannulated 5–10 mm. above the entry of the intestinal lymphatics with polyethylene tubing. The iron preparations were injected deep in the gluteal region in doses corresponding to 3 mg. Fe/kg. The injected leg was mechanically exercised throughout the observation period and the flow of lymph was continuously collected in test tubes. Arterial blood samples (7 ml.) were taken 6–8 times during an experiment and after each sample, the same volume of Ringer's solution was given intravenously. Control experiments showed that the sampling of blood did not affect the analytical values if the sampling was not repeated more than eight times at 15 min. intervals.

The analytical procedures for the determination of iron in serum, unsaturated iron-binding capacity (UIBC) and lymph iron were those described by Lindvall and Andersson (1961). The analytical values for iron in serum include plasma-bound iron and circulating iron preparation. The sum of iron in serum and UIBC does thus not represent true total iron binding capacity and is therefore denoted "TIBC." Paper electrophoresis of lymph collected during the experiments, as well as controls consisting of lymph mixed with iron-sorbitol preparation, was carried out on Whatman No. 1 paper in veronal buffer of pH 8·6. The paper strips were stained for iron with acid potassium ferrocyanide and scanned by a EEL densitometer.

Results

In preliminary experiments the iron concentration of the serum was studied after intramuscular injection of iron-sorbitol into normal cats

with or without the lymph vessels from the injected leg being ligated. It was observed that the iron in serum rose in both instances but the rate of rise with ligated lymph vessels was slower. Thus at least part of the dose was absorbed directly into the blood stream.

In subsequent experiments the lymph flow was collected and blood samples taken at regular intervals during 2 hr. after the injection of the iron-sorbitol or the iron-dextran preparations. In these experiments,

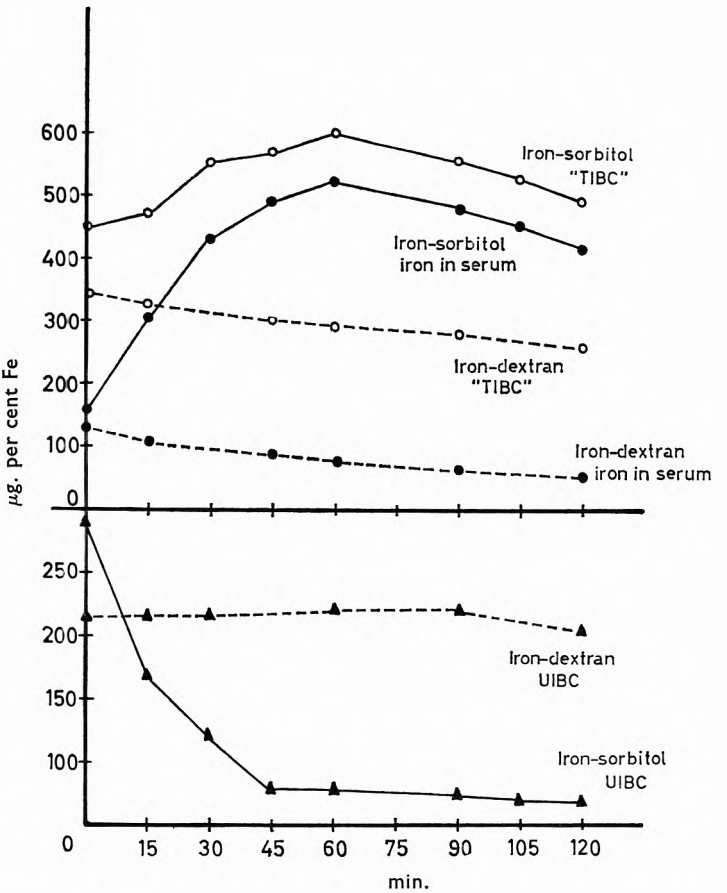

FIG. 1. Cats 3·0 and 3·2 kg., cannula in the thoracic duct. Iron-sorbitol (———) and iron-dextran (– – – –) respectively were injected i.m. at zero time. Dose 3 mg. Fe/kg. UIBC = unsaturated iron binding capacity "TIBC" = sum of iron in serum and UIBC.

where the lymph from the injection site was prevented from entering the blood, analysis showed that with iron-sorbitol a large elevation of the values for iron in serum occurred with a simultaneous decrease in unsaturated iron-binding capacity. With iron-dextran no such changes were noted, indicating that it was not absorbed by the blood. A typical experiment is illustrated in Fig. 1.

Fig. 2. shows the result from analysis of the lymph from the same animals as are represented in Fig. 1. The iron content of the lymph starts to rise almost immediately with iron-sorbitol while for iron-dextran there seems to be a certain delay in the absorption of iron. In some experiments the absorption of iron-dextran into the lymph, once started, proceeded with practically the same rate as that of iron-sorbitol, while in others, the iron-dextran was absorbed much more slowly. This was not related to the flow of lymph. Consequently iron-sorbitol absorption in the lymph

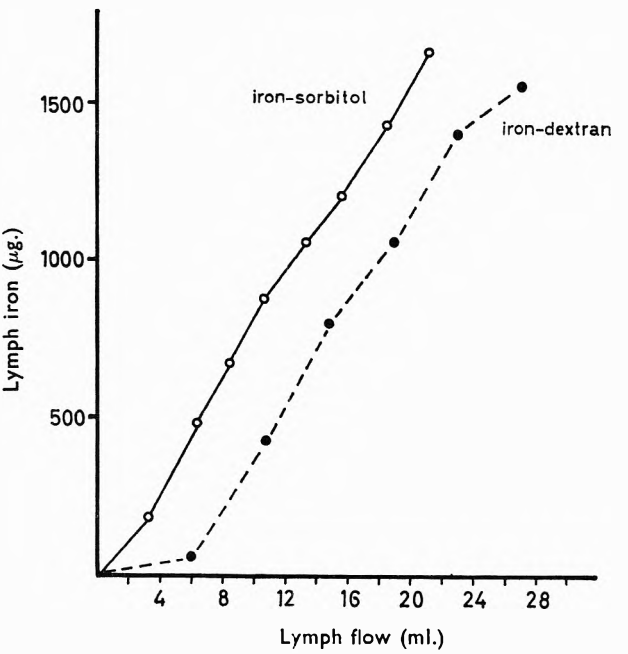

FIG. 2. Same cats as in Fig. 1. Iron in lymph after i.m. injection of iron-sorbitol (———) and iron-dextran (– – – –).

ranged from 10–16 per cent of the dose whereas iron-dextran absorption was more variable and ranged from 1–15 per cent during the same 2 hr. period.

Because of the complexity of the electrophoretic staining pattern of iron-sorbitol, no definite conclusions can be drawn about the iron component of the lymph except that the fastest moving fraction of iron-sorbitol with the lowest molecular weight (Lindvall and Andersson 1961), seen in control studies where the preparation was mixed with normal lymph, seems to be missing.

DISCUSSION

Molecular size is of predominant importance in the absorption of a compound from an intramuscular depot since it determines the route and to some extent also the rate of absorption. This has been shown for toxins and snake venoms by Barnes and Trueta (1941) who found that venoms

of molecular weight 5,000 were absorbed directly by the blood stream, while toxins and venoms with molecular weights above 20,000 were taken up and transported by the lymphatics. Everett and others (1954) demonstrated that the lymphatics play an insignificant role in the absorption from a subcutaneous site of inorganic iron, whereas protein-bound iron was absorbed almost exclusively by this route. Beresford, Golberg and Smith (1957) presented evidence that the iron-dextran complex is absorbed lymphatically after intramuscular injection. Though no direct data on the molecular weight have been given, Beresford and others state that the molecular dimensions of the iron-dextran complex are such as to make the absorption by the lymph the most important contribution.

The iron-sorbitol complex used in this study has been found to be composed of several fractions with different molecular weights. Our colleague, F. R. Eriksson tells us they all, however, fall below 5000.

The rapid decline in unsaturated iron-binding capacity (UIBC in Fig. 1) after iron-sorbitol injection indicates that at least one fraction immediately reacts with the transferrin. Since the total iron-binding capacity is increased, additional amounts of iron—not reacting with transferrin—must have entered the blood. Because of the rapid elimination of iron-sorbitol from the bloodstream (Lindvall and Andersson 1961) it is not possible to estimate directly the amount of iron-sorbitol absorbed by the blood. Indirectly, however, a rough estimate may be made by subtracting from the total dose those quantities of iron retained in the muscle (20 per cent) and those recovered in the lymph (16 per cent). If allowance is made for some iron being retained in the lymph nodes, 50–60 per cent of the injected dose of iron-sorbitol seems to have been absorbed by the blood in the 2 hr. experimental period.

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MULTIPLE EFFECTS OF γ-RESORCYLIC ACID ON INTERMEDIARY METABOLISM

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γ-Resorcylic acid inhibited glutamic-pyruvic and glutamic-oxaloacetic transaminase activities in extracts of rat liver, kidney, brain and heart. Its effects on the distribution of radioactivity from [3-^{14}C] pyruvate among the soluble metabolic intermediates of chopped preparations of the rat tissues were consistent with this interference with transaminase enzymes. However, when [^{14}C] glucose and [1,4-^{14}C] succinate were incubated with isolated mitochondria and with a soluble fraction prepared from rat liver, the resorcylic acid produced marked changes in the incorporation of the radiocarbon into malic acid and fumaric acids, into an oligosaccharide fraction and into various phosphate compounds concerned in glycolytic reactions.

THE modes of action of antirheumatic drugs are obscure. Relatively little is known about the mechanisms by which such chemically diverse substances as steroids, salicylates and antimalarials produce their beneficial effects on the inflammatory processes which form a major part of the reaction of the body in rheumatic disease. One method of approach is to study the effects of the drugs on cellular metabolism and to attempt the correlation of any defined biological action with their anti-inflammatory properties. As a necessary preliminary the metabolic actions of the antirheumatic drugs must be discovered and explored. γ-Resorcylic acid (2,6-dihydroxybenzoic acid) has been reported to be an effective agent in the treatment of rheumatic fever (Reid, Watson, Cochran and Sproull, 1951). Its most pronounced biochemical action is an inhibition of rat serum glutamic-pyruvic transaminase activity *in vitro* (Steggle, Huggins and Smith, 1961). The present paper is concerned with a more detailed investigation of this property with particular reference to the effects of the drug on tissue metabolism. The inhibitory activity against the enzyme has been shown to occur in extracts of various rat tissues. The observed effects of resorcylic acid on the distribution of radioactivity from labelled pyruvate among the soluble metabolic intermediates of chopped preparations of the rat tissues were consistent with the hypothesis that the drug primarily affects transaminase enzymes. However, when labelled glucose and succinate were used in conjugation with rat liver preparations such as homogenates, mitochondria and a soluble fraction, it became evident that resorcylic acid produced multiple effects on intermediary metabolism.

EXPERIMENTAL

Tissue Preparations

Adult rats (wt. 200–250 g.) of the Wistar strain, maintained on M.R.C. cube diet No. 41, were killed by cervical fracture. Chopped suspensions

of liver, kidney, brain and heart muscle were prepared according to the directions of Smith and Moses (1960). Liver homogenates were prepared in 0·25M sucrose (5 g. wet weight of liver in 5 ml. sucrose solution) using a Potter-Elvehjem homogeniser with a Teflon pestle. Liver mitochondria, separated from 4 g. wet weight of liver by the method of Schneider and Hogeboom (1950), were suspended in 1 ml. of 0·25M sucrose. A soluble fraction from liver was prepared by centrifuging a 1:5 homogenate in 0·25M sucrose for 30 min. at 105,000 *g* in a Spinco preparative ultracentrifuge and discarding the pellet.

Radioactive Experiments

A solution of radioactive substrate (5μc in 5μl.) was added to 200μl. samples which contained 1–2 mg. dry weight of the tissue preparation. Resorcylic acid solution (10μl.) was added to produce a final concentration of 5 mM and an equivalent quantity of the appropriate incubation medium was added in the corresponding control experiments. The radioactive substrates, [^{14}C] glucose (72·90μc/μmole); sodium [3-^{14}C] pyruvate (3·62μc/μmole) and [1,4^{14}C] succinic acid (10·80μc/μmole) were obtained from The Radiochemical Centre, Amersham, Bucks. The chopped preparations were incubated for 3 hr. at 37° in the medium of Hastings, Teng, Nesbett and Sinex (1952) and the homogenates and subcellular fractions for 30 min. at 37° in 0·01M phosphate buffer containing (m-mole/l.): KCl, 10; $MgSO_4.7H_2O$,2; cytochrome C, 0·03 and ATP, 1. All the incubation media, with the exception of that used for the mitochondria, contained 10 mM glucose. At the end of the incubation period the tissue preparations were killed by the addition of 200μl. of boiling ethanol. The radioactively labelled intermediates were extracted, separated by two-dimensional paper chromatography, visualised by radio-autography and the ^{14}C measured by the techniques described previously (Smith and Moses, 1960).

Measurement of Transaminase Activities

The tissues (liver, kidney, brain or heart muscle) were homogenised in a Waring blendor for 3 min. in 2–3 volumes of 0·01M phosphate buffer at pH 7·6. After centrifuging for 60 min. at 11,000 *g* the supernatant was separated, dialysed for 18 hr. against 0·01M phosphate buffer and the pH readjusted to 7·6. Glutamic-pyruvic and glutamic-oxaloacetic transaminase activities were measured in the dialysates by the method of Reitman and Frankel (1957).

Results

The results in Table I show that 5 mM resorcylic acid significantly inhibited glutamic-pyruvic transaminase activity in extracts from rat liver, kidney, brain and heart muscle. Although the inhibition of the glutamic-oxaloacetic activity in the tissue extracts was less pronounced, all the tissue preparations showed significant effects with the drug.

The percentages of radiocarbon' from [3-^{14}C] pyruvate which were incorporated into the soluble metabolic intermediates of the four tissues

in the presence or the absence of resorcylic acid are given in Table II. In the absence of the drug the qualitative patterns of incorporation of the isotope were similar for all the tissues. Most of the radioactivity was found in alanine and lactic acid which were derived from the pyruvate

TABLE I

INHIBITION OF GLUTAMIC-PYRUVIC AND GLUTAMIC-OXALOACETIC TRANSAMINASE ACTIVITIES IN RAT SERUM BY 5 mM γ-RESORCYLIC ACID

The results which are expressed as mean inhibitions per cent (± S.E.M.) have been analysed by the *t*-test and values of P are included. The minimum acceptable level of significance has been taken as P = 0·02. The number of observations are given in brackets.

Tissue	Glutamic-Pyruvic	P	Glutamic-oxaloacetic	P
Liver	81·5 ± 3·1 (8)	0·001	6·8 ± 0·6 (6)	0·001
Kidney	42·4 ± 4·1 (8)	0·001	22·7 ± 3·2 (5)	0·01
Brain	63·3 ± 1·3 (8)	0·001	9·9 ± 1·9 (6)	0·01
Heart	17·7 ± 0·8 (8)	0·001	11·5 ± 2·0 (6)	0·01

itself by transamination and dehydrogenation reactions respectively. A considerable proportion of the isotope was also incorporated into the amino-acids, glutamic and aspartic, which were formed from their corresponding α-keto acids (α-ketoglutaric and oxaloacetic) by transamination. The occurrence of radiocarbon in these compounds, as well as in citric and malic acids, is evidence that the pyruvate carbons entered

TABLE II

METABOLISM OF [3-^{14}C] PYRUVATE BY ISOLATED RAT TISSUES IN THE PRESENCE OR THE ABSENCE OF 5 mM γ-RESORCYLIC ACID

Results expressed as the total per cent ^{14}C incorporated from the labelled substrate into the sum of all the separated soluble intermediates; the ^{14}C in the residual substrate being excluded

Soluble Intermediate	Liver		Kidney		Brain		Heart	
	None	Resorcylate	None	Resorcylate	None	Resorcylate	None	Resorcylate
Alanine	61	31	30	25	18	9	51	32
γ-Aminobutyric acid	0	0	1	0	9	5	0	0
Aspartic acid	3	8	9	11	6	5	3	2
Glutamic acid	9	24	30	33	38	42	6	7
Citric acid	1	1	0	0	7	5	10	12
Lactic acid	25	33	25	29	14	28	19	37
Malic acid	1	0·5	4	3	3	2	6	6
Phosphates	1	2	1	2	0·4	1	2	2
Unidentified compounds	0·3	2	0	0	4	3	2	2

the tricarboxylic acid cycle. The distribution of the radioactivity from the labelled pyruvate among the soluble intermediates is illustrated in the radioautogram (Fig. 1).

The major effect produced by resorcylic acid was a substantial reduction in the formation of labelled alanine accompanied by increased incorporations of radioactivity into the other fractions.

The results in Table III show the distribution of radioactivity from labelled succinate among the soluble intermediates of various preparations of rat liver. These comprise a chopped preparation, a homogenate and a

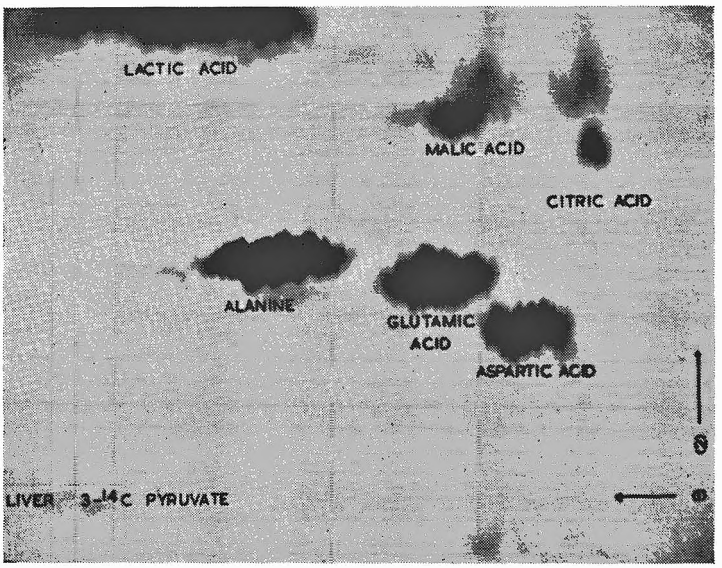


FIG. 1. Radioautogram of paper chromatogram showing distribution of radioactivity from [3-^{14}C] pyruvate among the soluble metabolic intermediates of a chopped preparation of rat liver. In this, and the subsequent figures, the solvent systems used for chromatography are as follows: (1) phenol-water; (2) butanol-propionic acid-water. The chromatograms represent extracts prepared from one-fifth of the reaction mixture. Exposed 14–21 days. U.K. represents unidentified compounds.

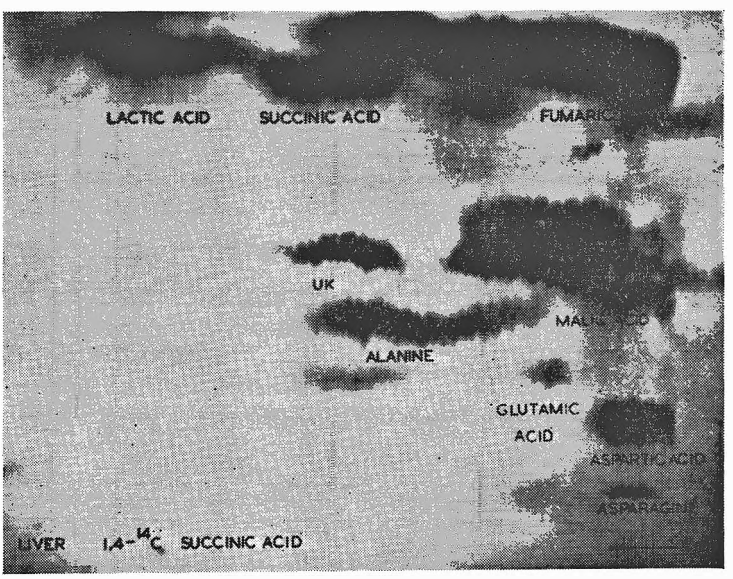


FIG. 2. Distribution of radioactivity from [1,4-^{14}C] succinate among the soluble metabolic intermediates of a chopped prepation of rat liver.

suspension of mitochondria. The radioautogram (Fig. 2) shows the distribution of ^{14}C in the chopped preparation. The soluble fraction prepared from rat liver did not metabolise the labelled succinate. In the control experiments, malic and fumaric acids were the compounds which contained the major proportion of the radioactivity with smaller amounts

TABLE III

METABOLISM OF [1,4-^{14}C] SUCCINATE BY RAT LIVER PREPARATIONS IN THE PRESENCE OR THE ABSENCE OF 5 mM γ-RESORCYLIC ACID

(Results expressed as in Table II)

Soluble Intermediate	Chopped preparation		Homogenate		Mitochondria	
	None	Resorcylate	None	Resorcylate	None	Resorcylate
Alanine	1	0·3	8	4	1	0·5
Aspartic acid	2	1	20	17	25	7
Asparagine	0·1	0	0	0	5	0
Glutamic acid	0·1	0·2	8	6	2	1
Glutamine	0	0	1	0	19	0
Citric acid	0	0	0	0	1	4
Fumaric acid	22	22	15	23	5	21
Lactic acid	0·4	1	3	6	16	1
Malic acid	74	75	34	42	7	63
Phosphates	0	0	0	0	12	0
Unidentified compounds	0·3	0	1	1	5	0

being found in aspartic acid, glutamic acid, alanine and lactic acid. A consistent effect of the resorcylic acid in all the preparations was a reduction of the incorporation of ^{14}C into the amino-acids (alanine, aspartic and glutamic). However, the most prominent action of the drug was concerned with the tricarboxylic cycle acids in the mitochondrial

TABLE IV

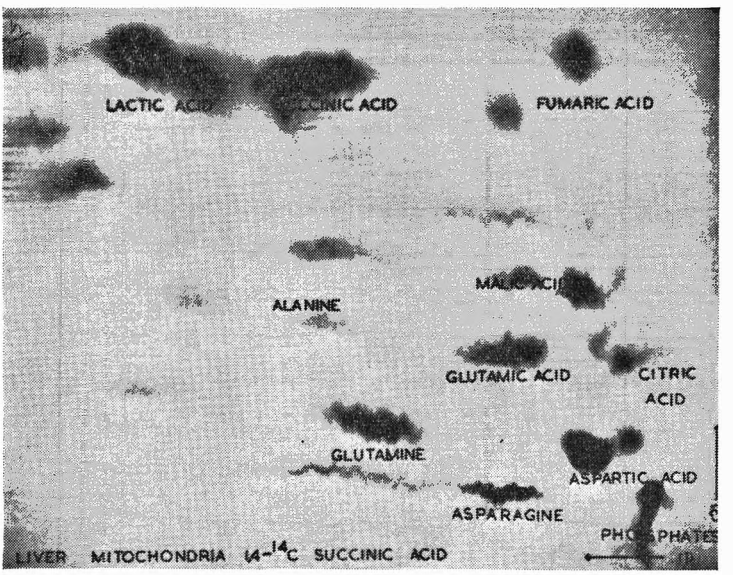
METABOLISM OF [^{14}C] GLUCOSE BY RAT LIVER PREPARATIONS IN THE PRESENCE OR THE ABSENCE OF 5 mM γ-RESORCYLIC ACID

(Results expressed as in Table II)

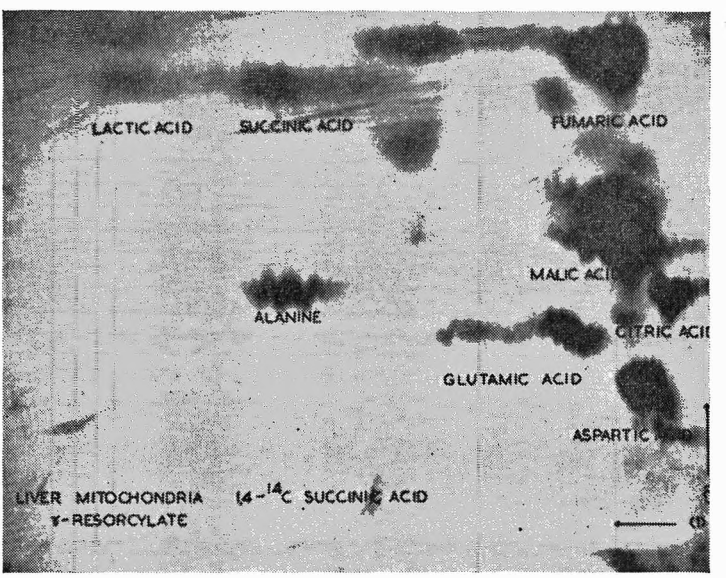
Soluble Intermediate	Chopped preparation		Homogenate		Soluble fraction	
	None	Resorcylate	None	Resorcylate	None	Resorcylate
Alanine	9	7	13	7	16	10
γ-Aminobutyric acid	14	9	0	0	1	1
Aspartic acid	14	16	0	0	16	13
Glutamic acid	14	14	0	0	0·5	0·3
Glutamine	0	0	0	0	0·5	0
Oligosaccharides	0	0	36	67	0	0
Citric acid	1	1	0	0	0	0
Lactic acid	7	5	14	0	37	26
Malic acid	0	0	0	0	1	1
Phosphates	30	32	37	16	24	48
Unidentified compounds	9	16	0	9	1	2

experiments. Here, the formation of the labelled malic acid and fumaric acid were substantially increased and this effect is illustrated in the radioautograms (Fig. 3).

Table IV shows the distribution of radioactivity from labelled glucose among the soluble intermediates of the liver preparations. The mitochondria are excluded because they did not metabolise this labelled substrate. In the absence of resorcylate, the ^{14}C was incorporated mainly into various phosphate compounds, which are intermediates in glycolysis, into lactic acid and into the amino-acids, alanine, aspartic acid,



(a)



(b)

FIG. 3. Incorporation of ^{14}C from [1,4-^{14}C] succinate into metabolic intermediates of a mitochondrial suspension from rat liver. (a) control; (b) in the presence of 5 mM γ-resorcylic acid.

glutamic acid and γ-aminobutyric acid. The radioautogram (Fig. 4) was made from the chopped preparation of liver. The liver homogenate differed from the other preparations in forming a high proportion of a labelled oligosaccharide fraction, which on acid hydrolysis yielded only radioactive glucose, and in the virtual absence of the amino-acids (aspartic, glutamic and γ-aminobutyric acids) which are derived by way of the tricarboxylic acid cycle. A separate experiment using [^{14}C] glucose as the only substrate showed that liver homogenates were capable of forming these labelled amino-acids. It must therefore be presumed that

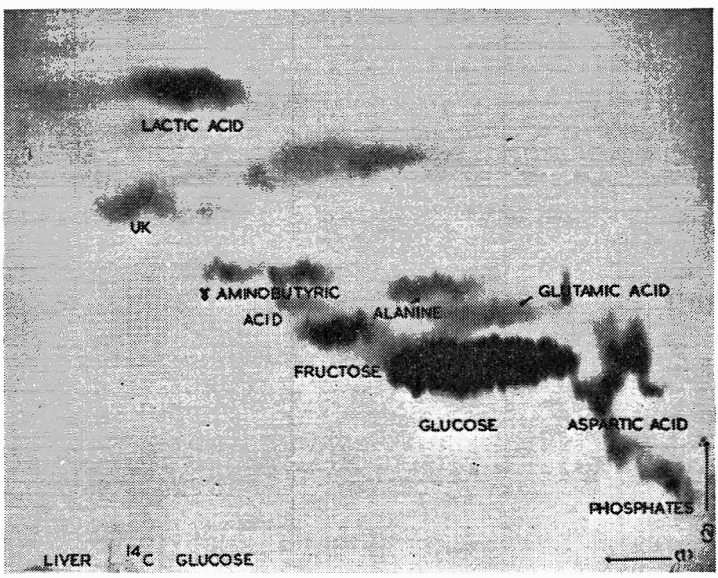


FIG. 4. Distribution of radioactivity from [^{14}C] glucose among the soluble metabolic intermediates of a chopped preparation of rat liver. The glucose spot contains about 90 per cent of the ^{14}C activity of the paper; the fructose was a contaminant of the original labelled glucose.

the presence of a relatively large amount of unlabelled glucose (10 mM) in the experiments recorded in Table IV prevented detectable amounts of isotope from the labelled substrate being incorporated into the aspartic, glutamic and γ-aminobutyric acids. The effects of resorcylic acid, which were common to all three liver preparations, were a reduction of the formation of labelled alanine and lactic acid. In addition, the drug caused substantial increases in the accumulation of ^{14}C in the oligosaccharide fraction in the homogenate and in the phosphate compounds in the soluble fraction.

DISCUSSION

The results show that the inhibitory activity of resorcylic acid against rat serum glutamic-puruvic transaminase is also evident in extracts prepared from isolated rat tissues. The drug also inhibited glutamic-oxaloacetic transaminase activity in the extracts although to a smaller

extent. The interference with glutamic-pyruvic transaminase activity is reflected in the altered distribution of radioactivity from labelled pyruvate among the soluble metabolic intermediates of the isolated tissues. Thus, resorcylic acid caused a substantial reduction in the formation of radioactive alanine in the chopped preparations of rat liver, kidney, brain and heart. The diminished conversion of the labelled pyruvate to alanine caused a higher degree of incorporation of radiocarbon into substances, such as lactic acid, which are formed from pyruvate carbons by metabolic pathways insensitive to the action of resorcylate. A similar, but smaller reduction in the incorporation of radioactivity into alanine also occurred in the liver preparations incubated either with the labelled glucose or with the labelled succinate in the presence of the resorcylic acid. In the mitochondrial suspensions incubated with the radioactive succinate, the resorcylic acid caused a decreased incorporation of the isotope into aspartic acid which suggested that the drug also inhibited the glutamic-oxaloacetic transaminase in this particular experimental system.

In the liver preparations the resorcylic acid produced several effects which appeared to be distinct from its inhibition of transaminase enzymes. The most striking action, illustrated in Fig. 3, was a large increase in the incorporation of ^{14}C into the malic and fumaric acid fractions of rat liver mitochondria incubated with labelled succinate. These changes may reflect corresponding increases in the pool sizes of the acids due to an inhibition of malic dehydrogenase. Alternatively, the resorcylic acid may have altered the permeability of the mitochondrial membranes causing an increased escape of the tricarboxylic cycle acids formed initially inside the subcellular particles.

A further effect of the drug was concerned with the liver homogenate supplied with labelled glucose (Table IV). This preparation converted a large proportion of the incorporated radioactivity into an oligosaccharide fraction probably through the mediation of hepatic maltotransglucosylase (cf. Stetten and Stetten, 1960). This enzyme is capable of forming a series of malto-oligosaccharides from glucose and suitable acceptors, for example maltose and maltotriose. The relatively large amount of labelled oligosaccharide found in the liver homogenate may be due to the availability of these acceptor substances formed during glycogen breakdown in this preparation. In the chopped liver preparation which was incubated for a much longer period, 3 hr. as opposed to 30 min. for the homogenate, the oligosaccharide acceptor substances may have been further degraded to smaller molecules and in the soluble fraction from liver they were probably removed during the preparative procedures. The effect of resorcylic acid in almost doubling the extent of incorporation of radioactivity from the labelled glucose into the oligosaccharide fraction could follow either from a direct action on an enzyme system including the maltotransglucosylase or more indirectly by increasing the amounts of suitable acceptor substances, such as maltotriose, in the homogenate.

In the soluble fraction of the liver a large fraction of the ^{14}C was incorporated into phosphate compounds formed during glycolysis. The presence of resorcylic acid increased the proportion of radiocarbon into

the phosphates suggesting that the drug may have inhibited a reaction concerned with the glycolytic breakdown of the labelled substrate.

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MICELLE FORMATION BY LECITHIN IN SOME ALIPHATIC ALCOHOLS

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Lecithin has been studied in methanol, ethanol, butanol, and hexanol using light scattering and viscosity techniques. A new type of light scattering cell was employed. Micelles were present in all solvents, the micellar weights being 2,300; 7,100; 18,000. and 22,000 in the four alcohols respectively. It appeared that as the polarity of the solvent decreased, the micellar size increased. Dissymmetry measurements showed that no dimension of the micelles exceeded 270Å ($\lambda/20$), but high observed depolarisations showed that the particles were anisotropic. Viscosity measurements also gave an idea of the shape of the micelles, giving intercepts of plots of specific viscosity/solute volume fraction against solute volume fraction of 4·29, 4·26, 4·26, and 3·56 for the four alcohols respectively. Using the dimensions of the lecithin molecule, and the number of monomers present in the micelle, calculations of the theoretical viscosity intercept have been made for each solvent, and compared with values obtained experimentally.

Micelle formation by lecithin in benzene has been studied (Elworthy, 1959) using osmotic pressure, diffusion, and viscosity measurements. The micelles had a laminar structure with the phosphorylcholine head groups situated in the centre of the micelle, and the hydrocarbon chains extending outwards into the solvent. The micellar weight was 57,000. There are presumably repulsive forces between the polar head groups and the solvent, giving a reversal of the type of micelle structure found in water, in which the hydrocarbon chains form the interior of the micelle, and the polar groups are on the outside.

As a continuation of the study of micelle formation by lecithin in organic solvents, it was decided to study lecithin in solvents which should provide intermediate conditions between water and benzene. A series of aliphatic alcohols was chosen, varying in dielectric constant from methanol (33·6) to hexanol (14·3). Price and Lewis (1929) have reported that monomers were present in ethanol, using ebullioscopic measurements.

The light scattering method was used for determining micellar weight, and to give an idea of particle shape. Viscosity measurements also gave an idea of shape.

Experimental

Materials

Lecithin was prepared from fresh chickens' egg yolks by treatment with alumina to remove ninhydrin reacting materials, followed by chromatography on silica to remove lysolecithin (Elworthy and Saunders, 1957). Two samples were used in this work; sample one had N, 1·8; P, 3·8 per cent; I No. 73; sample two had N, 1·8; P, 3·8 per cent; I No. 60.

Analar benzene was fractionally crystallised, dried, fractionally distilled, stored over sodium, and had n_D^{25} 1·4979 (1·4981). Analar toluene was dried, fractionally distilled, stored over sodium, and had n_D^{25} 1·4940 (1·4941). The alcohols were dried (Vogel, 1950), fractionally distilled, and stored over anhydrous calcium sulphate. Methanol had n_D^{15} 1·3315 (1·3306), ethanol had n_D^{20} 1·3619 (1·3614), butanol had n_D^{15} 1·4020 (1·4012), and hexanol had n_D^{20} 1·4181 (1·4179). Figures given in brackets are literature values of refractive index (Timmermans, 1950, Hodgmen, 1957).

Viscosity Measurements

Viscosities of solutions relative to solvent were measured in a suspended level dilution viscometer.

Light Scattering Measurements

The light scattering photometer previously described (Elworthy, 1960) has been considerably modified. Light from a stabilised 250 W mercury vapour lamp was made parallel by a lens system, and the green line

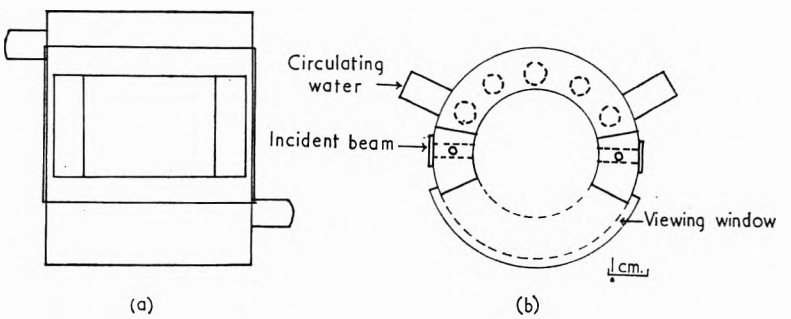

FIG. 1. Light scattering cell. (a) Front view; (b) from above.

(5461Å) isolated by means of an interference filter together with a neodymium glass to remove the last traces of the sodium lines. Final collimation of the beam was achieved by passing it through two 2 mm. wide slits placed 32 cm. apart; the beam passing through the cell measured 2 × 26 mm.

The light scattering cell was of a new design (Fig. 1). It was cylindrical with two parallel flattened faces to accommodate the windows through which the incident beam passed. The scattered light was viewed through a curved glass window, allowing observation between 45 and 135° to the incident beam. A constant temperature was achieved by circulating water through a series of tubes cut in the back wall of the cell. The two principal factors found to reduce stray light were, firstly, complete blackening of the inside of the cell by the Relonol process, and secondly, making the channels inside the cell, through which the incident beam entered and left, sufficiently long so that the actual points where the beam struck the glass windows could not be seen through the curved viewing window.

The eleven-stage photomultiplier receiving the scattered light was mounted on a broad tufnol arm which rested on a tufnol plate. The arm was pivoted beneath the centre of the cell, giving a very smooth movement of the photomultiplier around the cell. The signal was amplified (Ottewill and Parreira, 1958) and read on a galvanometer.

A polished perspex block was used as a light scattering standard, being calibrated from Ludox solutions using Maron and Lou's (1954) procedure. As a check on the calibration, the Rayleigh's ratios of benzene and toluene were determined, giving $16{\cdot}0 \times 10^{-6}$ and $17{\cdot}9 \times 10^{-6}$cm.$^{-1}$ respectively, which agreed with literature values (Ottewill and Parreira,

TABLE I

LIGHT SCATTERING AND VISCOSITY DATA

Solvent	Methanol	Ethanol	Butanol	Hexanol
dn/dc, ml. g.$^{-1}$	0·135	0·118	0·087	0·071
Micellar weight	2,300	7,100	18,000	22,000
Monomers in micelle	3	9	24	28
Depolarisation	0·345	0·476	0·552	0·509
Dissymmetry Z_{45}	1·01	1·00	1·00	1·00
$\left(\frac{\eta sp}{c}\right)_{\phi = 0}$	$4{\cdot}2_0$	$4{\cdot}2_0$	$4{\cdot}2_0$	$3{\cdot}5_6$

1958). The molecular weight of a National Chemical Laboratory's sample of polystyrene was found to be 370,000 as against 390,000 quoted.

All solutions were clarified by filtering through No. 5 sintered glass filters until dust free.

Specific Refractive Index Increments (dn/dc)

These were determined with a Hilger-Rayleigh interference refractometer using Bauer's (1945) technique for monochromatic light. The instrument was checked using sodium chloride solutions as standards.

Depolarisations

Measurements were made in the usual way with a polaroid disc (Stacey, 1956). As high depolarisations are reported in this paper, particular care was taken in checking the technique. A comparison of the sensitivity of the photomultiplier to light of horizontal and vertical polarisation was made, and corrections applied for its greater sensitivity to the horizontal component. The contribution of the solvent to both components was subtracted from the values for solutions. The depolarisation of dilute Ludox solutions was found to be 0·01, benzene gave $\rho = 0{\cdot}41$, and toluene $\rho = 0{\cdot}42$, in good agreement with literature figures (Ottewill and Parreira, 1958). All solutions were tested for fluorescence as described by Brice, Nutting, and Halwer (1953). No fluorescence was observed in any of the systems studied.

RESULTS

The results of the light scattering measurements are given in Fig. 2 and Table I. T is the observed turbidity, c is the concentration in g. ml.$^{-1}$, and $H = 32\pi^3 n_0^2 (dn/dc)^2/3\lambda^4 N$, where n_0 is the refractive index of the solvent,

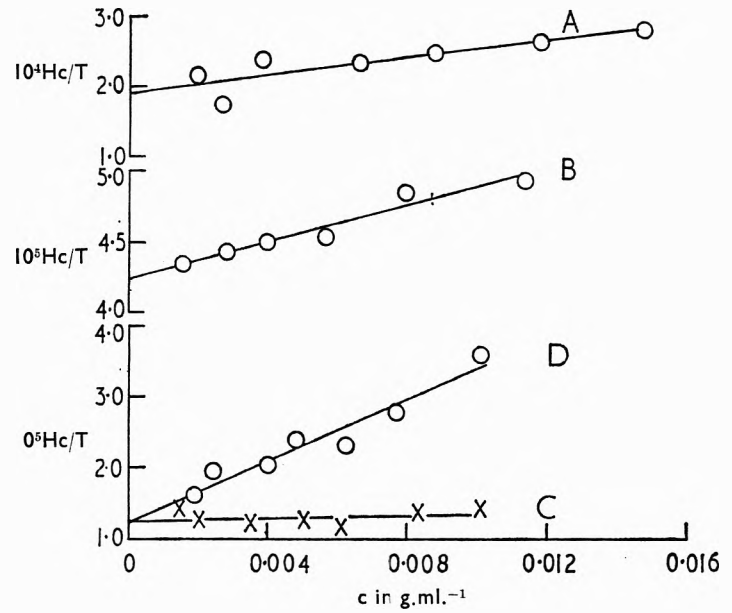

FIG. 2. Graphs of Hc/T against c. A, methanol; B, ethanol; C, butanol; D, hexanol.

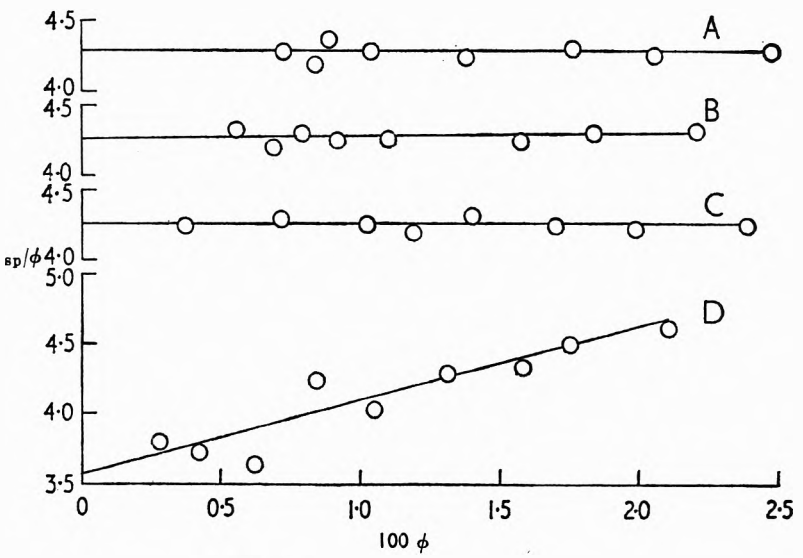

FIG. 3. Graphs of η_{sp}/ϕ against ϕ. A, methanol; B, ethanol; C, butanol; D, hexanol.

and λ the wavelength of the light used. For the calculation of micellar weight, the Cabannes factor, calculated from the depolarisation, was applied to the Hc/T value at zero concentration. All measurements were made at 20°.

There was very little slope of the Hc/T against c plots for methanol, ethanol, and butanol, but a moderate slope for hexanol. This may indicate a greater interaction between solute and solvent in hexanol than in the three lower alcohols. Graphs of concentration against turbidity gave straight lines passing through the origin (after subtraction of the solvent turbidity from the turbidity of each solution). There were none of the breaks associated with critical micelle concentrations. If these are present, they are at concentrations too small to be measured accurately by the present technique.

The viscosity results are given in Fig. 3 as graphs of η_{sp}/ϕ against ϕ, where η_{sp} is the specific viscosity and ϕ is the volume fraction of solute

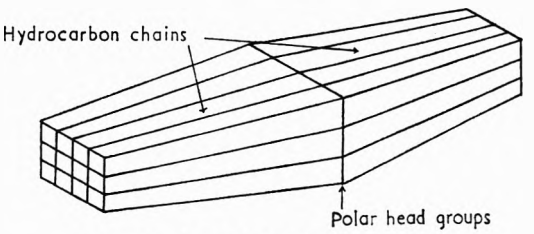

FIG. 4. Model of lecithin micelle.

The slope of the graphs for the three lowest alcohols is very small, but the hexanol system shows a definite slope.

No differences between the properties of the two lecithin samples used were observed.

DISCUSSION

It can be seen from Table I that the micelle size increases on passing from methanol to hexanol, that is, increasing as the dielectric constant of the solvent decreases. As the solvent series is ascended, there are probably increased repulsive forces between the solvent and the polar head group of the lecithin molecule which tend to aid the formation of larger micelles. As there is a small aggregate of monomers present in methanol, it appears that there is only a small lack of affinity between this solvent and the polar head groups. Ethanol appears to have still less affinity, causing the polar heads to be tucked inside the micelle. This type of structure was observed in benzene (Elworthy, 1959), which is a much less polar solvent than the alcohols; the observed micellar weight of 57,000 emphasises how the micelles grow in passing into a non-polar solvent. In water the type of micellar structure described is reversed, the hydrocarbon chains being inside the micelles. The lecithin-methanol system would appear to represent a half way point between the type of micelles formed in aqueous systems, and those present in the higher alcohols and benzene.

The micellar size in benzene decreased from 57,000 at 25° to 43,000 at 40°. This trend may explain why in ethanol at 20° we have found small micelles, while Price and Lewis (1929) reported that monomers were present at the boiling point.

The observed dissymmetries (intensity of light scattered at 45°/intensity scattered at 135°) were close to unity, indicating that no dimension of any of the micelles studied exceeded 270Å ($\lambda/20$). However, from the high observed depolarisations, some asymmetry is indicated. Anisotropic particles composed of a uniform material have been treated as ellipsoids (Rayleigh, 1918), having different degrees of polarisability along their different axes, which leads to cases where this type of particle gives higher depolarisations than small isotropic ones.

The large observed depolarisations may also be a consequence of the arrangement of monomers in the micelle, which will have a general structure, except for those present in methanol, as in Fig. 4. It is tentatively suggested that the high depolarisations may be due to the hydrocarbon chains being roughly parallel to one another, and allowing a much greater polarisation to occur along the axis of the bundle of hydrocarbon chains. It is not suggested that the bimolecular leaflet structure occurs in methanol, as there is present only a small aggregation of monomers.

Large Cabannes' factors had to be applied to the Hc/T intercepts in the calculation of the micellar weights. Measurements in benzene (Elworthy and McIntosh unpublished results) also gave a large depolarisation ($\rho = 0{\cdot}265$), giving M = 50,000 with dn/dc = 0·038. In view of the smallness of dn/dc, this result agrees reasonably well with that found by diffusion (57,000). Diffusion measurements in methanol gave 2,000 for the micellar weight (Elworthy, unpublished results).

From the viscosity experiments, values of $(\eta_{sp}/\phi)_{\phi=0}$ (abbreviated to ν) were obtained. Values of this function should be 2·5 if the particles are unsolvated and spherical (Einstein, 1906, 1911). From the micellar weights we can calculate ν for certain model structures. The simplest model to choose at the present stage of the work is a rod-like structure as shown in Fig. 4. The length of each lecithin molecule was taken as 35Å, and the head group area as 55Å^2, from molecular models. In methanol we assume that the monomers lie side by side, and in the other solvents that a bimolecular leaflet is present. The ratio of the length to the breadth of this rod-like structure can be related to ν (Mehl, Oncley, and Simha, 1940). This treatment gave $\nu = 3{\cdot}6$ for methanol, 4·4 for ethanol, 3·4 for butanol, and 3·1 for hexanol. The calculated values of ν are of the correct order, but generally lower than the observed values. This may be due to deformation of the particles on flow, yielding rather higher viscosities than predicted on a volume fraction and asymmetry basis, or to solvation of the particles. No solvation was apparent in benzene, where ν was 2·8. Viscosity studies will be continued using a Coutte viscometer.

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THE PATTERN, SENSITIVITY AND PRECISION OF THE RESPONSE TO INSULIN IN RANDOM BRED, INBRED AND HYBRID STRAINS OF MICE

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The study of the pattern of the response to insulin, its sensitivity and precision in hybrid, random bred and inbred mice has confirmed that while the qualitative and quantitative differences in response for each strain are specific and must be determined empirically, there is considerable general correlation in this species of the average weight of the strain of mouse and the sensitivity and precision of its response.

The British Pharmacopoeia of 1932 introduced as an official test for the assay of insulin, the convulsive responses in mice previously deprived of food. Such responses were therefore considered a suitable tool for the investigation of specific differences between random bred, inbred and hybrid strains of mice.

Material and Methods

The test animals were bred at the Laboratory Animals Centre and issued soon after weaning so that mice aged six weeks ± seven days could be used for the first test. The weight distribution limit of 5 g. imposed in the official routine assay could not be applied to these less readily available strains without severely curtailing the numbers in each group.

TABLE I

Mean and standard deviation of the average of the mean weights of the mice used in each of the first tests analysed in table II

Strain	M	F
LAC grey	23·8 ± 3·2	20·6 ± 2·2
A2G	18·4 ± 2·8	15·7 ± 2·1
C57Br/cd	18·3 ± 1·9	14·5 ± 1·2
CBA	17·5 ± 2·9	14·7 ± 1·8
DBA/1	13·0 ± 1·1	12·1 ± 1·6
$A2DB/1F_1$	17·8 ± 3·6	14·1 ± 1·5
$A2CF_1$	18·8 ± 1·6	15·6 ± 1·0
$CA2F_1$	17·9 ± 0·5	15·8 ± 0·4
$A2BrF_1$	19·9 ± 2·5	16·6 ± 0·8
$BrA2F_1$	16·4 ± 1·3	14·4 ± 0·7

Mice from each strain were therefore segregated first by sex and then by weight into groups. The several groups of mice for each strain were randomised into boxes—for any one strain three for males and three for females. Doses of insulin were adjusted so that each mouse received the exact equivalent per kg. mouse of the dose allotted to it, to correct within strain variation in weight. The average of the mean weights of mice used for the first tests and the standard deviation of the average for each strain is given in Table I. Whenever sufficient mice were available three groups

TABLE II

THE COMPARISONS OF DEFINITE AND SEVERE RESPONSES TO DOSES OF INSULIN IN MICE: 1. DIFFERENCES IN PATTERN OF RESPONSE BETWEEN THE SEXES OF EACH STRAIN; 2. DIFFERENCES IN PATTERN OF RESPONSE BETWEEN ALL OTHER STRAINS AND THE CONTROL LAC GREY AT DOSES GIVING A SIMILAR PERCENTAGE OF TOTAL REACTIONS. P = PROBABILITY, D = DIFFERENCE, −P. >05, +P.05, ++P.01, +++P.001

Expt.	Strain	Severe Reaction		Definite Reaction		No Reaction		Percentage reaction	Difference between sex response pattern			Difference between strains response pattern and LAC grey response pattern		
		M	F	M	F	M	F		χ^2	P	D	χ^2	P	D
1	LAC Grey ..	8	19	90	130	125	75	55·2	24·2	<0·001	+++			
	A2G	37	74	59	75	128	82	57·3	24·4	<0·001	+++	72·7	<0·001	+++
2	LAC Grey ..	3	14	31	53	54	26	55·8						
	A2G	22	39	27	27	40	31	52·0	6·1	0·05	+	37·4	<0·001	+++
	DBA/1	24	23	17	21	40	20	58·6	2·0	0·5	−	35·8	<0·001	+++
	$A2DB/1F_1$ ..	10	15	19	25	41	30	45·4	3·5	0·2	−	13·9	ca. 0·02	++
3	LAC Grey ..	27	41	126	143	90	53	70·2	13·4	ca. 0·001	+++			
	$A2CF_1$	27	47	40	38	48	25	67·6	12·6	0·01–0·001	+++	40·1	<0·001	+++
	$CA2F_1$	23	59	42	33	53	26	66·5	26·1	<0·001	+++	56·5	<0·001	+++
	$BrA2F_1$	51	46	26	34	43	40	65·4	1·4	0·7	−	88·9	<0·001	+++
4	LAC Grey ..	4	5	39	52	50	30	55·6						
	$A2BrF_1$	16	25	20	22	51	42	53·2	2·9	0·2	−	34·9	<0·001	+++
5	LAC Grey ..	2	1	18	29	30	20	50·0						
	C57Br/cd ..	22	16	8	4	20	27	51·5	3·2	0·05–0·1	−	55·7	<0·001	+++
6	LAC Grey ..	2	7	27	28	21	15	64·0						
	CBA	6	21	16	20	23	7	67·7	17·2	<0·001	+++	15·9	0·01	++

$A2DB/1F_1$ = A2G (F) × DBA/1 (M) $A2CF_1$ = A2G (F) × CBA (M) $CA2F_1$ = CBA (F) × A2G (M) $BrA2F_1$ = C57Br/cd (F) × A2G (M)
$A2BrF_1$ = A2G (F) × C57Br/cd (M)

of 10 males and three groups of 10 females, that is 60 mice were used for each strain per test, and in no test was each group less than 8 or the total number of mice less than 48.

One batch of crystalline insulin, kindly supplied by Dr. G. A. Stewart of the Wellcome Chemical Works, Dartford, was used for all tests and about six strains of mice were tested on one day, always including the LAC grey mice as control. All doses of insulin used were in the range 500–2,812 milliunits/kg. with a dose ratio 1 : 1·334. The middle dose was chosen to give a response of about 50 per cent.

The mice were allowed to convulse in a room thermostatically controlled at 20° and illuminated by one central strip light. Glass jars measuring 17 cm. in diameter by 12 cm. in depth were used to house the mice under test and not more than five or less than four mice were put into any one jar. Frank convulsions were recorded as severe and the convulsed mice were injected with 0·5 ml. of 15 per cent glucose and returned to their original container. Convulsions causing hind leg paralysis and general immobility were recorded as definite and the mice remained with the non-reactors in the test jar. This simplified the final recording. Reactions were noted for 2 hr. because the lower temperature used compared with that used for the official test made the interval between injection of insulin and reaction longer.

Results

Differences in Pattern of Response to Injections of Insulin in Random Bred LAC Grey Mice, Inbred and Hybrid Strains

For doses of insulin to which between 50 and 70 per cent of the mice injected gave positive responses in strains LAC grey, A2G, CBA, A2CF, and CA2F, there was a significant difference between the sexes in their pattern of response, the females being the more reactive (see Table II). The hybrid strains $A2CF_1$ and $CA2F_1$ followed the same pattern as their parent strains A2G and CBA. The hybrid strains $A2DB/1F_1$, $A2BrF_1$ and $BrA2F_1$ for which there was no sex difference in the response pattern each had one parent with little or no sex difference, namely DBA/1 or C57Br/cd.

All strains were found to differ significantly from LAC grey mice in the pattern of the quality of their response because this strain had always a higher proportion of definite than severe reactions (see Table II). In other words the LAC grey strain showed qualitatively less response than all others.

Among hybrid strains the pattern of response for the $BrA2F_1$ mice, which were tested at the same time as the hybrid mice $A2CF_1$ and $CA2F_1$ (Table II, experiment 3), was significantly different in quality from these ($P = 0{\cdot}02$ and $P = 0{\cdot}001$). There were more severe reactions than definite reactions, that is, qualitatively more marked response.

Finally for the strains of experiment 2 in Table II, parents with their hybrid, the pattern of response of the hybrid mice $A2DB/1F_1$ was not in these tests found to be significantly different from that of either parent ($P = 0{\cdot}05$–$0{\cdot}1$ for each parent), although differences approach significance.

TABLE III

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 THE FIRST TESTS ONLY: 1. WITHIN STRAIN COMPARISON FOR ALL FIRST TESTS MADE: 2. COMPARISON BETWEEN LAC GREY MICE, INBRED STRAINS AND HYBRID STRAINS; 3. COMPARISON BETWEEN A2G MICE, DBA/1 MICE AND THEIR HYBRID A2DB/1F:; 4. COMPARISON BETWEEN DBA/1 MICE AND HYBRID A2DB/1F$_1$. P = PROBABILITY, D = DIFFERENCE

Strain	No. of tests	Approx. ED50 milliunit/kg. mouse	Mean slopes	Within strain comparison		Between LAC grey mice and other strains		Between A2G, DBA/1 and their hybrid mice		Between DBA/1 and A2DB/1F$_1$	
				P	D	P	D	P	D	P	D
LAC grey	6	1,880	1·018	0·5	–						
A2G	6	953	1·929	0·5	–	0·01–0·001	+++				
LAC grey	3	2,335	0·742	0·3	–						
CBA	3	1,125	0·905	0·1–0·2	–	0·2–0·3	–				
LAC grey	3	1,875	0·904	0·99	–						
A2CF$_1$	3	1,190	1·815	0·2–0·3	–	0·1–0·2	–				
CA$_2$F$_1$	3	1,100	0·985	0·8–0·9	–	ca. 0·99	–				
BrA2F$_1$	3	885	1·825	0·99	–	ca. 0·05	+				
LAC grey	2	1,965	0·929	0·99	–						
A2BrF$_1$	2	1,110	1·625	0·2–0·3	–	0·7–0·8	–				
LAC grey	2	1,845	1·280	0·8–0·9	–						
A2G	2	885	1·962	0·1–0·2	–	0·1–0·2	–				
DBA/1	2	875	2·842	ca. 0·5	–	0·02–0·05	+	0·1–0·2	–		
A2DB/1F$_1$	2	890	1·283	ca. 0·8	–	0·99	–	0·1–0·2	–	0·01–0·02	++

Parallelism of the Regression Between the Convulsive Response and the Dose of Insulin for First Tests only by means of χ^2 Summation and using Random Bred, Inbred and Hybrid Mice

It is important that the within strain analyses (Table III) indicate that for first tests on each strain the regression lines between convulsive response and insulin dose are parallel. There are, however, significant

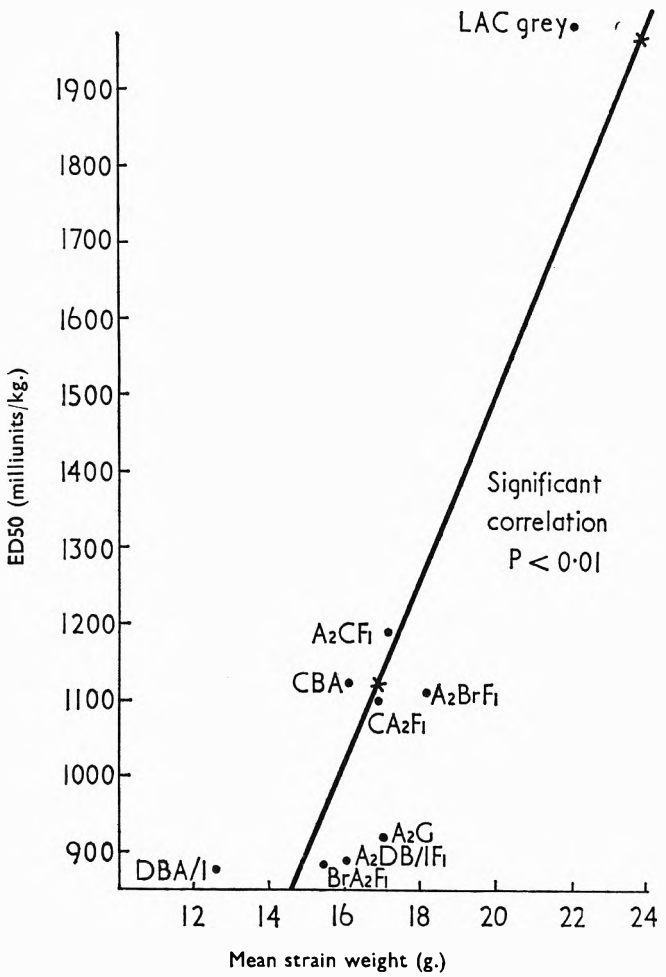

FIG. 1. Correlation of the mean strain weights and the ED50 milliunits for insulin.

differences in the precision of response between strains. The regressions between the convulsive response and the insulin dose for DBA/1 and $BrA2F_1$ mice and for the first group of A2G mice are significantly steeper than that for LAC grey mice (see Table III). The amount of within strain variation in slope may affect the probability of parallelism between

two strains. Thus of the hybrid strains $A2CF_1$ and $BrA2F_1$ the former, with a mean slope of 1·815, having greater within strain variation of slope (or less probability of parallelism) does not show a significant difference in slope from the LAC grey mice, while the latter strain $BrA2F_1$ with a mean slope of 1·825 shows a significant difference. The results for the hybrid mice $A2CF_1$, as also for the second group of A2G mice tested, show that, in spite of considerable variation, that is, less probability of

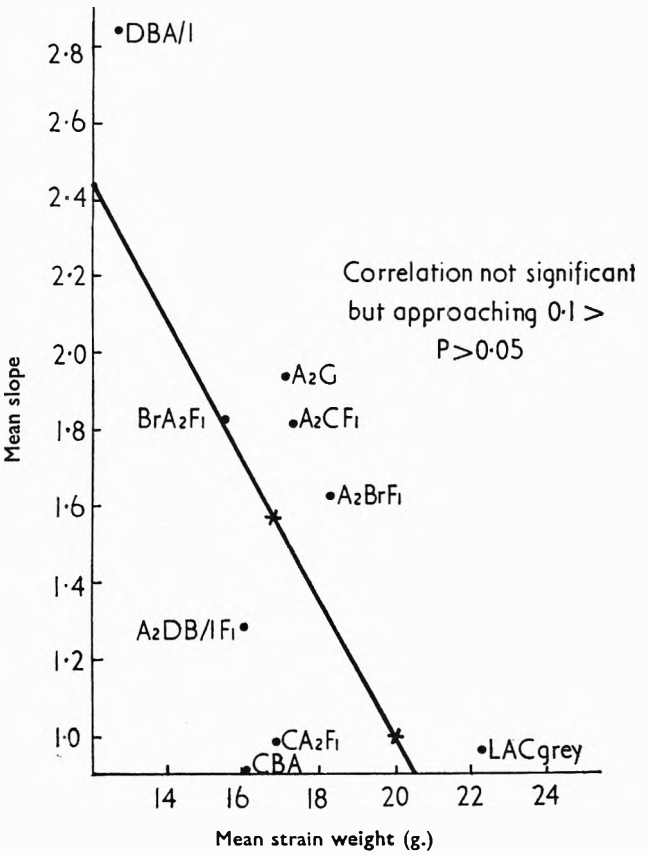

FIG. 2. Correlation of the mean strain weights and the mean slope of the regression of the response to insulin.

within strain parallelism, the difference in slope between the regression for both these groups of mice and the LAC grey mice is towards significance ($P = 0·1–0·2$).

The group of strains comprising hybrid mice with their parents is also interesting. The slope of the regression for the hybrid is less than that of either parent and significantly less than that of the DBA/1 strain. The within strain variation for this hybrid, however, is much less than that for either parent strain.

Correlation of Mean Strain Weights, and ED50 milliunits/kg. of Insulin and of Mean Slope of the Regression of the Response to Insulin. Also the correlation of ED50 milliunits/kg. and Mean Slope in the same Strains of Mouse

The combined test results are plotted against mean strain weight in Figs. 1 and 2 and show that strains derived by mutation or selection in this species (*Mus musculus*) react with insulin in such a way that there is a

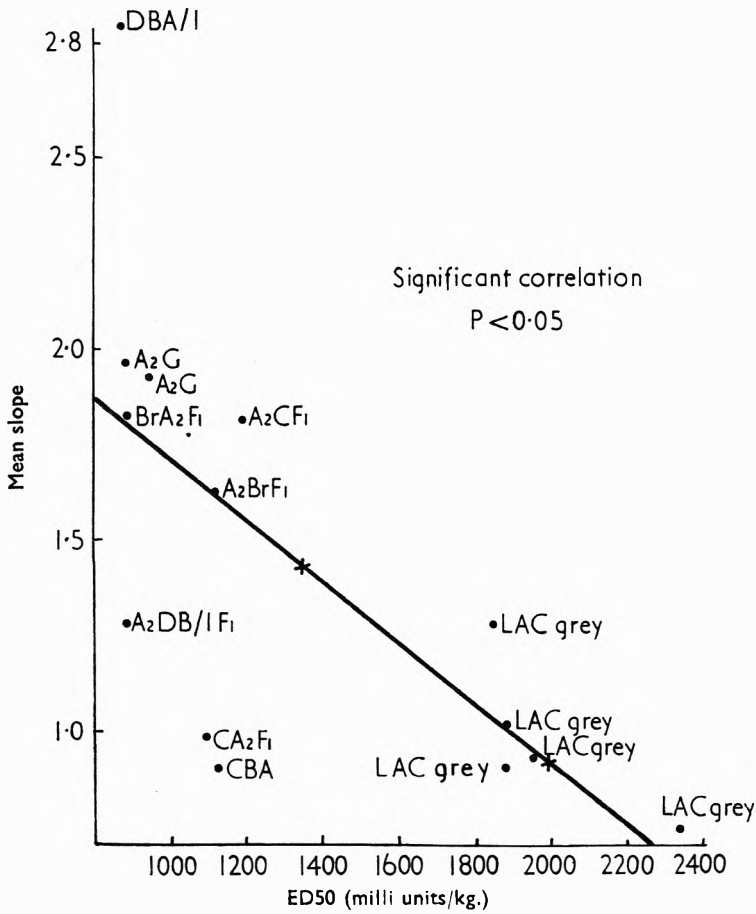

FIG. 3. Correlation of the ED50 and mean slope of the regression in the same strains of mouse.

significant general correlation of ED50 and the mean weight of the strain used, and an almost significant correlation of mean slope of the regression of this reaction and the mean weight of the strain. The results are in contrast to those for within strain variation published by Young and Stewart (1952) in that the lighter strains are more sensitive and more precise, whereas in the analysis of a larger number of mice from a random bred strain the heavier mice were the more sensitive.

Young and Stewart made no statement about the precise age of these mice. The third figure showing the correlation of mean slope and ED50 for these strains is interesting, emphasising the unique position of the DBA/1 strain and also the difference between the F_1 strains $A2CF_1$ (maternal strain A2G) and $CA2F_1$ (maternal strain CBA), each more closely resembling its maternal parent (cf. Walton and Hammond, 1938, and McClaren and Michie, 1956).

Discussion

As the response to doses of insulin in mice has been an official assay test since 1932 it has been often investigated. The present work which has been done in a constant environment such as was deemed necessary in the work of Sellar and Smart (1959), shows that the response is in pattern, sensitivity and precision specific to the strain of mouse employed.

The strain specificity in pattern of reaction is evident both in differences between male and female convulsion rates and in the predominance of either severe or definite reactions rates in the reactions of any one strain.

The sensitivity of the mice to insulin is strain specific and with the strains used the ED50 varied from approximately 900 milliunits/kg. mouse to approximately 2,000 milliunits. From the correlation of the average weights of the mice used its value would seem to be inversely related to the average weight of the strain of mouse employed.

Although there is a general correlation of the sensitivity of the mice and the slope of the regression between their response and the dose of insulin received, the specific reactions of individual strains are still manifest. This is well illustrated by the plots of the results from the hybrid $A2DB/1F_1$ and its parents on the correlation graph. The hybrid plot is well outside that of its parents (cf. Chai, 1960). All have approximately the same sensitivity but the parent strains show greater precision of response, that of DBA/1 being significantly greater than the hybrid. There is no doubt that the general correlations are the outcome of the genetic relationship between the strains.

It would appear that the sensitivity and pattern of response may bear some relation to each other. The very insensitive LAC grey mice have a pattern of response significantly different from all other strains at doses giving similar percentages of reaction. At all doses studied the mice of this strain give more definite than severe reactions, a qualitative difference of less response than all other strains.

The within strain variation in precision, that is, in the parallelism of the regression between its convulsive response to doses of insulin, is not dependent on whether the strain is inbred or hybrid. The hybrid strains $A2CF_1$ and $CA2F_1$ with the hybrid strains $A2BrF_1$ and $BrA2F_1$ illustrate this. For both pairs of hybrids the strain out of A2G females is the more variable.

Specifications most suitable for biological assay, as illustrated by response to doses of insulin, cannot be said to appertain either to all hybrid mice, to all inbred mice or to all random bred mice. Each strain of mouse must be assessed empirically in the environment where it will be

used, and for routine insulin assay this was not the environment I was able to provide. The random bred strain used in this work is entirely unsuitable for insulin assay and would probably be unsuitable in any environment, but this does not condemn all other random bred strains. Of the hybrid and inbred strains tested, the BrA2F$_1$, DBA/1 and A2G strains were those most suitable in the conditions which I imposed.

Similar conclusions are illustrated in the work of Chai (1960), on hormone response in mice, and in other work on this subject which he reviews. It has been further confirmed in the work on the response in hybrid, inbred and random bred mice to pentobarbitone sodium (Brown, 1961) and to histamine acid phosphate (Brown, 1959).

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SLEEPING TIME RESPONSES OF MICE—RANDOM BRED, INBRED AND F_1 HYBRIDS—TO PENTOBARBITONE SODIUM

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The sensitivity and the precision of the sleeping time responses to pentobarbitone sodium in 10 per cent ethanol in mice varies with the strain of mouse. In any one strain sleeping time response in animals dosed by weight with pentobarbitone sodium is related to the weight of the animals. As mice aged, the sleeping time for all strains differed between the sexes, the males sleeping longer than the females. If the same mice were reinjected with pentobarbitone sodium the ageing effect was accelerated. It has been confirmed that the responses of F_1 hybrids must be determined experimentally, and may resemble that of either parent, or fall between or outside those of their parents. In choosing animals for the estimation of pharmacological responses, therefore, their strain, weight, age and sex are each of importance, and should be as controlled as their environment before and during tests.

Work on sleeping time responses in mice was undertaken to investigate some practical questions that arise in pharmacological assay. It had been asked whether when choosing animals for use in the assay of pharmacological responses similar age or similar weight was the more important consideration. It was hoped that the study of one particular reaction in random bred, inbred and hybrid mice using mice of known age, sex and weight might throw further light on the relationship between these variables and indicate which, if any, is the most important.

Material and Methods

Pentobarbitone sodium was chosen as the sleeping drug for it is the hypnotic of choice when long sleep in animals is required. I have followed a usual convention of describing the characteristic central depression which follows anaesthetic doses of this drug as "sleeping time". It was freshly dissolved for each test at a concentration of 5 mg./ml. in normal physiological saline containing 10 per cent ethanol. For the preliminary tests in random bred P/LAC mice a single dose of 60 mg./kg. was used while in all other experiments sleeping times produced by 55 and 66 mg./kg. mouse were measured. The injections were intraperitoneal.

The test animals were bred at the Laboratory Animals Centre and issued soon after weaning so that mice aged 5 weeks $\pm$ $3\frac{1}{2}$ days were available for the first experiments. They were housed not more than five in each metal box $14 \times 6 \times 4\frac{1}{2}$ in. in size and were fed on diet 41B *ad lib.* from hoppers. They were watered from plastic water bottles with metal drinking tubes and kept in a room thermostatically controlled at 70° F. $\pm$ 2°. For every experiment equal numbers of male and female mice, strictly segregated, were distributed by random numbers into the mouse boxes and usually 10 of each sex were used at each dose of pentobarbitone

TABLE I

RESULTS OF ANALYSIS SHOWING THE EFFECTS OF SEX ON THE WEIGHTS AND ON THE SLEEPING TIMES OF P/LAC MICE INJECTED ON EACH OCCASION WITH 60 MG./KG. OF PENTOBARBITONE SODIUM IN SALINE CONTAINING 10 PER CENT OF ETHANOL

Age	Mice: Normal M	Mice: Normal F	Mice: Treated M	Mice: Treated F	No. of previous treatments	Results of analysis of variance showing sex differences: Between mean weights M>F	Between mean weights P	Between mean log of sleeping times M>F	Between mean log of sleeping times P	Differences of sleeping time adjusted for body weight M>F	Differences of sleeping time adjusted for body weight P
5 weeks	28	30			0	–	>0·25	–	>0·25	–	0·6–0·7
6 weeks	18	20			0	++	<0·01	–	>0·25	–	ca. 0·5
			28	29	1	+	<0·05	++	<0·01	+++	<0·001
8 weeks	10	10			0	+++	<0·001	+	ca. 0·02	++	ca. 0·01
			10	10	2	+++	<0·001	+++	<0·001	+++	<0·001
12 weeks	10	10			0	+++	<0·001	–	0·1	++	ca. 0·01
			10	10	3	+++	<0·001	+++	<0·001	+++	<0·001

M = male. F = female.

sodium, or a total of 40 mice per strain for any one test. The mice were ear punched for individual identification. Experimental sleeping time was measured as the time between the animals receiving their injection and that time when they were able to right themselves when placed on their backs. Analyses were made with logarithms of these times and of the weights of the animals used. The possibility of using the reciprocals, the squares or square roots as functions of time in preference to the logarithms was investigated and rejected. A constant temperature sleeping tray was not used, but a tray lined with foam rubber which conveniently took 120 mice and shielded them from draughts was found satisfactory for the comparison of three strains of mice on one day. Repeat sleeping tests were made at intervals of two weeks or longer and not more than four tests were made on any one set of mice. As the temperature was not controlled in the experimental laboratory, comparison could not be made between the absolute values of sleeping times obtained on different days. Mice which died as the result of repeated injections or for any unknown cause were not replaced and unless otherwise stated all analyses were made on the actual numbers used.

Results

The effect of Sex and Body Weight in Random Bred P/LAC Mice on Sleeping Time Responses to Pentobarbitone Sodium

The results obtained in these experiments were analysed as shown in Table I. Preliminary work on the sleeping times of the five week old mice, 28 males and 30 females, had shown that for male mice the correlation coefficient of the logarithms of weight and sleeping times was $-0{\cdot}525$, while for female mice it was $-0{\cdot}616$, and for five week old mice irrespective of sex $-0{\cdot}564$. It therefore seemed reasonable not only to analyse results for the variance between sex, weight (or log weight) and log sleeping time but also to determine the variance between the mean logarithms of the sleeping time of each group of mice adjusted by co-variance for weight. Inspection of Table I shows the advantage of the dual analysis in that differences which appear on analysis of variance are confirmed and become more definite with co-variance analysis. A difference between male and female mean sleeping time arises with age and the ageing effect is accelerated in mice that have received more than one injection.

The Homogeneity of the Variance of the Sleeping Time Response of Groups of Mice including Inbred, Hybrid and Random Bred Strains

In order to determine differences between sleeping time responses for groups of mice by analyses of variance and co-variance it was necessary to determine by Bartlett's test whether the variance of the responses of the groups of mice used was homogeneous. The results obtained indicated much heteroscedasticity within some of the nine groups of mice used. However, the control strain A2G has a large degree of within strain homogeneity of variance at the usual level of significance $P = 0{\cdot}05$, while one

TABLE II

RESULTS OF THE ANALYSIS OF VARIANCE SHOWING THE EFFECTS OF THE SEX AND STRAIN OF MICE AND THE DOSE OF PENTOBARBITONE SODIUM IN 10 PER CENT ETHANOL ON THEIR SLEEPING TIME RESPONSES

GROUPS	Strains M & F	Age in weeks	Significant differences P = 0·001 = +++ Sex	Strain
1	A2G, A	5	——	+++
		8	+++	+++
		11	+++	——
		14	+++	——
3	A2G, A	5	——	——
2	A2G, CBA, LAC Grey ..	5	——	+++
		8	+++	+++
		11	+++	+++
6	A2G, BrCF	5	——	+++
		8	+++	+++
		12	+++	+++
7	A2G, A2DB/1F$_1$	5	——	——
		9	+++	——
		13	+++	——

GROUPS	Strains M only	Age in weeks	Significant differences P = 0·001 = +++ Strain
4	A2G, C57BL, C57Br/cd ..	5	+++
		7	+++
		9	+++
		11	+++
5	A2G, C3H	5	——
		7	+++
		10	——
		13	——
7	A2G, DBA/1, A2DB/1, F$_1$ ..	5	——
		9	——
		13	+++
8	A2G, DB/1A2F$_1$	5	——
		12	+++

TABLE III

MEAN SLEEPING TIMES OF MICE y ADJUSTED FOR THE WEIGHTS OF THE ANIMALS AT TWO DOSES OF PENTOBARBITONE SODIUM IN 10 PER CENT ETHANOL RESPECTIVELY 66 AND 55 MG./KG. MOUSE; ALSO b THE SLOPES OF THE REGRESSION BETWEEN RESPONSE AND DOSE; AND THE PRECISION OF THE RESULTS MEASURED BY λ. GROUPS CORRESPOND WITH THOSE IN TABLE II

		A2G			A					
Test	Group 1	y	b	λ	y	b	λ			
1	M	2·223 1·956	0·256	0·277	2·124 1·856	0·257	0·244			
	F	2·243 2·047	0·188	0·507	2·068 1·902	0·159	0·664			
2	M	2·247 2·082	0·159	0·436	2·200 1·912	0·181	0·598			
	F	2·147 2·059	0·085	0·955	2·102 1·878	0·215	0·354			
3	M	2·306 2·126	0·173	0·439	2·320 2·075	0·235	0·415			
	F	2·168 1·997	0·164	0·317	2·198 1·823	0·360	0·272			
4	M	2·274 2·134	0·134	0·629	2·366 2·072	0·282	0·290			
	F	2·138 1·985	0·147	0·551	2·170 2·072	0·094	1·126			
		A2G			CBA			LAC Grey		
	Group 2	y	b	λ	y	b	λ	y	b	λ
1	M	2·224 2·081	0·137	0·583	2·232 2·047	0·178	0·295	2·002 1·852	0·144	0·700
	F	2·188 1·994	0·186	0·494	2·172 1·978	0·186	0·248	2·069 1·978	0·087	1·059
2	M	2·182 2·017	0·159	0·470	2·296 2·108	0·181	0·219	2·063 1·949	0·110	0·806
	F	2·051 1·867	0·177	0·280	2·037 1·875	0·156	0·484	1·994 1·831	0·157	0·606
3	M	2·229 2·051	0·171	0·406	2·244 2·112	0·127	0·561	2·170 1·910	0·250	0·425
	F	2·175 1·904	0·260	0·323	2·098 1·940	0·152	0·387	2·031 1·878	0·147	0·637

		A2G			A		
Test	Group 3	y	b	λ	y	b	λ
1	M	2·167 1·981	0·179	0·397	2·075 1·858	0·208	0·546
	F	2·075 1·877	0·190	0·569	1·981 1·762	0·210	0·612
		A2G			$BrCF_1$		
	Group 6	y	b	λ	y	b	λ
1	M	2·353 2·158	0·187	0·356	2·129 2·035	0·090	1·504
	F	2·308 2·048	0·250	0·323	2·227 1·952	0·264	0·472
2	M	2·289 2·146	0·137	0·820	2·191 2·079	0·108	0·646
	F	2·260 1·919	0·328	0·213	2·072 1·835	0·228	0·437
3	M	2·275 2·116	0·153	0·496	2·364 2·116	0·238	0·247
	F	2·218 1·958	0·250	0·349	1·972 1·748	0·215	0·426
		A2G			$A2DB/1F_1$		
	Group 7	y	b	λ	y	b	λ
1	M	2·268 2·132	0·131	0·619	2·271 2·040	0·222	0·596
	F	2·397 2·172	0·216	0·305	2·274 2·114	0·154	0·495
2	M	2·246 2·106	0·134	1·005	2·281 2·024	0·247	0·143
	F	2·160 1·958	0·204	0·510	2·081 1·833	0·239	0·427
3	M	2·297 2·168	0·124	0·944	2·376 2·088	0·277	0·287 0·425
	F	2·152 2·004	0·142	0·700	2·061 1·820	0·232	

whole group and portions of seven other groups were sufficiently homoscedastic to be analysed. Only those differences significant at a probability level of 0·001 were allowed.

The Effects on the Sleeping Time Responses of the Dose of Pentobarbitone Sodium and the Sex and Strain of Mice as Determined for Inbred, Hybrid and Random Bred Mice

The results of the analyses of variance showed that there was a significant difference between the sleeping times resulting from doses of respectively 55 and 66 mg. of pentobarbitone sodium per kg. mouse for all strains of mice used.

TABLE IV

VALUES OF y, b AND λ FOR THE MALES OF GROUPS 5, 6, 8 AND 9, WITH 66 AND 55 MG./KG. OF PENTOBARBITONE SODIUM IN 10 PER CENT ETHANOL

		A2G			C57BL			C57Br/cd		
Test	Group 4	y	b	λ	y	b	λ	y	b	λ
1	M	2·354 2·058	0·284	0·278	2·183 1·990	0·185	0·788	2·077 1·784	0·281	0·406
2	M	2·246 2·106	0·134	0·586	2·175 2·007	0·161	0·679	2·016 1·863	0·147	0·739
3	M	2·249 2·120	0·124	0·463	2·081 1·951	0·125	0·993	2·151 1·985	0·159	0·277
4	M	2·286 2·095	0·183	0·374	2·135 1·990	0·139	0·779	2·196 2·017	0·172	0·360
		A2G			C3H					
	Group 5	y	b	λ	y	b	λ			
1	M	2·358 2·151	0·199	0·347	2·221 1·976	0·235	0·544			
2	M	2·396 2·134	0·252	0·172	2·202 1·978	0·215	0·414			
3	M	2·329 2·141	0·176	0·439	2·331 2·152	0·172	0·540			
4	M	2·273 2·012	0·251	0·107	2·382 2·071	0·299	0·416			
		A2G			DBA/1			A2DB/1F$_1$		
	Group 7	y	b	λ	y	b	λ	y	b	λ
1	M	2·270 2·134	0·131	0·619	2·217 2·006	0·203	0·508	2·275 2·044	0·222	0·596
2	M	2·225 2·092	0·128	1·058	2·259 1·940	0·306	0·361	2·263 1·905	0·344	0·325
3	M	2·254 2·146	0·104	1·129	2·193 1·920	0·262	0·488	2·330 2·097	0·224	0·354
		A2G			DBA2F$_1$					
	Group 8	y	b	λ	y	b	λ			
1	M	2·246 2·018	0·219	0·533	2·323 1·881	0·425	0·331			
3	M	2·228 2·048	0·173	0·448	2·313 2·146	0·160	0·409			

The effect of the age and re-injection of the mice is shown in Table II, indicating that with immature mice there is no difference between the reaction of the sexes while with re-injected older mice there is always a difference. Adult males have a greater response to pentobarbitone sodium dosage than adult females whatever the strain of mice. Definite strain differences in sensitivity to pentobarbitone sodium were found in groups 2, 4 and 6, and are also indicated in Table II.

The analyses of co-variance that were made on these groups confirmed the sex and dose differences and elucidated the strain differences. The strains LAC grey, $BrCF_1$, C57BL and C57Br/cd showed less response to pentobarbitone sodium consistently than the A2G strain, as in some tests did strains A and DBA/1. It is therefore clear that the control strain A2G is one of the more sensitive strains.

The Effect of the Strain of Mouse used on the Precision of the Sleeping Response to Two Doses of Pentobarbitone Sodium

Preliminary work indicated a straight line regression between the logarithms of the sleeping time response and the logarithms of three pentobarbitone sodium doses covering the range from 55 to 66 mg./kg. mouse in A2G and C3H males. It was therefore decided to use the index of precision $\lambda = \frac{\text{standard deviation}}{\text{slope of regression}}$ to determine strain differences.

Table III and IV give the sleeping time responses adjusted for the weights of the animals, the slopes of the regression lines, and the index of precision for some of the strains used. For those strains where both male and female mice are considered, only the LAC greys differ consistently from the A2G strain and these are less precise. Where males only are considered C57BL and C3H strains are consistently less precise, and DBA/1 and the $A2DB/1F_1$, strains more precise than the A2G strain.

Discussion

It will be necessary in discussing the responses of mice to injections of pentobarbitone sodium in 10 per cent ethanol that the homogeneity of the variances of the responses of those animals receiving injections should be considered as well as the effects of weights, age, sex and strain of the mice on the response and whether the reaction varies with the dose of pentobarbitone sodium which they receive.

The heteroscedasticity of the groups of mice was such that to make valid deductions from the various analyses, significant difference was defined at a level of probability of 0·001. The possibility of reducing this variance error by using litter mates (Mandl, 1955) or larger groups of mice was found impracticable for inbred strains.

In primarily injected random bred P/LAC mice of similar age the response of the mice and the logarithms of their weights was frequently related. The effect of the weight of mice used on the slope of the dose-response curve, was shown by Young and Stewart (1952) in an extensive analysis of a series of insulin tests in mice. The relation between the

weights of the animals and the response expected cannot be ignored, therefore, even when animals of similar age are used in any one test.

The importance of age as related to sex was found for all mice tested and has previously been noted in the tolerance of rats to barbiturates. Holck, Karan Mills and Smith (1937) found adult female rats to be more sensitive to barbiturates in contrast to the greater sensitivity of male mice. This work was extended by Homberger, Etsten and Himwich (1947), Cameron, Cooray and De (1948), also Brodie (1956) and Edgren (1957). The difference in reaction in rats has been shown to be partially sex hormone dependent, which explains its relation to age.

In work with hexabarbitone in mice, Jay, Jnr. (1958) and Brodie (1956) obtained results which stressed differences in strain responses but did not appear to find marked sex differences. Jay also found that the relation between the responses obtained in different strains can alter with dose, or the precision of the response varied with the strain. I found from a study of the index of precision for males and females that the LAC grey strain was less precise than the control, and from a study of males only that C57BL and C3H strains were less precise and DBA/1 and the hybrid A2DB/1F_1 more precise.

Michie (1955), working with pentobarbitone sodium in mice, described variance differences between strains which led him to advocate the use of random bred F_1 hybrid mice in preference to inbred mice for pharmacological work. In some of his work he used one sex only, and if both were used he considered sexes apart in statistical analysis. But he makes no mention of a sex difference in reaction time. In this work I have considered as significant only differences at the 0·001 level of probability. I have found that adult mice for all strains differ in their sleeping time according to their sex. Also the precision of their response is distributed haphazardly between inbred and F_1 hybrid strains, only the F_1 strains, which were out of DBA/1 mice mated to A2G mice, being more precise than the A2G strain. The random bred strain which I studied was less precise. In all tests the variation between strains emphasised by Chance (1957), and due to environment, was eliminated as far as possible by the manner of their similar treatment.

It was shown by Chai (1960) that the response of an F_1 hybrid to hormone may or may not fall between the responses of its parents. Both F_1 crosses out of DBA/1 and A2G strains resembled the DBA/1 strain more nearly than the A2G strain in their responses to pentobarbitone sodium dosage. The ability to forecast the usefulness of any F_1 hybrid for a particular pharmacological assay will depend on prior knowledge of the responses of its parents, but the confirmatory experimental results may be disappointing, as I have shown with insulin assay (Brown, 1961).

The effect on the responses produced by the route of injection of the substance (Bacharach, Clark, McCulloch and Tomich, 1959), has not been studied in this work and the variance of the responses may well have been more homogeneous with other injection routes.

Hypotheses may be postulated to account for the significant differences in strain response occurring at all ages and in sex response differences

apparent in adult animals. These hypotheses will have some bearing on differences due to weights and ages of the animals. Species, strain and sex differences in the metabolism of hexabarbitone have been shown by Quinn, Axelrod and Brodie (1958). If mice that sleep a shorter time catabolise the pentobarbitone sodium more quickly than others this may be due to differences in liver action (Bunsfield, Child, Basil and Tomich, 1960), liver size, or to the absorption of breakdown products by large deposits of fat in the body (Hong and Cho, 1959). These hypotheses may be tested by experiment.

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THE EFFECT OF TEMPERATURE ON THE ACTION OF PENICILLIN ON *ESCHERICHIA COLI*

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At least two lethal mechanisms operate when penicillin is allowed to act on growing cultures of *E. coli*. These effects may be observed by studying penicillin action at 44°.

In the course of studies on the mode of action of penicillin on bacteria it has become apparent that at least two distinct mechanisms of cell destruction are operative. One mechanism is clearly an interference with the synthesis of cell wall material, an essential process for normal cell division, and the second, the exact nature of which has yet to be determined, may be a non-specific poisoning or an interference with protein synthesis. In this paper, experiments are described in which these two effects, normally shown to occur simultaneously (Hugo and Russell, 1960), may be separately observed by adjustment of the temperature at which the antibiotic is allowed to act.

Methods

The organism was *Escherichia coli* type I (formerly NCTC 5934). Penicillin was a commercial sample of Benzylpenicillin B.P. with no added buffer or wetting agent. Chemicals were of analytical reagent quality. Nutrient broth consisted of peptone (Oxoid) 10 g., meat extract (Lab Lemco) 10 g., sodium chloride 5 g., water to 1 litre; final pH, after sterilising by heating at 115° for 30 min., 7·2. Viable counts were made by serial dilution of 1 ml. quantities in 9 ml. of sterile distilled water and plating in a nutrient agar, made by incorporating 2 per cent w/v of agar in the nutrient broth described above. Cultures containing 0·33M sucrose, 0·25 per cent w/v $MgSO_4.7H_2O$ and penicillin showed typical giant forms. These are attributed to cells deficient in a rigid component, the laying down of which is prevented by penicillin; they swell into spherical forms (spheroplasts) and were counted in a counting chamber, using interference or phase-contrast microscopy.

Results

A typical experiment consists in adding 0·5 ml. of a 17 hr. culture of *E. coli* (approximately 3×10^8 viable organisms) to nutrient broth containing sucrose and magnesium sulphate and 5,000 u/ml. penicillin and incubating with rotation at 37°. At the end of 5 hr. about a third of the inoculum has been converted to spheroplasts; about two-thirds has been killed by a mechanism other than inhibition of cell wall synthesis and potential lysis (Hugo and Russell, 1960). If this experiment is repeated at 44° spheroplast formation is almost completely inhibited and the lethal effect is enhanced (Fig. 1).

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The effect of temperature on the growth of *E. coli* from an inoculum of 2×10^8 cells was measured from growth curves, and from these the mean generation time and lag phase was calculated. These are summarised in Table I.

Stability of Spheroplasts at Various Temperatures

Whether spheroplasts are formed but do not survive above 37° was next investigated. Spheroplasts were induced by exposure of *E. coli* at 37° to

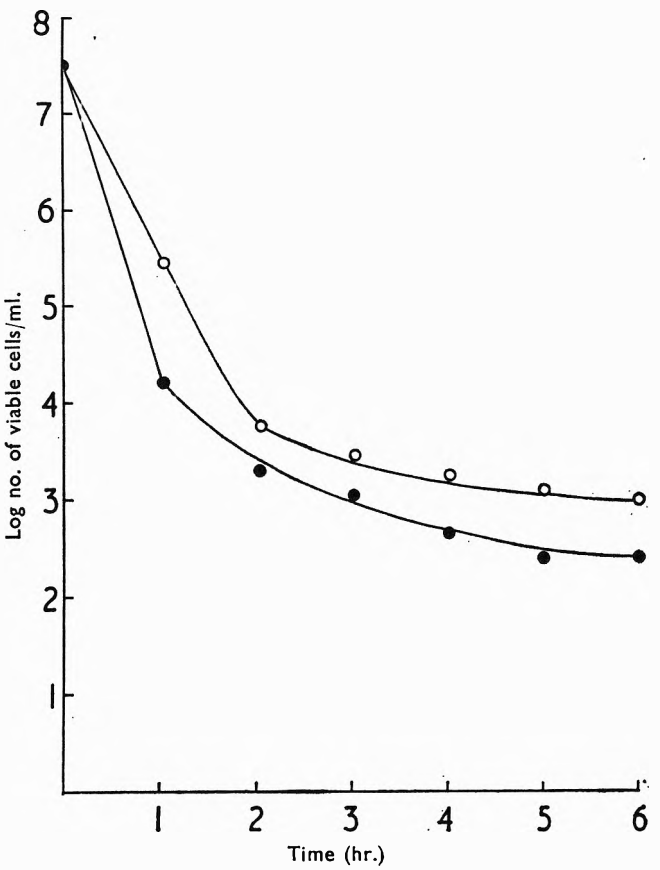

FIG. 1. The effect of temperature on the action of 5000 u/ml. penicillin on *E. coli* in nutrient broth containing 0·33M sucrose and 0·25% per cent w/v $MgSO_4 \cdot 7H_2O$.
37° ○—○ 44° ●—●

5,000 u/ml. penicillin. Aliquots were examined at intervals after storage at various temperatures.

Table II shows that over a period of 3 hr., the spheroplasts are stable at all the temperatures tested; in view of the reports (e.g., Gebicki and James, 1958) that spheroplasts are susceptible to mechanical and thermal shock, the stability over this period at 55° is surprising. Since spheroplasts were formed before exposure at various temperatures a second

experiment was made. Accordingly, 0·5 ml. of a 17 hr. culture of *E. coli* rotated 37° was added to 10 ml. of sucrose-Mg^{++}-broth, containing 5,000 u/ml. penicillin, and incubated at 37°. At intervals, aliquots were removed and transferred to 44°. Total incubation was for 5 hr., when

TABLE I

THE DURATION OF THE LAG PHASE AND THE MEAN GENERATION TIME OF *E. coli* AT 37° AND 44° WHEN GROWN IN A NUTRIENT BROTH CONTAINING 0·33M SUCROSE AND 0·25 PER CENT W/V $MgSO_4.7H_2O$

Temperature	Lag phase (min.)	Mean generation time (min.)
37°	30	27
44°	30	21

TABLE II

EFFECTS OF TEMPERATURE ON STABILITY OF SPHEROPLASTS INDUCED AT 37°

Temperature at which stored	Stability of spheroplasts after storage for	
	3 hr.	18 hr.
4°	+++	+++
18°	+++	+++
37°	+++	+
44°	+++	+
55°	++	nt

+++ Very stable. ++ Stable. + Less stable. nt Not tested.

TABLE III

THE EFFECT OF PRE-INCUBATION AT 37° ON SPHEROPLAST COUNTS AT 44°

Incubation at 37° (hr.)	Subsequent incubation at 44° (hr.)	Spheroplasts induced by 5,000 u/ml. penicillin after total incubation at 5 hr.*	
		1st expt.	2nd expt.
0	5	0	0
1	4	65	86
2	3	110	120
3	2	90	112
4	1	76	115

* Figures refer to percentage of number of spheroplasts induced after 5 hr. at 37°.

the tubes were examined by interference microscopy. Once spheroplast formation has been induced at 37°, the effect of 44° on subsequent spheroplast stability is negligible (Table III).

In an experiment in which the aliquots were transferred from 44° to 37°, spheroplast formation is not subsequently induced by penicillin.

Effect of Using an Inoculum Grown at 44°

The cells were grown at 44° for 17 hr. in nutrient broth or in this medium containing 0·33M sucrose and 0·25 per cent w/v $MgSO_4.7H_2O$. 0·5 ml. of the inoculum was then added to 10 ml. tubes of sucrose-Mg^{++}-broth, penicillin 5,000 u/ml., previously warmed to 44°. No difference in the pattern of response was noted from that seen at 37°.

Effect of Penicillin on Actively-dividing Cells at 44°

In the experiments described above, cells were not allowed to divide at 44° before treatment with penicillin. In the following experiment, bacteria were inoculated into sucrose-Mg^{++}-broth previously warmed to 44°, and incubated at 44° for 0, 30, 60 or 90 min. Penicillin to give

TABLE IV

THE EFFECT OF PREINCUBATION AT 44° ON SPHEROPLAST INDUCTION AT 37°

Tube No.	Incubation at 44° (hr.)	Subsequent incubation at 37° (hr.)	Presence (+) or absence (−) of spheroplasts
1	0	5	+
	0	24	+
2	1	4	−
	1	23	−
3	2	3	−
	2	22	−
4	3	2	−
	3	21	−
5	4	1	−
	4	20	−
6	5	0	−
	5	19	−

TABLE V

ADDITION OF PENICILLIN TO ACTIVELY-DIVIDING CELLS AT 44°

Min. at 44° before addition of penicillin	Viable cells when penicillin added	Counts 5 hr. later	
		Viable	Spheroplast
	per ml.	per ml.	per ml.
0*	$2{\cdot}3 \times 10^7$	$4{\cdot}4 \times 10^9$	10^6
30	$2{\cdot}3 \times 10,$	$3{\cdot}1 \times 10^9$	10^6
60	4×10^7	$2{\cdot}8 \times 10^9$	$6{\cdot}9 \times 10^6$
90	$1{\cdot}2 \times 10^8$	$1{\cdot}2 \times 10^9$	4×10^7†

* Viable cells/ml. at 0 min. in all tubes = $2{\cdot}1 \times 10^7$.
† Aggregates of spheroplasts.

5,000 u/ml. was then added, and the tubes incubated for a further 5 hr. at 44°. The results are shown in Table V. Spheroplasts were now seen at 44° but only when the bacteria have been kept at this temperature for 60 or 90 min. before the addition of penicillin.

DISCUSSION

When an aliquot of a 17 hr. culture of *E. coli* is added to sucrose-Mg^{++}-broth at 44° containing 5,000 u/ml. penicillin the bacteria are killed without being induced to first form spheroplasts. This is in contrast to the action of penicillin at 37°. Thus a separation of the two effects of penicillin is accomplished.

The lack of induction of spheroplasts cannot be attributed to instability at 44° of any spheroplasts which may be formed, as the spheroplasts are

as stable at 44° as at 37° (Table II); similarly, it cannot be attributed to the inability of the organism to grow at this temperature, for as shown in Fig. 2, cell division is rapid at 44° in the absence of the antibiotic; the fact that the stationary phase is soon reached is probably due to the so-called "M" concentration effect (Topley and Wilson, 1948).

The predominance of the direct lethal effect of penicillin might be thought to be due to the sudden transference of an aliquot of an inoculum grown at 37° to sucrose-Mg^{++}-broth containing penicillin at 44° but this seems most unlikely since similar results were obtained with an inoculum grown at 44° and also, cells incubated for 30 min. at 44° before the addition of penicillin were not induced to form spheroplasts at 44° (Table V).

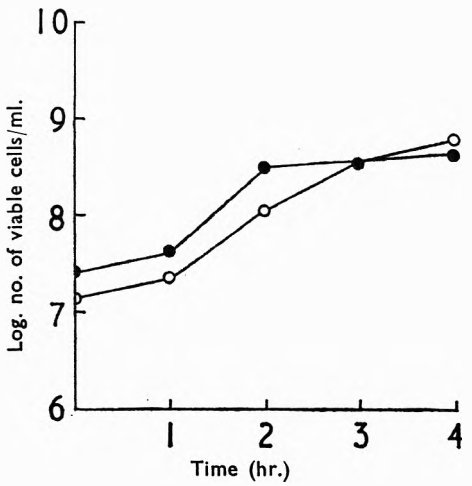

FIG. 2. The effect of temperature on the growth of *E. coli* in nutrient broth containing 0·33M sucrose and 0·25 per cent w/v $MgSO_4 \cdot 7H_2O$.

37° ○—○ 44° ●—●

Table IV shows that when the bacteria are exposed to penicillin at 44° for a period even as short as 1 hr., they are not induced to form spheroplasts on subsequent transference to 37°.

The calculated temperature coefficients per degree and per 7 and 10 degrees for the rate of kill at 37° and 44° for the first hr. (Fig. 1) are 1·07, 1·60 and 1·95 respectively. It is interesting to note that the calculated rate of kill for a 10° rise in temperature is almost doubled, which may account for the predominance of the lethal effect at 44°. It is also of importance to realise that once spheroplast formation has been induced at 37°, subsequent transference to 44° has no apparent significant effect on spheroplast stability or the increase in spheroplast diameter (Table III).

Spheroplast formation at 44° could be induced only when the bacteria had been allowed to divide at this temperature (60 or 90 min.) before the addition of penicillin, but even under these conditions the percentage conversion of rods into spheroplasts was low (about 17 or 33 per cent).

This result provides further proof of the existence of an effect of penicillin other than that involving cell wall synthesis (Hugo and Russell, 1960, 1961).

In an investigation into the effects of the external conditions on the action of penicillin on *Staphylococcus aureus*, Garrod (1945) found that the antibiotic was more effective at 42° than at 37°, although growth of the organism at the higher temperature had ceased. Similarly, Knox and Collard (1952) found that a penicillinase-producing *Bacillus cereus* was 100 times as sensitive to penicillin at 42° as it was at 37°, but the increased sensitivity could be related to a decreased production of penicillinase.

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SOME 1-ALKYL-3-AROYL UREAS

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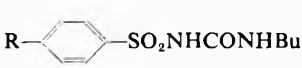
Some 1-alkyl-3-aroylureas and a few related types have been synthesised for examination as anticonvulsants. Maximum activity was found in 3-benzoyl-1-n-butylurea and 1-n-butyl-3-*o*-toluoylurea.

In 1956 preliminary reports on the promising hypoglycaemic activity of the 3-arylsulphonyl-1-n-butylureas (cf. I) of the "carbutamide" type (I; R = NH_2) led us to synthesise some formally related 3-aroyl-1-n-butylureas (II; R′ = n-Bu) for biological study.



(I)

(II)

Our first two compounds (II; R = H or *p*-NH_2, R′ = n-Bu) were found by Dr. A. David and his colleagues (Department of Pharmacology, Godalming) to be devoid of hypoglycaemic activity. The former compound, however, proved to be an anticonvulsant agent against both leptazol and electroshock-induced convulsions in rats, an observation which led us to a wider study of the group. Acyl-, diacyl- and phenacylureas had previously been synthesised by such workers as Volweiler and Tabern (1936), Blicke and Centolella (1938), Stoughton (1938) and Stoughton, Dickison and Fitzhugh (1939) and Spielman, Geiszler and Close (1948), who had noted their formal similarity to "open-chain" barbiturates and had consequently examined them for sedative, hypnotic and anticonvulsant properties. While our work was in progress Budesinsky, Emr, Muzil, Perina and Zikmund (1959) described the preparation of a few compounds of type (II) for study as hypoglycaemic agents.

The 3-aroyl-1-n-butylureas listed in Table I (see Experimental) were synthesised, except where otherwise indicated, by condensation of n-butylurea with an aroyl chloride on the steam bath, generally in a solvent such as benzene and in the presence of a hydrogen chloride abstractor such as pyridine or phenazone, or by reaction between the aroyl amide and n-butyl isocyanate in the presence of triethylamine. Their biological study revealed peak anticonvulsant activity when R was H or *o*-Me. Attempts to increase potency still further by replacement of the n-butyl group (R′) by alkyl groups containing 3 to 6 carbon atoms or by benzyl or cyclohexyl groups proved wholly unsuccessful.

The 1-butyl-3-*o*- and -*p*-nitrobenzoylureas (above) were reduced to the corresponding amino compounds. 3-*o*-Acetamidobenzoyl-1-n-butylurea (II; R = *o*-NHAc, R′ = n-Bu) was obtained from the amine (III) by reaction with acetic anhydride in benzene at room temperature. Its attempted preparation by heating the amine with glacial acetic acid led

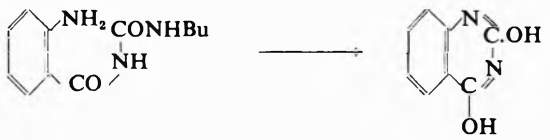
TABLE I

UREAS

R.CO.NH.CO.NH.R′

R	R′	Method	Yield per cent	m.p. °C	Formula	Found				Required			
						C	H	Cl	N	C	H	Cl	N
$(C_6H_5)_2CH$-	n-C_6H_9-	b	73	61–62	$C_{21}H_{22}N_2O_2$	61·3	10·4	—	13·2	61·6	10·4	—	13·1
Cl_3C-	n-C_6H_9-	b	30	66–68	$C_7H_{11}Cl_3N_2O_2$	32·2	4·4	41·6	11·0	32·1	4·2	40·7	10·7
Furyl	n-C_6H_9-	b	80	102–104	$C_{10}H_{14}N_2O_3$	57·0	6·7	—	13·1	57·2	6·7	—	13·3
C_6H_5-	i-C_3H_7-	c	71	116–117	$C_{11}H_{14}N_2O_2$	64·3	7·1	—	13·9	64·1	6·8	—	13·6
	n-C_4H_9-	c, e	67, 37	91–92	$C_{12}H_{16}N_2O_2$	65·7	7·4	—	13·2	65·4	7·3	—	12·7
	n-C_5H_{11}-	d	78	76–78	$C_{13}H_{18}N_2O_2$	67·2	7·8	—	12·0	66·6	7·7	—	12·0
	i-C_5H_{11}-	d	75	109–110	$C_{13}H_{18}N_2O_2$	67·1	7·6	—	12·1	66·6	7·7	—	12·0
	n-$C_{15}H_{31}$-	d	70	87–88	$C_{24}H_{40}N_2O_2$	67·7	8·1	—	11·2	67·7	8·1	—	11·3
	cyclohexyl	d	72	162–163	$C_{14}H_{18}N_2O_2$	68·4	7·6	—	11·1	68·3	7·4	—	11·4
	$C_6H_5.CH_2$-	d	76	168–169	$C_{15}H_{14}N_2O_2$	71·3	5·7	—	10·8	70·8	5·6	—	11·0
o-$CH_3.C_6H_4$-	n-C_6H_9-	d	55	114–116	$C_{13}H_{18}N_2O_2$	—	—	—	12·5	65·4	7·3	—	12·7
	i-C_6H_9-	c	56	139–140	$C_{13}H_{18}N_2O_2$	65·8	7·3	—	12·5	65·4	7·3	—	12·7
	n-C_7H_{15}-	c	55	90–91	$C_{16}H_{24}N_2O_2$	66·5	7·9	—	11·8	66·6	7·7	—	12·0
	i-C_7H_{15}-	d	72	114–115	$C_{15}H_{16}N_2O_2$	66·8	7·8	—	12·0	66·6	7·7	—	12·0
	s-C_8H_{17}-	d	60	93–95	$C_{16}H_{16}N_2O_2$	66·4	7·6	—	11·8	66·6	7·7	—	12·0
	t-C_8H_{17}-	d	60	144–145	$C_{15}H_{16}N_2O_2$	67·1	7·9	—	11·8	66·6	7·7	—	12·0
	i-C_9H_{11}-	d	80	109–111	$C_{14}H_{14}N_2O_2$	—	—	—	11·5	67·7	8·1	—	11·3
m-$CH_3.C_6H_4$- ..	n-C_7H_{15}-	d	63	64–66	$C_{16}H_{24}N_2O_2$	—	—	—	11·9	66·6	7·7	—	12·0
p-$CH_3.C_6H_4$-	n-C_7H_{15}-	d	80	133–134	$C_{16}H_{24}N_2O_2$	66·5	8·1	—	11·7	66·6	7·7	—	12·0
p-$CH_3O.C_6H_4$- ..	n-C_7H_{15}-	d	63	118–119	$C_{16}H_{24}N_2O_3$	62·4	7·3	—	11·3	62·4	7·2	—	11·2
o-$Cl.C_6H_4$-	n-C_7H_{15}-	d	50	115–116	$C_{16}H_{23}ClN_2O_2$	57·0	6·0	14·0	11·1	56·6	5·9	13·9	11·0
p-$Cl.C_6H_4$-	n-C_7H_{15}-	b	52	139	$C_{16}H_{23}ClN_2O_2$	56·7	6·2	14·0	11·0	56·6	5·9	13·9	11·0
o-$NO_2.C_6H_4$-	n-C_7H_{15}-	a	40	137–139	$C_{15}H_{23}N_3O_4$	54·5	5·9	—	15·9	54·3	5·7	—	15·8
p-$NO_2.C_6H_4$-	n-C_7H_{15}-	a, e	60, 38	150–152	$C_{15}H_{23}N_3O_4$	54·4	5·6	—	15·6	54·3	5·7	—	15·8
o-$NH_2.C_6H_4$-	n-C_7H_{15}-	–	—	134–136	$C_{15}H_{25}N_3O_2$	61·6	7·4	—	18·1	61·3	7·3	—	17·9
o-$CH_3.CO.NH.C_6H_4$- ..	n-C_7H_{15}-	–	—	131–132	$C_{17}H_{26}N_3O_3$	61·0	6·8	—	15·3	60·6	6·9	—	15·2
p-$NH_2.C_6H_4$-	n-C_7H_{15}-	–	—	167–168	$C_{15}H_{25}N_3O_2$	61·4	7·1	—	17·9	61·2	7·3	—	17·9
$C_6H_5.O.CH_2$-	i-C_5H_{11}-	c	80	104–105	$C_{20}H_{24}N_2O_3$	60·9	6·6	—	12·0	61·0	6·7	—	11·9
$C_6H_5.O.CH_2$-	n-C_7H_{15}-	c	80	108–109	$C_{22}H_{28}N_2O_3$	62·6	7·6	—	10·8	62·4	7·3	—	11·2
$C_6H_5.C(C_2H_5)_2$- ..	n-C_6H_{13}-	e	30	112–113	$C_{17}H_{26}N_2O_2$	70·0	9·0	—	9·6	70·3	8·8	—	9·7
$C_6H_5.CH:CH$- ..	n-C_4H_9-	b	62	131–132	$C_{14}H_{18}N_2O_2$	68·7	7·1	—	11·2	68·3	7·4	—	11·4

to the formation of 2,4-dihydroxyquinazoline (IV) by elimination of butylamine:



(III) (IV)

The last product (IV) has been described by Diels and Wagner (1912) who obtained it by a similar reaction involving the treatment of *o*-aminobenzoylurea with mineral acid.

Replacement of the aroyl group of (II) by diethylacetyl, trichloroacetyl, furoyl, phenoxyacetyl, diethylphenylacetyl, and cinnamoyl groups yielded compounds with virtually no anticonvulsant activity. The formally related 1-n-butyl-3-(1-hydroxy-2,2,2,trichloroethyl)urea (V) was obtained by condensing chloral hydrate with n-butylurea. Reaction between n-butyl isocyanate and benzhydrazide yielded 1-benzoyl-4-n-butylsemicarbazide (VI).

$CCl_3.CHOH.NH.CO.NHBu$ (V)

$Ph.CO.NH.NH.CO.NHBu$ (VI)

Experimental

The compounds listed in Table I were prepared by reaction of the acid chloride with the substituted urea or by reaction of the acid amide with the appropriate alkyl isocyanate. These methods are illustrated below with specific examples [(a) to (e)].

(a) 1-*n-Butyl*-3-p-*nitrobenzoylurea*. A mixture of n-butylurea (1·3 g.) and *p*-nitrobenzoyl chloride (1·86 g.) was heated on the steam bath for 2 hr. The residual solid was crystallised from ethanol to yield the *product* (1·6 g.) as cream-coloured needles, m.p. 150–152°.

(b) 1-*n-Butyl*-3-*furoylurea*. A solution of n-butylurea (11·6 g.) in dry benzene (100 ml.) was treated with furoyl chloride, pyridine (0·2 ml.) was added as catalyst and the mixture heated under reflux for 3 hr. The benzene was boiled off and the residual gum stirred with water. The resultant solid (16·8 g.) had m.p. 102–104° after crystallisation from ethanol.

(c) 1-*n-Butyl*-3-*phenoxyacetylurea*. A mixture of n-butylurea (11·6 g.), and phenoxyacetyl chloride (17 g.), in benzene (50 ml.), was treated with pyridine (7·9 g.) and the mixture heated under reflux for 2 hr. Benzene and pyridine were distilled off at reduced pressure, the solid residue was stirred with water and just acidified with hydrochloric acid. The *product* (20 g., m.p. 108–109°) crystallised from ethanol in prismatic needles.

(d) 1-*n-Butyl*-3-p-*toluoylurea*. A mixture of n-butylurea (19·5 g.), *p*-toluoyl chloride (25·8 g.) and phenazone (37·6 g.) in benzene (100 ml.) was heated under reflux for 4 hr. The solvent was boiled off and the residual solid stirred with water, collected and washed with water. The *product* (31·3 g.), had m.p. 133–134° after crystallisation from methanol.

(e) 3-*Benzoyl*-1-*n-butylurea*. A mixture of n-butyl isocyanate (12·9 g.), benzamide (12·1 g.) and triethylamine (2·5 ml.), was heated on the steam bath for 10 hr. The semi-solid residue was stirred with water and acidified with hydrochloric acid. The resultant solid was collected, washed with water and crystallised from ethanol to yield the *product* (8·1 g.), m.p. 91–92°.

3-o-*Aminobenzoyl*-1-*n-butylurea*. To a suspension of 1-n-butyl-3-*o*-nitrobenzoylurea (28 g.) in 50 per cent ethanol (200 ml.) was added ferrous sulphate (4 g.) and iron powder (50 g.) and the mixture was heated with stirring under reflux for 6 hr. It was filtered hot and the residue extracted with two 100 ml. portions of boiling ethanol. The combined filtrate and washings were boiled with charcoal and filtered. Concentration of the filtrate yielded the *product* (19·6 g.), m.p. 134–136° after crystallisation from aqueous methanol.

A solution of the foregoing amine (1·2 g.) in acetic acid (10 ml.) was heated under reflux for 1 hr., when excess of acid was removed at reduced pressure. The residual solid (0·6 g.) after boiling with ethanol had m.p. 356–358°. Found: C, 59·6; H, 3·7. Calc. for 2,4-dihydroxyquinazoline, $C_8H_6N_2O_2$: C, 59·3; H, 3·7 per cent.

A stirred solution of the *amine* (2 g.) in benzene (20 ml.) was treated with acetic anhydride (2 ml.), added dropwise. After 30 min. the mixture was diluted with light petroleum (b.p. 60–80°) to precipitate 3-o-*acetamidobenzoyl*-1-*n-butylurea* (1·1 g.), m.p. 131–132° after crystallisation from ethyl acetate-light petroleum (b.p. 60–80°).

1-*n-Butyl*-3-(1-*hydroxy*-2,2,2, *trichloroethyl*)*urea*. A mixture of chloral hydrate (16·6 g.) and n-butyl-urea (11·6 g.) was heated on the steam bath for 5 min. The *product* had m.p. 136–138° after crystallisation from ethyl acetate-light petroleum (b.p. 60–80°). Found: C, 32·0; H, 4·6; N, 10·6; Cl, 40·1. $C_7H_{13}Cl_3N_2O_2$ requires C, 31·9; H, 4·9; N, 10·6; Cl, 40·4 per cent.

1-*Benzoyl*-4-*n-butylsemicarbazide*. A suspension of benzhydrazide (13·6 g.) in toluene (50 ml.) was treated with n-butyl isocyanate (9·9 g.) and the mixture heated under reflux for 2 hr. The solids (20 g.) were collected and had m.p. 163–165° after crystallisation from methanol-ethyl acetate. Found: C, 61·3; H, 7·7; N, 17·8. $C_{12}H_{17}N_3O_2$ requires C, 61·2; H, 7·3; N, 17·9 per cent.

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A NOTE ON THE ANTIBACTERIAL ACTION OF SOME HALOGEN SUBSTITUTED CHALKONES

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Results of bacteriological tests with some halogen substituted chalkones corroborates previous reports that the bacteriostatic action of $\alpha\beta$-unsaturated ketones increases appreciably with the introduction of hydroxyl and halogen substituents.

It has been shown by Geiger and Con (1945) that $\alpha\beta$-unsaturated ketones exhibit bacteriostatic activity against Gram-positive organisms. Similar observations were made by Kamoda and Ito (1954) on their bactericidal action. More recently Schraufstatter and Deutsch (1948) demonstrated an increase in the bacteriostatic action of $\alpha\beta$-unsaturated ketones with the introduction of hydroxyl and halogen substituents. Thus 2,2',3,3',5-tribromochalkone has been shown to be active against *Staphylococcus aureus* at a dilution of 1 in 640,000. Based on these findings a number of halogen substituted chalkones were synthesised to study their antibacterial action. The syntheses of some of these, and their characterisation by the preparation of their derivatives have been described (Gudi,

TABLE I

Reaction of various groups of samples with peroxide-sulphuric test

Name of the compound 500 mg./100 g. acetone	Diameter of zone of inhibition in mm.	
	Staph. aureus	*E. coli*
2-Bromo-4-methyl-2'-hydroxychalkone	17·0	Nil
2-Bromo-4-methyl-2'-hydroxy-$\alpha\beta$-dibromochalkone	17·0	16·0
2-Bromo-4-methyl-2'-hydroxy-4'-methoxychalkone	17·0	16·0
2,5'-Dibromo-2'-hydroxy-4'-methoxychalkone	21·0	24·0
2-Bromo-2'-hydroxy-4'-methoxychalkone	17·0	17·0
2-Bromo-4-methyl-2'-hydroxy-5'-methylchalkone	16·5	16·5
2-Bromo-4-methyl-2'-hydroxy-5'-chlorochalkone	Nil	Nil
4-Bromo-2-methyl-2'-hydroxy-5'-chlorochalkone	17·0	20·0
4-Bromo-2,5'-dimethyl-2'-hydroxychalkone	17·5	20·0
4-Hydroxy-4'-nitro-diphenylsulphone	17·0	18·0

Hiremath, Badiger and Rajagopal, 1961; Kadiwall, Hirayakkannawar, Badiger and Rajagopal, 1961). The present paper records the results of bacteriological tests made with some of these chalkones.

Evaluation of Bacteriostatic Activity by a Paper Disc Method

The tests were made following the procedure of Gould and Bowie (1952) employing the two organisms *S. aureus* and *Escherichia coli* kindly made available by the Haffkine Institute, Bombay.

The paper discs, prepared from Whatman filter paper No. 1, each measured 14 mm. in diameter. They were sterilised in a petri dish in an air oven at 120° for 1 hr. Hot agar was poured in $5\frac{1}{2}$ in. petri dishes to about 7–8 mm. depth and allowed to cool to room temperature. The

agar plates were inoculated uniformly from a culture of the organism grown for 18 hr. in nutrient broth, by flooding the surfaces and then removing the excess by a sterile capillary pipette. The open plates were then allowed to dry in an inverted position in an incubator for 30 min. The sterilised paper discs were then placed on the culture medium with sterile forceps and a drop of a solution of each compound, previously prepared by dissolving it in acetone, was placed on the paper disc. A control disc using acetone only was also used. This showed no zone of inhibition with either organism. The plates were incubated overnight at 37°. The activity was measured by the diameter of the areas of inhibition, and included that of the disc in addition to the surrounding zone.

The chalkones listed in Table I compare in their antibacterial properties with 4-hydroxy-4-nitrodiphenylsulphone.

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BOOK REVIEW

NAME INDEX OF ORGANIC REACTIONS. By J. E. Gowan and T. S. Wheeler. Pp. 293 (including Index). Longmans, Green & Co. Ltd., London, 1960. 50s.

When the first small version of "Name Index of Organic Reactions" was published by the Society of Chemical Industry in 1950 notes of this kind proved to be invaluable as a reference to organic reactions associated with their discoverers. This must have been generally recognised since the first edition found immediate favour and two subsequent reprints were rapidly exhausted.

The original aim was to give an idea of the nature of the reaction and also a few references from which further information could be gathered. The authors pointed out at that time that the undesirable custom was growing up of labelling organic reactions, even those which are uncommon, with the discoverer's name. This practice, which has caused some obscurity, has nevertheless persisted and publications on organic chemistry are continually referring to reactions solely by the name of the discoverers or persons who have developed them. It is therefore necessary for the chemist to be familiar with the name reaction or to have ready access to information about it. The new edition of "Name Index of Organic Reactions" has been written to serve this purpose.

This book is far more than a revised version of the old; it has been completely rewritten and is now comprehensive, containing 739 named reactions and also molecular rearrangements. A clear and concise description of each reaction, illustrated by general or where necessary specific equations, is given. In addition, references to original papers and articles published on the reaction are included, many of which have appeared within the last ten years.

All the reactions are in alphabetical order, enabling easy cross reference to similar reactions to be made. A general index is included and also a valuable index of the type of reaction, for example, acetylation, decarboxylation or reduction; this enables the reader to consult alternative ways of performing the reaction.

Readers familiar with the first edition will realise the immense value of this new book. It will be an indispensable companion for anyone who reads the literature on organic chemistry whether as a student, teacher or research worker. The book, well bound and printed, is inexpensive for the wealth of information on organic chemistry included by the authors. N. ROBINSON.

LETTERS TO THE EDITOR

Amphetamine Toxicity in Aggregated Mice

SIR,—Amphetamine sulphate is known to be much more toxic to grouped mice than to mice housed singly in individual cages (Gunn and Gurd, 1940; Chance, 1946). It has been shown that environmental temperature and the degree of aggregation are two important factors influencing toxicity (Chance, 1946; Hogn and Lasagna, 1960) and most of the work reported in the literature has been carried out at room temperatures of 23·9–26·7° (75°–80° F.).

During the course of testing compounds for their action against grouped amphetamine toxicity in this laboratory, the importance of room temperature became very apparent. At 26·7° the LD50 for amphetamine in grouped mice was found to correspond with that reported in the literature. However, a reduction in the environmental temperature to 21·1° (70° F.) produced approximately a 4-fold decrease in the toxicity.

With aggregated mice, especially at the higher room temperature the injection of amphetamine at dose levels below 100 mg./kg. gave rise to a period of marked hyperactivity. Death was preceded by a stage of apparent exhaustion and did not normally occur less than 1½ hr. from the commencement of the experiment. It was seldom accompanied by convulsions. In isolated mice death followed a period of severe convulsions and normally occurred within 30 min. of the injection. Thus as found by Hogn and Lasagna (1960) there was a distinct difference in time to death and also in the appearance of mice before death between grouped and isolated mice.

Amphetamine is hyperthermic and a significant association has been found to exist in grouped mice between increased lethality and actions on motor activity and rectal temperature (Greenblatt and Osterberg, 1961). They found no corresponding association in isolated mice. In view of the hyperactivity which occurs and since the LD50 is markedly dependent on environmental temperature, it appeared possible that death in grouped mice could follow hyperpyrexia and heat exhaustion. Experiments were therefore made in which the body temperature of mice was measured every 20 min. for a period of 2 hr. after the injection of amphetamine sulphate.

Female Schofield albino mice of weight 20–22 g. were used. They were placed in groups of 5 in metal boxes 9 × 15 × 11 cm. deep, covered by a wide-mesh lid. Before the experiment they were housed in normal stock cages and allowed free access to food and water. The tests were made at room temperatures of 21–21·5° and 26–26·5°, and were terminated after 7 hr. At the commencement of the experiment, shortly after being placed in the metal boxes, the temperature of each mouse was measured using a thermocouple. The probe was inserted in the rectum to a constant depth of 3 cm. and was removed after each reading. After the injection of amphetamine the temperature of each mouse was recorded every 20 min. for a period of 160 min. and then every hr. up to 6 hr. Amphetamine sulphate was dissolved in distilled water and given by intraperitoneal injection at a constant volume of 10 ml./kg. Seven dose levels were used ranging from 8·8 mg./kg. to 100 mg./kg., the ratio between successive doses being 1·5.

When the test was carried out at 26° the body temperature of grouped mice given amphetamine at dose levels below 100 mg./kg. was found to rise 1·5–4·5° in the first 20 min. The maximum temperature rise over the same period at

21° was 3°. In some mice, after the initial sharp rise, the temperature gradually levelled out and returned slowly to or slightly below the control value, normally reaching this point 120–240 min. following the injection. In this case, the animals did not show signs of exhaustion and always survived the duration of the experiment. In other animals, however, the body temperature continued to rise, frequently to a point above 42°, before dropping sharply at a time when the animal showed signs of exhaustion. Most of these animals died. An example of both types of effect of amphetamine on body temperature is given in Fig. 1, where the dose of amphetamine was 13·2 mg./kg. and the room temperature 26°. Here the mean control temperature of the 5 mice was 38·4° and rose to 41·0° at 20 min., remaining at this level for a further 60 min.

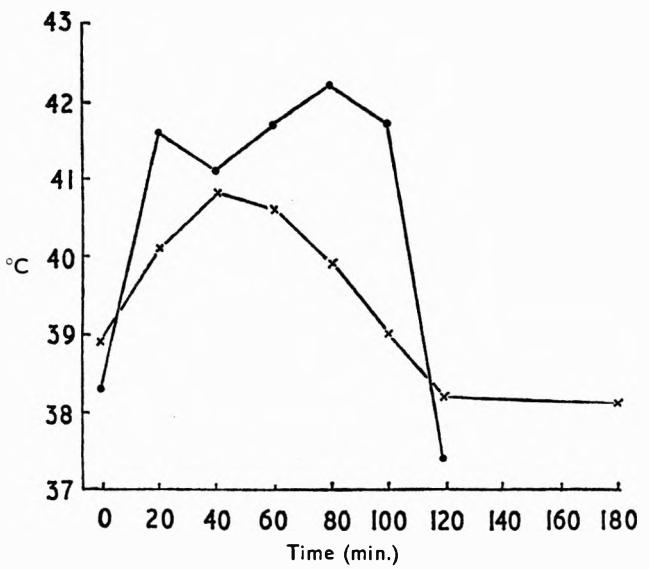

FIG. 1. Temperatures of 2 individual mice from a group of 5 given amphetamine sulphate 13·2 mg./kg., i.p. at a room temperature of 26° C. ●—● Dead at 126 min. X—X Survived for 7 hr.

After the administration of 100 mg./kg. of amphetamine to grouped mice at 26° a total mortality occurred within 34 min. of injection and was preceded by a period of violent convulsions similar to that found with isolated mice. At this room temperature the LD50 for amphetamine to grouped mice was about 25–30 mg./kg. whilst at 21° it was greater than 100 mg./kg.

At the conclusion of the experiments both at 21° and 26° it was found that, at dose levels below 100 mg./kg., all those mice whose recorded temperature had remained below 41·8° survived for the duration of the experiment. With one exception, all mice whose temperature had risen above 42·4° died. The one mouse which survived, following a rise in temperature to 43·1°, was in an exhausted state at the end of the experiment and had a body temperature of 32·2° at 6 hr. Thus from the temperature rise in individual mice it became possible to forecast with reasonable accuracy whether or not a particular animal would die. These results indicate that hyperpyrexia may be an important factor contributing to the increased toxicity of amphetamine to aggregated

mice, for although with isolated mice at dose levels in the region of the LD50 there was a marked increase in body temperature in no case did it rise above 41·8°.

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September 18, 1961.

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Neuromuscular Blocking Action of Succinyldicholine Stereoisomers

SIR,—An interesting fact has come to light during an investigation of the neuromuscular blocking action of a series of succinyldicholine compounds. On the cat gastrocnemius preparation, the isomers of succinyldi-(β-methyl)-choline had very little activity compared with that of suxamethonium; the L(+)-isomer was 1·35 times as effective as the D(−)-isomer on a molar basis, and the DL meso-mixture occupied an intermediate position (Table I).

TABLE I

COMPARISON OF POTENCY ON CAT GASTROCNEMIUS PREPARATION

Substance	Isomer	No. of mols of substance equivalent to 1 mol. of suxamethonium
Succinyldi-(β-methyl)choline	L(+)-	887
	D(−)-	1,200
	DL-meso-	913

The nature of this neuromuscular blocking action was further investigated, using the isolated innervated biventer cervicis muscle of the chick (Ginsborg and Warriner, 1960). Two points of note emerged. By contrast with all isomers of both the methiodide and the ethiodide series of succinyldi-(α-methyl)choline, both the L(+)- and the D(−)-isomers of succinyldi-(β-methyl)-choline were curare-like (Fig. 1), that is, they produced a reduction in twitch height without causing contracture. On a molar basis (+)-tubocurarine was 175 and 400 times as potent as the L(+)- and D(−)-isomers respectively. On the other hand the DL-meso-mixture produced a typical suxamethonium-like response, that is, it caused a contracture, which was found to be referable entirely to the meso content of the mixture. (Suxamethonium was 270 times as potent as the mixture in this action.)

The chick nerve-muscle preparation was selected since both the reduction of twitch height and the contracture are simultaneously available for comparison. In fact it appears that for suxamethonium-like neuromuscular blocking agents reduction of twitch height always runs parallel to contracture. Occasionally, however, a reduction of twitch height is observed shortly after the application

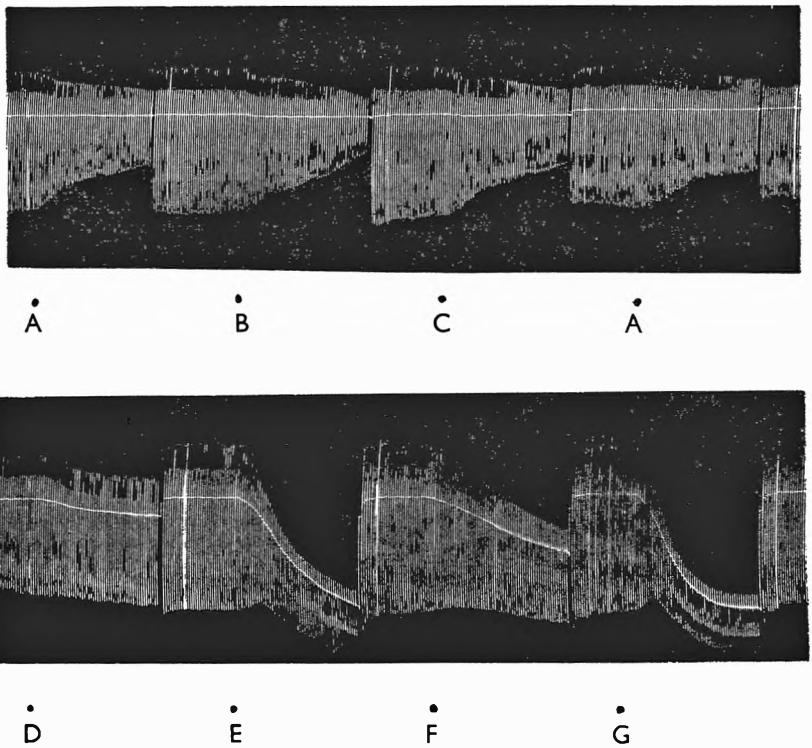

FIG. 1. Record of twitch produced by supra-maximal stimuli applied at a frequency of 12/min. Doses were given as follows:

A. 120 μg. of (+)-tubocurarine chloride.
B. 40 mg. L(+)-succinyldi-(β-methyl)choline iodide.
C. 40 mg. D(−)-succinyldi-(β-methyl)choline iodide.
D. 4 μg. suxamethonium iodide.
E. 2 mg. DL-meso-mixture succinyldi-(β-methyl)choline iodide.
F. 1 mg. DL-meso-mixture succinyldi-(β-methyl(choline iodide.
G. 8 μg. suxamethonium iodide.

of the drug, but this is rapidly swamped by the onset of contracture (Fig. 1: E and G).

All the succinyldicholine compounds used were synthesised by Mr. J. W. Clitherow in the Pharmaceutical Chemistry department of Chelsea School of Pharmacy.

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August 31, 1961.

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