REVIEW ARTICLE

MECHANISMS OF ACTION OF SCHISTOSOMICIDAL AGENTS*

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More than 25 years ago, A. J. Clark[1] pointed out that drugs may bring about their effects on living cells by affecting enzymes. In the present article the validity of this hypothesis will be considered by analysing the biochemical actions of organic trivalent antimonials on the adult stages of the parasitic worm, *Schistosoma mansoni*. In addition, the metabolic effects of another group of schistosomicidal agents will be reviewed.

Schistosomes invade the portal and mesenteric veins, the veins of the urinary bladder and the liver sinuses of man and of other mammals. They undergo a life cycle involving certain snails as intermediate hosts and penetrate the mammalian skin when the latter comes in contact with water containing the larvae which have been shed by the snails. Schistosomiasis is caused by three species, *Schistosoma mansoni*, *Schistosoma haematobium* and *Schistosoma japonicum*, and is endemic in Egypt, other parts of Africa, Central and South America, the Middle East, China, Japan and some Pacific islands. It has been estimated[2] that over 100 million human beings suffer from schistosomiasis.

Schistosomes do not depend primarily on respiratory metabolism for survival [3–6] although the oxygen tension of their environment is relatively high[7,8]. When schistosomes are cultured under completely anaerobic conditions, they remain alive for a period of at least five days[9]. Furthermore, administration of cyanine dyes to the host causes an almost complete inhibition of the oxygen uptake of the parasites; yet, these dyes have no chemotherapeutic activity against schistosomes[6]. Survival and reproduction of schistosomes depend almost entirely on the anaerobic utilisation of carbohydrate. The rate of this utilisation is extremely high. In one hour, schistosomes metabolise an amount of glucose equal to one-fifth of their dry weight[4]. In contrast to other helminths, schistosomes convert glucose quantitatively to lactic acid[4]. In this respect the metabolism of the parasite resembles that of the host; as in vertebrate tissues, lactic acid is formed *via* the Embden-Meyerhof scheme of phosphorylating glycolysis (Table I) and the occurrence of enzymes involved in this series of reactions can be demonstrated in the parasite[10–14].

Antimonials

So far, no completely satisfactory chemotherapeutic agent against infections produced by schistosomes is known. However, trivalent

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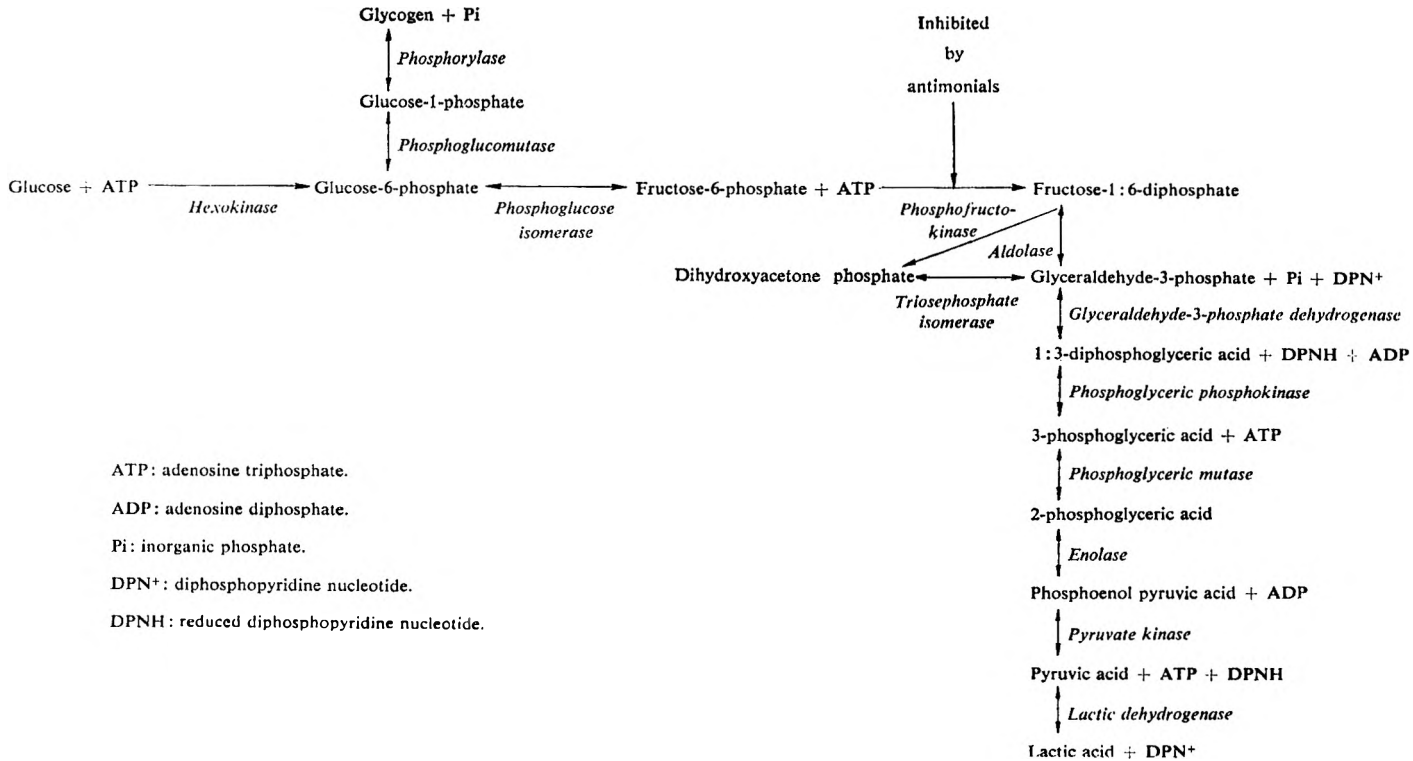
TABLE I

REACTIONS AND ENZYMES INVOLVED IN GLYCOLYSIS OF GLUCOSE AND OF GLYCOGEN



ATP: adenosine triphosphate.

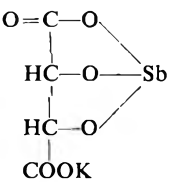
ADP: adenosine diphosphate.

Pi: inorganic phosphate.

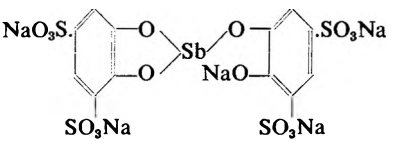
DPN+: diphosphopyridine nucleotide.

DPNH: reduced diphosphopyridine nucleotide.

organic antimonials are quite effective in the treatment of schistosomiasis although they are rather toxic[15–17]. Among these compounds potassium antimony tartrate (tartar emetic, I) and sodium antimony biscatechol disulphonate (stibophen, "fuadin," II) are used most frequently.



(I) Antimony potassium tartrate



(II) Stibophen

Low concentrations of these compounds markedly reduce the rate of glycolysis of the intact worms[4] and of homogenates of the parasites. Stibophen inhibits glycolysis of worm extracts[11] when glucose, glucose-6-phosphate or fructose-6-phosphate are used as the substrate. However, lactic acid formation from hexosediphosphate is not affected by this compound. Similar results have been obtained with antimony potassium tartrate. These observations indicate that antimonials inhibit glycolysis by blocking the formation hexosediphosphate from fructose-6-phosphate. This reaction is catalysed by phosphofructokinase and is the result of the phosphorylation of fructose-6-phosphate by adenosine triphosphate (Table I). Direct measurements of the effects of antimonials on schistosome phosphofructokinase have shown that these compounds markedly inhibit the activity of this enzyme[11]. It should be noted that the phosphofructokinase of the host has a much lower sensitivity to antimonials than the enzyme of the parasite. For example, the concentration of potassium antimony tartrate required to inhibit the mammalian enzyme to an extent of 50 per cent is 80 times higher than that which has a similar effect on the enzyme of the parasite[11]. Even with the highest concentration of stibophen used (1×10^{-2} M) no inhibition of the mammalian enzyme was observed. This selective effect of antimonials demonstrates that the enzymes which have the same catalytic function in the parasite and in the host are not identical with each other; in addition, the toxicity of antimonials for the host cannot be ascribed to an inhibition of phosphofructokinase activity. Differences at various levels in the nature of homologous glycolytic enzymes of *S. mansoni* and of its mammalian host have been demonstrated also in the cases of hexokinase[12], of phosphoglucose isomerase[13] and of lactic dehydrogenase[10,18,19]. Such differences suggest possibilities for interfering with the functional integrity

of enzymes of the parasite without affecting those catalysing the same reactions in the host.

If, in schistosomes, the rate of the phosphofructokinase reaction were limiting the rate of glycolysis of the parasite, inhibition of the activity of phosphofructokinase by antimonials would account for the inhibitory action of the latter on glycolysis. Since addition of purified rabbit muscle phosphofructokinase[20] causes an increase in the rate of glycolysis of schistosome homogenates, it is evident that the reaction catalysed by phosphofructokinase is determining the glycolytic rate of these preparations. Furthermore, the inhibitory effect of stibophen and of antimony tartrate on lactic acid production by schistosomes is abolished by the addition of mammalian phosphofructokinase[14]. Thus, lactic acid production from glucose by schistosome extracts is susceptible to inhibition by trivalent antimonials and this metabolic defect is corrected specifically by the addition of an excess of mammalian phosphofructokinase. This suggests that the reaction catalysed by phosphofructokinase might be the rate-limiting step of glycolysis in schistosome homogenates. Yet, under optimal conditions the rate of conversion of fructose-6-phosphate to fructose-1 : 6-diphosphate is more rapid than the rate of the next step in glycolysis[14], the formation of 2 moles of triosephosphate from 1 mole of fructose-1 : 6-diphosphate, a reaction catalysed by aldolase. Therefore, the rate of the reaction catalysed by phosphofructokinase is not the limiting factor. In view of these results the possibility has been explored whether the concentration of the product of the phosphofructokinase reaction could affect the rate of glycolysis. This was tested by determining the effect of the concentration of fructose-1 : 6-diphosphate on the activity of schistosome aldolase. Optimal activity is observed at a relatively high concentration of the substrate. If the concentration of the substrate is reduced below a certain critical level, a very sharp decline in the activity of aldolase occurs[14]. For example, if during glycolysis of schistosomes the molar concentration of fructose-1 : 6-diphosphate does not exceed 5×10^{-4}, even a slight decrease below this concentration, due to inhibition of phosphofructokinase activity by antimonials, would markedly reduce the activity of aldolase, resulting in an inhibition of glycolysis. Conversely, addition of mammalian phosphofructokinase would increase the rate of hexosediphosphate formation giving rise to a significant increase in aldolase activity, thereby increasing the rate of lactic acid production. Finally, inhibition of hexosediphosphate formation by antimonials could be abolished by supplying an excess of phosphofructokinase. These changes are in agreement with those observed experimentally[14]. Therefore, antimonials reduce the rate of glycolysis of schistosomes by an inhibition of the activity of phosphofructokinase; this inhibition brings about a decreased formation and thus a lower concentration of fructose-1 : 6-diphosphate which in turn results in a decrease in the activity of aldolase.

These observations were made using cell-free preparations of the worms; thus, the problem arises whether similar mechanisms operate also in the intact parasite. Utilisation of glucose and production of lactic acid by

intact schistosomes are reduced by antimonials in concentrations similar to those which inhibit the activity of the parasite's phosphofructokinase[4,14]. If antimonials were producing in intact worms an increase in the concentration of the substrate and a decrease in the concentration of the product of the phosphofructokinase reaction, this would supply evidence for the inhibition of this enzyme. Potassium antimony tartrate, in a concentration of 1×10^{-4} molar, markedly inhibits the activity of schistosome phosphofructokinase and the rate of glycolysis of worm extracts. With the same concentration of the antimonial, survival of the worms *in vitro* is reduced from 30 days to less than eight hours, and a definite reduction in the motility becomes evident after one to two hours. At this period the concentration of fructose-1:6-diphosphate in the worms is reduced markedly while fructose-6-phosphate accumulates[14]. These changes indicate an inhibition of phosphofructokinase activity during the relatively brief exposure of the parasite to potassium antimonyl tartrate. A similar reduction in the concentration of di- and an increase in that of the monophosphate ester of fructose is observed in worms obtained from mice which have received subcurative doses of stibophen[14], that is, a dosage regime producing a slight shift in the distribution of the worms from the mesenteric to the portal veins[21]. These alterations in the concentrations of phosphate esters in schistosomes indicate an inhibition of phosphofructokinase activity after exposure of the worms to antimonials within the host.

Criteria for Drug Enzyme Inhibition

The selective action of trivalent antimonials on phosphofructokinase of *S. mansoni* raises the question about the relationship between this drug-enzyme interaction and the chemotherapeutic effect of antimonials in schistosomiasis. While a multitude of enzymes are affected by drugs, it has been demonstrated only in a relatively few instances that such effects are responsible for the pharmacological or chemotherapeutic action of a particular drug. This in no way invalidates Clark's drug-enzyme theory, but can be ascribed to many inherent experimental difficulties and to frequent neglect in relating the effects of drugs on isolated biological systems to their action on the intact organism. In a discussion of this problem, Hunter and Lowry[22] have directed attention to certain requirements which must be met before rigorous proof can be accepted that a drug acts by inhibiting a particular enzyme. These criteria will be applied to the inhibitory effect of antimonials on phosphofructokinase of schistosomes.

1. *The enzyme concerned should be inhibited in the intact cells.* Exposure of schistosomes to low concentrations of potassium antimonyl tartrate *in vitro* or administration of subcurative doses of stibophen to the host produces an accumulation of the substrate and a reduction in the concentration of the product of the phosphofructokinase reaction, indicating that the activity of the enzyme is inhibited within the intact schistosomes.

2. *The inhibition of the enzyme should quantitatively explain the effects of the drug.* Inhibition of phosphofructokinase is responsible for the

reduction in the rate of glycolysis of *S. mansoni.* Since glycolysis supplies the major, if not exclusive, source of energy for the schistosomes it is quite conceivable that inhibition of glycolysis accounts for the death of the worms.

3. *Enzyme inhibition must occur with an amount of drug no greater than that necessary to produce the drug action.* This criterion has been met also. Survival of the parasites *in vitro* is reduced from 30 days to eight hours by exposure to antimonials in concentrations which produce an inhibition of schistosome phosphofructokinase activity to an extent of over 50 per cent.

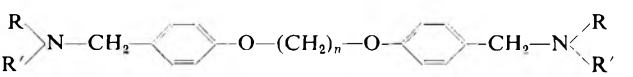
4. *If an isolated enzyme is inhibited by a concentration as low as that producing an effect on the intact organism it must be established that other cell constituents do not bind a substantial fraction of the drug.* Given concentrations of an antimonial inhibit phosphofructokinase activity of crude schistosome homogenates to the same degree as do those of purified preparations of this enzyme; therefore, it appears that there is no significant binding of antimonials by other constituents of schistosome cells.

On the basis of these considerations it is concluded that inhibition of phosphofructokinase activity can account for the schistosomicidal activity of trivalent antimonials. While the possibility cannot be excluded that these drugs may interfere also with other mechanisms essential for the survival of the parasite, these observations have revealed, within the metabolism of schistosomes, a vulnerable point which is susceptible to inhibition by a group of chemotherapeutic agents.

Alkyldibenzylamines

While antimonials have a marked inhibitory effect on a glycolytic enzyme of schistosomes another series of schistosomicidal agents exert their action through a different mechanism.

McCowen, Callender, Rennel, and Lawlish[23] have reported the amoebicidal activity of a series of alkyldibenzylamines of general formula III.



(III)

In an attempt to determine the antiparasitic spectrum of this series of compounds, it was noted that fairly low concentrations of these substances markedly reduce the survival of *S. mansoni in vitro*[24]. Incubation of schistosomes with these compounds produces paralysis of the worms; this is preceded by marked hyperactivity of the worms. On the basis of this observation the antischistosomal properties *in vitro* of some alkyldibenzylamines have been determined.

Secondary amines have considerably higher antischistosomal activity than the corresponding tertiary amine analogs. Another structural factor which has a significant effect on antischistosomal activity is the length of the central carbon chain. Optimal activity is observed with 6 carbons and a progressive decrease in activity occurs with either shortening or lengthening of the central carbon chain. When the latter

contains 6 carbons the nature of the substituent group of the secondary amine has no appreciable effect on activity. Quaternisation of the nitrogens abolishes activity.

Because of the significance of glycolysis as a source of energy for the parasite, the possibility has been investigated whether the action of dibenzylamines on the worms is associated with an interference in carbohydrate metabolism. To this end glucose utilisation and lactic acid production by schistosomes have been determined during incubation for one hour with sub-effective concentrations of alkyldibenzylamines, that is with a concentration below the one which produces changes in muscular activity during the period in which carbohydrate utilisation is measured. In the presence of a diamine, glucose utilisation is reduced significantly, but lactic acid formation is inhibited to a much lesser degree[25,26], indicating that under these conditions lactic acid must have been produced from an endogenous source. This has been confirmed by the observation that changes in the motility of schistosomes, produced by dibenzylamines, are preceded by a marked increase in glycogenolysis of the worms[26]. Following the formation of glucose-6-phosphate the pathways and enzymes concerned with the production of lactic acid are identical for glucose and for glycogen (Table I). As formation of lactic acid from glycogen is not inhibited, it appears that alkyldibenzylamines interfere either with the formation of glucose-6-phosphate from glucose or with the uptake of glucose by schistosomes. Even high concentrations of alkyldibenzylamines have no inhibitory effect on the activity of hexokinase or on the rate of glycolysis of cell-free homogenates or extracts of schistosomes; nor do they stimulate the activities of phosphorylase, of phosphoglucomutase or ATPases of the worms[26]. Because of the absence of any direct effect of high concentrations of alkyldibenzylamines on enzymes involved in the carbohydrate metabolism of the parasite it is concluded that these compounds interfere with the active transport of glucose into the worm and that the increased glycogenolysis of intact schistosomes produced by these compounds is secondary to the lack of utilisable exogenous glucose. It is noteworthy that the rate of glycolysis of cell free extracts of schistosomes is three to five times higher than that of the intact organism. Therefore, the rate of glycolysis of the latter is limited by the rate of its glucose uptake.

Because of the dependence of schistosomes on a high rate of carbohydrate metabolism, the parasite must be vulnerable to interference with glucose transport. Since antimonials interfere with another phase of the carbohydrate metabolism of the worms, i.e. with their phosphofructokinase activity, the susceptibility of schistosomes to *simultaneous* inhibition at two levels of their carbohydrate metabolism has been tested. During exposure of the worms to low concentrations (1 μg./ml.) of both an alkyldibenzylamine and of stibophen, survival of the parasite is reduced to a much greater degree in the presence of both these compounds than with the same concentration of each compound alone[26]. Therefore, the schistosomes are vulnerable to simultaneous interference at two distinct and critical levels of their carbohydrate metabolism.

Conclusions

Investigations of the metabolic effects of two groups of schistosomicidal agents have revealed that trivalent organic antimonials interfere specifically with the activity of a single glycolytic enzyme of the parasite while alkyldibenzylamines exert an inhibitory effect on the transport of glucose into the intact worm. It is concluded that a biochemical approach to chemotherapeutic problems can provide a better understanding of the mode of action of drugs and eventually may contribute to the rational, as opposed to the empirical, development of effective pharmacological and chemotherapeutic agents.

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RESEARCH PAPERS

DIKETOPIPERAZINES—A NEW GROUP OF CENTRAL NERVOUS SYSTEM-DEPRESSING AGENTS

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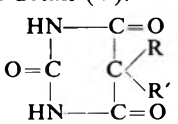
The pharmacology of Ph. 481, *N*(3′:4′dimethoxyphenylethyl)-2:6-diketopiperazine HCl is presented. The compound is shown to augment the effect of barbiturates in mice and of ether in rats. It specifically inhibits the conditioned escape response in rats. It is practically devoid of anticonvulsive, analgesic or antitussive activity, and it diminishes the tone of the skeletal muscles without interfering with neuromuscular transmission or polysynaptic spinal reflexes. It has slight antispasmodic activity on smooth muscles.

Some one hundred hitherto unknown *NN*′-substituted 2:6- and 2:4-diketopiperazines were synthesized. The starting point of this programme was the barbiturate molecule (I), especially its 2-dihydrogenated congener mysoline (II). We decided to investigate the extent to which the pharmacological properties in this type of molecule would be preserved if its main features were introduced into the piperazine instead of the pyrimidine nucleus, the position of two keto groups being 2:5- (III) or 2:6- (IV). The derivatives of type III with two alkyl-, aryl- or aralkyl-groups, attached to one of the carbon atoms were found to be hardly if at all active. The activity on the central nervous system was as a rule higher in the compounds with one or two *N*-atoms alkylated. Details of the chemistry of the whole series of compounds are given elsewhere.[1] Several of them were shown to possess central depressant properties. In this paper the pharmacological activities of a representative compound *N* (3′:4′dimethoxyphenylethyl)-2:6-diketopiperazine HCl (Ph. 481) are given in detail (V).



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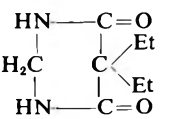

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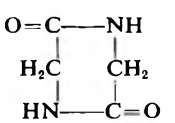

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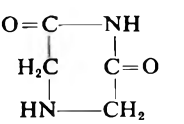

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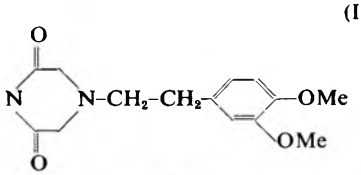

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Experimental Methods

Toxicity in Mice and Rats

The volume of the intravenous, intraperitoneal and oral doses in mice amounted to 0·1 ml./10 g., that of the intraperitoneal injection in rats to 0·1 ml./100 g. of body weight. To estimate the intravenous toxicity, the mice were first subjected for ten minutes to a temperature of 36 ± 1°. The injections were then given into the tail vein, the duration of injection being kept constant at 20 ± 2 seconds. The oral doses were given in a gum solution by stomach tube. After administering the drugs the animals were kept at a temperature of 24 ± 2° for an observation period of three days. The LD50 values and their 95 per cent confidence limits were computed with the graphical method described by Litchfield and Wilcoxon.[2]

Central Nervous System Activity

Observations on non-narcotised mice, rats, rabbits and dogs. We studied the influence of the drug on behaviour and over-all motility.

Anticonvulsive activity in mice. We investigated the influence of Ph. 481 on leptazol convulsions (60 mg./kg. i.v.), on electroconvulsions according to the method described by de Jongh,[3] on nicotine convulsions (0·5 mg./kg. i.v. injected nicotine bitartrate), on picrotoxin convulsions (4 mg./kg. i.v.), and on strychnine convulsions (1 mg./kg. i.v.).

Analgesic activity. For the experiments in rats we used a modification of the method of D'Amour and Smith[4] as described by de Jongh and Knoppers.[5] A modification of the hot plate method of Eddy and Leimbach[6] was used for observations in mice. The criteria of analgesic activity were described in a previous paper from our laboratory.[7]

Augmentation of hynoptic action of pentobarbitone sodium in mice (TNO strain). The investigation was carried out according to the method described previously[8]. After an intravenous dose of 20 mg./kg. of pentobarbitone sodium, only five out of 300 mice slept. Ph. 481 was given orally 30 minutes before the barbiturate injection.

Augmentation of the ether effect in rats. White rats weighing 170–200 g. were used. Ether was rapidly blown into the container through an injection needle fixed to the lid[9]. The rats were removed from the container after a fixed interval and then placed on their backs. The duration of the narcosis was determined by the return of the righting reflexes. Ph. 481 and the saline solution were injected intraperitoneally immediately before exposure to the ether.

Tremorine syndrome in mice. The following reactions could be observed in all animals 30 minutes after the intravenous injection of 28 mg./kg. tremorine: tremor, dacryorrhoea, salivation and diarrhoea. Ph. 481 was given orally one or two hours before the tremorine injection.

Mescaline intoxication in mice. An intravenous injection of 160 mg./kg. of mescaline caused death in 33 out of 45 animals (73·5 per cent). The typical responses are a kind of clonic seizure followed by respiratory

arrest within one or two minutes. Ph. 481 was administered by the oral route one or two hours before the mescaline injection.

Conditioned escape reaction in rats. The method of the pole-climbing response as described by Cook and Weidley[10] was slightly modified[11]. The drug was given by the intraperitoneal and oral routes and the influence on the conditioned and unconditioned responses was observed at 30 minutes, 1, 2, 4, 6 and 24 hours after the injection.

Mono- and Polysynaptic Reflexes in Cats

Decapitated cats were used. The knee jerk reflex was elicited with an electro-magnetically driven hammer as described in a previous paper[12]. The crossed extensor reflex was elicited by stimulating the femoral nerve on the other side with supramaximal condenser discharges. The stimuli were given alternately to the patellar tendon and the femoral nerve at 15 second intervals. The substances to be tested were injected into the femoral vein and the movements of the leg were recorded by an isotonic lever.

Blood Pressure and Respiration in Cats and Rats

Cats. Allobarbitone-narcotised, decerebrated and decapitated cats were used. The drugs were injected into the femoral vein.

Rats. The blood pressure was measured with a modified tail plethysmograph on unnarcotised animals[13]. Ph. 481 was injected intraperitoneally. The observation period was six hours.

The respiration was measured in unnarcotised rats by a method described previously[14]. The observation period was for two hours after intraperitoneal injection of the drug.

Body Temperature in Rats

The body temperature of rats weighing 150–200 g. was recorded by thermocouple at intervals of 15 minutes once before and ten times after intraperitoneal injection of the drug. The average fall during the observation period exceeded a value of 1·5° only once in 30 experiments. This value was used as an all-or-none criterion for a hypothermic effect to be used for estimating an ED50 value according to Litchfield and Wilcoxon[2].

Pupil Diameter in Mice

We used the method described by Pulewka[15] with white mice weighing 16–19 g. The pupil diameters were measured immediately before and 10, 30, 60, 120 and 240 minutes after the intravenous injection of the compound.

Antitussive Activity in Rats

We used a modification of the method of Winter and Flataker[16]. Rats weighing 120–160 g. were exposed to an air flow loaded with 0·02 per cent SO_2. Details of the method will be reported. The criterion of antitussive activity was the complete suppression of cough.

The Isolated Phrenic Diaphragm Preparation in the Rat

The Bülbring[17] preparation was used, with direct and indirect stimulation.

Motility of the Intestine in the Cat

Decerebrated cats were used. A rubber balloon filled with saline and connected to a polyethylene tube was inserted into an isolated loop of the ileum, at a pressure of 5–10 mm. of water, and movements recorded graphically.

RESULTS

Toxicity in Mice and Rats

The results are given in Table I.

TABLE I

LD50 VALUES WITH 95 PER CENT CONFIDENCE LIMITS (MG./KG.)

Species	Route	LD50	Number of animals
Mouse	i.v.	520 ± 62	105
Mouse	i.p.	600 ± 282	95
Mouse	oral	> 1000	60
Rat	i.p.	414 ± 80	65

Central Nervous System Activity

Observations on non-narcotised mice, rats, rabbits and dogs. Mice and rats treated with the highest sublethal doses showed signs of muscular relaxation and highly reduced activity. There was a distinct prostration and atonia. With the more toxic doses the righting reflexes were lost, the animals showed signs of cyanosis and died gasping. These phenomena were especially evident after intravenous injection. Bleeding from the nose was observed in the rats after the highest doses (500 mg./kg.).

After intraperitoneal doses of 20–320 mg./kg. the activity in rabbits was reduced. This reduction was sometimes preceded by a short period of excitation. After the highest doses the animals had tremors and strong excitatory stimuli were needed to make them walk. All symptoms were reversible, even with the highest dose. With all doses a distinct acceleration of the respiration was seen soon after the injections, the respiration returning to normal after 30 minutes.

The compound was injected intravenously into dogs in a dose range of 2·5–160 mg./kg. Doses of 10 mg./kg. caused an inhibition of the spontaneous activity. Defecation and vomiting occurred after doses of 40 mg./kg. interfering with the "tranquillisation".

Anticonvulsive activity. The results of these experiments are given in Table II.

Analgesic activity. Ph. 481 was injected intraperitoneally into rats in a dose range of 20–160 mg./kg. After 80 mg./kg. two of ten animals showed a significant prolongation of the reaction time. After 160 mg./kg. this increased to three.

Occasional analgesic effects could be observed in mice after intraperitoneal injections of at least 80 mg./kg. (ED50 = 101 ± 17 mg./kg.)

Augmentation of the hypnotic action of pentobarbitone sodium in mice. Ph. 481 was given in oral doses of 10–80 mg./kg. The ED50 amounted to 32 ± 19 mg./kg., a dose which made 50 per cent of the animals sleep after the injection of a non-hypnotic quantity of pentobarbitone sodium. None of the animals died during the observation period of one day.

Augmentation of the ether effect in rats. Duration of anaesthesia was increased up to three times with doses of Ph. 481 from 10–40 mg./kg. According to Wilcoxon's test[18] the difference is already statistically significant at a dose level of 10 mg./kg. (P = 0·014).

TABLE II

INFLUENCE OF PH. 481 ON DIFFERENT TYPES OF CONVULSIONS IN MICE

Type of convulsions	Ph. 481 mg./kg.	Number of animals protected (Total number of animals) after 1 hour	after 2 hours	after 4 hours
Electro	up to 320	0/30	—	—
	640	7/40	1/10	1/10
	1280	9/30	12/20	10/20
Leptazol	up to 320	0/20	—	—
	640	1/29	0/20	2/10
	1280	2/22	6/19	4/19
Nicotine	up to 640	0/20	0/20	3/10
	1280	12/30	11/19	4/19
Picrotoxin	up to 320	0/20	1/10	0/20
	640	2/20	3/10	0/10
	1280	2/20	3/10	3/10
Strychnine	up to1280	0/20	0/20	—

Tremorine syndrome in mice. Ph. 481 was given in oral doses of 320, 640 and 1280 mg./kg. The tremorine syndrome was not affected even by the highest dose.

Mescaline intoxication in mice. Slight protection was observed with doses of Ph. 481 of 320–1280 mg./kg.

Conditioned escape reaction in rats. The lowest intraperitoneal dosage affecting the conditioned response was 25 mg./kg. 140 mg./kg. completely blocked the conditioned without affecting the unconditioned response. Larger quantities also affected the unconditioned response. A dose of 280 mg./kg. completely blocked the conditioned response for four hours when the unconditioned response was apparent again. The quantities needed for oral effects were higher. 100 mg./kg. was slightly effective. For the full effect of 1 g./kg. was needed. Comparative investigations showed that Ph. 481 is much more specific in this test than meprobamate. No dosage of the latter compound inhibits the conditioned response without depressing the unconditioned one.

Mono- and Polysynaptic Reflexes in Cats

5 mg./kg. of mephenesin specifically affects the polysynaptic reflex, while the monosynaptic reflex is not influenced. Ph. 481 was given in

doses of 5–64 mg./kg. With the highest dose there was a reduction of both reflexes with a slight preference for the polysynaptic reflex.

Blood Pressure and Respiration in Cats and Rats

Cats. Allobarbitone narcotised cats. Doses upwards of 1 mg./kg. of body weight caused rapidly reversible hypotensive reactions. After 32 mg./kg. a fall in blood pressure of 70 mm. of Hg lasting 10–20 minutes was observed. The frequency of the respiration was distinctly augmented by dosages upwards of 16 mg./kg. A quantity of 64 mg./kg. caused death after acute hypotension and apnoea.

Decerebrated cats. Intravenous injections of 0·5–8 mg./kg. caused extremely short-lasting hypotensive reactions of 10–30 mm. of Hg. This hypotension increased with increasing doses and reached a value of 90 mm. of Hg after 128 mg./kg. Restoration to normal took about 6 minutes. The respiration is stimulated by dosages upwards of 4 mg./kg.

Decapitated cats. Intravenous doses of 0·5–8 mg./kg. caused reversible hypotensive reactions of about 20 mm. of Hg. Upwards of 8 mg./kg. this was preceded by acute hypertension, reaching a value of 20 mm. of Hg after 64 mg./kg. This dose also caused tachycardia.

Rats. The blood pressure was measured 1, 2, 4 and 6 hours after intraperitoneal injection of 20–160 mg./kg. No clear-cut changes of the blood pressure were seen.

The respiration was measured after 40–640 mg./kg. of Ph. 481 injected intraperitoneally. None of these doses caused a significant depression of the respiration.

Body Temperature in Rats

Ph. 481 was injected intraperitoneally in doses of 20–160 mg./kg. The ED50 for hypothermic potency amounted to 64 (40–120) mg./kg.

Pupil Diameter in Mice

Intravenous injections of 40, 80, 160 and 320 mg./kg. were given. Only with the highest dose did four of ten animals show a slight and fleeting mydriatic response.

Antitussive Activity in Mice

We gave intraperitoneal injections of 40–160 mg./kg. of Ph. 481. The ED50 = 70 (57–86) mg./kg. (the ED50 value of codeine being 26 (23–29) mg./kg.).

The Isolated Phrenic Diaphragm Preparation in the Rat

The volume of the bath was 125 ml. Doses of 25 mg. did not affect the response to direct and indirect stimulation of the diaphragm preparation.

Motility of the Intestine in the Cat

Doses upwards of 0·25 mg./kg. of Ph. 481 caused a reversible suppression of the movements of the intestine. This effect increased with increasing doses.

Discussion

It is largely a matter of opinion whether a substance such as Ph. 481 is to be considered a remote barbiturate analogue. It has some barbiturate-like properties, and in addition some new ones. Whereas the barbiturates do not specifically inhibit the conditioned avoidance response in rats, Ph. 481 does. It is remarkable that there is also an almost complete absence of anticonvulsive activity. Furthermore, the hypnotic anaesthetic potency is low, only sublethal doses suppressing the righting reflexes in experimental animals. Lastly, a remarkable hitherto unexplained pharmacological effect of the compound is the muscular relaxation which it causes. We were able to exclude curariform activity or specific inhibition of the polysynaptic spinal reflexes, but this is as far as we got in our search for an explanation.

Taking everything together, we feel inclined to include Ph. 481 in the category of the so-called tranquillisers. This is admittedly a rather poorly defined group, but it does seem that some importance can be attached to a specific inhibition of the escape response in rats. If this were taken as a sole criterion, the narcotics would be tranquillisers too. Absence of pronounced analgesic activity should therefore be another element in the definition of tranquillisers. As is shown in this paper, Ph. 481 meets this criterion.

We were struck by the observation that Ph. 481 has some anti-spasmodic activity, an action which may be related to the β-3:4-dimethoxyphenyl-ethylamine moiety, which can be recognised in both the molecules of papaverine and Ph. 481.

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AN INVESTIGATION INTO THE ACTION OF BASES ON CHLOROFORM*

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The reaction of a number of bases with chloroform has been studied by titration of the liberated halide ion. The results are interpreted in the light of strength of the base, and steric factors. The examination of solutions of strong bases such as piperidine and pyrrolidine in chloroform B.P. indicates that the base reacts with an impurity present. This paper provides additional confirmation, to that obtained by Caws and Foster, of the presence of reactive halogeno-hydrocarbons in this solvent. Methods are also described for assessing the quality of a sample of chloroform B.P., and for preparing chloroform free from these reactive impurities.

There are many references in the literature to impurities in chloroform and to methods for removing them[1–5]. Water, alcohols, carbonyl chloride, chlorine and hydrochloric acid are well-known impurities in chloroform. Recently Caws and Foster[6,7] showed that other impurities were present which cause a small error in the B.P. assay process for strychnine salts.

As early as 1862 interest was reported[8,9] in the decomposition of chloroform by alcoholic potash. An extensive investigation has been made[10] of the rates of reaction of all the chloro-, bromo-, and iododerivatives of methane (except CI_4), several halogeno derivatives of ethane and the chloro-derivatives of toluene with bases such as potassium hydroxide, tetra-alkyl ammonium hydroxides, ammonia and piperidine. Hine[11] investigated the kinetics of the basic hydrolysis of chloroform in aqueous dioxan and found that strongly basic reagents were more reactive to chloroform.

Observations on the reactions of a variety of organic bases with chloroform and other organic halides have also been recorded[12–18]. For example, phenylhydrazine, benzylamine, and trimethylamine react with chloroform to give the respective hydrochlorides, whilst piperidine reacts with alkyl bromides to give the hydrobromide.

$$R{\cdot}Br + 2C_5H_{10}NH = C_5H_{10}N{\cdot}R + C_5H_{10}NH{\cdot}HBr$$

Much of interest has arisen from the use of chloroform as a solvent for alkaloids. Watkins and Palkin[19] discussed chloroform as a suitable solvent for alkaloidal assays indicating that it caused appreciable errors when used. They suggested that these errors were due to a "partial neutralisation" of the alkaloid during its extraction and that these could be eliminated by prolonged refluxing of the solvent with the alkaloid, preferably brucine before extracting the base. The reaction between ephedrine and chloroform[20,21] yielding the hydrochloride is a well-known

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test for ephedrine. Klemperer and Warren[22] investigated the composition of water-soluble crystals which separated when strychnine or brucine was refluxed with chloroform. They claimed that these were dichloromethochlorides of each alkaloid having one ionisable chlorine atom per molecule. Caws and Foster[6,7] have shown that reaction between strychnine and chloroform B.P. occurs due to the presence of reactive impurities like chlorobromomethane in the solvent. They isolated strychnine chloromethobromide and showed that the chloroform recovered at this stage produced very little, if any, reaction with further quantities of strychnine. Gas chromatography indicated various samples of chloroform B.P. to contain as much as 0·5 per cent v/v chlorobromomethane and up to 0·1 per cent v/v methylene dichloride. These impurities were found to be much reduced in chloroform recovered from reaction with strychnine.

Now, in a reaction of the type:

$$B + A - X \rightarrow BA + X$$

where B is an organic base and A — X a reactive halogeno-compound, the relative reactivity of various bases B may correspond roughly to their strengths[11]. But, several types of reactions are known where steric factors, depending on the structure of both the base and the organic halide are more important considerations. In reactions such as:

$$RR'R''N: + A - X \rightarrow RR'R''\overset{+}{N}: A + X^-$$

involving quaternisation it is known that when groups R become increasingly more bulky the reaction is retarded. Brown[23] interprets this in terms of a Frontal (F) strain, which is the strain involved in the compression of groups in A by those in the base molecule B required for the formation of the cation $(B:A)^+$. The effect and extent of this hindrance in the reactions of various bases with halogen compounds has been extensively studied[24–32]. Thus in considering reactions involving bases reference to their dissociation constants as a guide to their reactivity must be treated with reserve. The use of acids of larger steric requirements than the proton, such as trimethyl boron[28–30] has resulted in a shift of emphasis from the consideration of polar to steric effects.

Experimental

When chloroform reacts with a base, ionised halogen is liberated. The reaction was studied therefore by measuring the amount of halide ion liberated after definite time intervals. For this purpose the Volhard method was found to give more reliable and reproducible results than the Mohr and adsorption indicator methods. The quantities of ferric alum, nitric acid and nitrobenzene used were kept constant.

Materials

Chloroform B.P. was used throughout the work except where otherwise stated.

Bases. Each of the following bases were distilled into a flask protected by anhydrous calcium chloride and soda lime, the first four being fractionated using a 12-inch rod and disc column. Piperidine 105–106°;

pyrrolidine 87–88°; *N*-ethylpiperidine 129°; 2-methylpiperidine 118°; *cyclo*hexylamine 135°; benzylamine 184°; 2-phenylethylamine 92–93°/19 mm.; *n*-butylamine 77–78°; di-*iso*propylamine 84°; triethylamine 88–89°. Storage, if necessary, was in dark glass bottles in separate desiccators over anhydrous calcium chloride and soda lime.

Methylene dichloride, redistilled, was obtained and its boiling point checked.

Chlorobromomethane was redistilled at 68–69°.

A proportion of base to chloroform of 0·01 to 0·03 moles was found to yield measurable quantities of halide ion. The base was weighed into a glass ampoule, followed by 2·50 ml. chloroform (weight per ml. 1·48 g.) and the ampoule sealed. Two ampoules were prepared for each base reacting with chloroform for each time interval. The reactions were studied at 20° ± 0·02° and 60° ± 0·02°. At 60° titrations were at two hourly intervals but for weak bases at 20°, intervals of twenty-four hours were necessary. The contents of an ampoule were collected, excess base neutralised with 6N nitric acid and 1·0 ml. of 6N acid was added in excess. A suitable volume of 0·02N silver nitrate solution and 0·5 ml. of saturated ferric alum solution was then added and the excess silver nitrate titrated with 0·02N ammonium thiocyanate in the presence of 1·0 ml. of nitrobenzene. Control experiments were made on all samples of chloroform. At no time did they require more than 0·02 ml. of the silver nitrate solution.

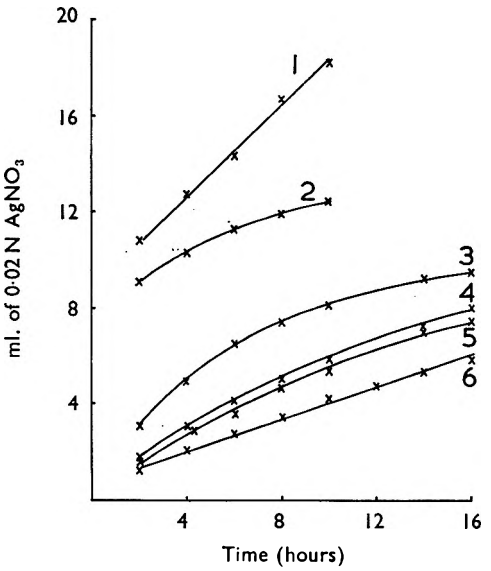


FIG. 1. Reaction between base and chloroform B.P. temperature 60°.

1. Pyrrolidine (11·12).
2. Piperidine (11·13).
3. 2-Methyl piperidine (10·99).
4. *n*-Butylamine (10·61).
5. 2-Phenylethylamine (9·83).
6. Benzylamine (9·34).

RESULTS

Reaction of Bases with Chloroform B.P.

These will be discussed in the light of the dissociation constants of the bases (Table I), and steric factors. The reactions between chloroform and the bases: benzylamine, 2-phenylethylamine, *n*-butylamine, 2-methylpiperidine, piperidine, pyrrolidine at 20° are recorded in Table II and at 60° in Figure 1. Table III summarises the results obtained for the bases: *N*-ethylpiperidine, *cyclo*hexylamine, di-*iso*propylamine and triethylamine.

The reactivity of benzylamine, 2-phenylethylamine, *n*-butylamine and 2-methylpiperidine increases with increasing pKa values. But di-*iso*-propylamine and triethylamine are considerably less reactive than is expected from their pKa values. A comparison of the rates of reaction of pyrrolidine, piperidine and 2-methylpiperidine indicates distinct differences, whilst their pKa values agree closely. *N*-Ethylpiperidine is also considerably less reactive than would be expected from its pKa value.

TABLE I

pKa VALUES OF BASES USED

Base	pKa
Piperidine	11·13
Pyrrolidine	11·12
Di-*iso*propylamine	11·05
2-Methylpiperidine	10·99
Triethylamine	10·80
*cyclo*Hexylamine	10·64
n-Butylamine	10·61
N-Ethylpiperidine	10·40
2-Phenylethylamine	9·83
Benzylamine	9·34
Strychnine	7·37

TABLE II

REACTION BETWEEN BASES AND CHLOROFORM B.P. TEMPERATURE 20°

Time	ml. 0·02N $AgNO_3$ required				Time	ml. 0·02N	$AgNO_3$ reqd.
Days	Benzyl amine	2-Phenyl-ethylamine	*n*-Butyl-amine	2-Methyl-piperidine	Hours	Piperidine	Pyrrolidine
1	1·17	0·88	1·27	1·96	2	1·46	3·06
2	1·67	1·48	1·93	3·35	4	2·66	5·05
3	2·19	2·08	2·80	4·46	6	3·85	6·43
4	2·77	2·58	3·31	5·50	8	4·77	7·28
5	3·27	—	3·84	6·26	10	—	7·90
6	3·67	3·59	4·50	6·93	12	6·46	—
7	—	3·94	5·18	7·38	14	7·00	8·52
8	—	—	5·40	7·72	16	7·32	8·75

TABLE III

REACTIONS BETWEEN BASES AND CHLOROFORM B.P. AFTER INTERVAL OF 24 HOURS AT 20°

Base	ml. 0·02N $AgNO_3$ required
N-Ethylpiperidine	0·02
*cyclo*Hexylamine	0·10
Di-*iso*propylamine	0·02
Triethylamine	0·20

Clearly another factor other than the strength of the base is involved. Steric effects explain these irregularities. Thus triethylamine and di-*iso*-propylamine have large steric requirements in their interaction with chloroform. Those of benzylamine, 2-phenylethylamine and *n*-butyl-amine are small since the nitrogen atom is not hindered by bulky groups. The low reactivity of *cyclo*hexylamine may be associated with the buckling

of the ring. The decrease in the rate of reaction of 2-methylpiperidine compared with piperidine is due to the position of the methyl group. Thus an increased F strain in the molecule of the adduct has to be overcome. The marked decrease in rate of reaction noted with *N*-ethylpiperidine is due to the ethyl group. The increased reactivity of pyrrolidine over piperidine results from the decreased F strain characterising the five-membered ring adduct compared with that present in the six-membered ring adduct formed with chloroform. Thus the two α-methylene groups of pyrrolidine are held back to a greater extent from the portion of the halogenohydrocarbon molecule co-ordinating with the nitrogen atom than similar groups in piperidine and therefore give less steric hindrance to the reaction involving pyrrolidine.

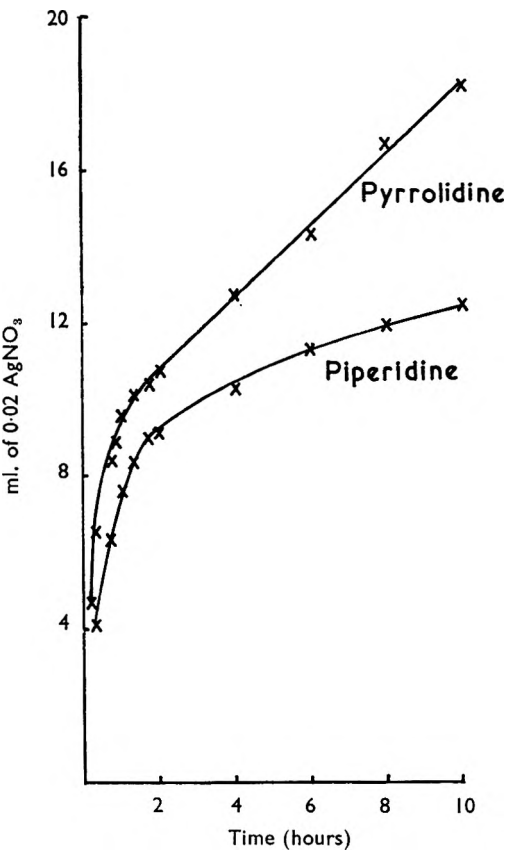


FIG. 2. Reaction between base and chloroform B.P. Temperature 60°.

The curves for piperidine and pyrrolidine (Fig. 1) indicate that, at 60°, both have a high initial rate of reaction with chloroform. Hence each reaction was investigated closely for the first two hours and the results, shown in Figure 2, seem to indicate the presence of a reactive impurity[7] in chloroform B.P. This is supported by the observation that the marked increase in the rates of reaction of piperidine and pyrrolidine with chloroform at 20° (Table II) compared with other bases is not so pronounced at 60° (Fig. 1). The explanation might well be that at the lower temperature all the chlorobromomethane in chloroform had not reacted completely with any one of the bases, whilst at the higher temperature both piperidine and pyrrolidine decompose all the chlorobromomethane during the first two hour period, resulting in further reaction being comparatively slow. Complete removal of chlorobromomethane probably results in a liberation of halide ion equivalent to approximately 9·0 ml. 0·02N silver nitrate solution.

Effect of Addition of Chlorobromomethane to Chloroform B.P.

To test the above assumptions it was decided to add successive quantities of 0·25 (K) 0·50 (L) per cent v/v chlorobromomethane to chloroform and re-examine the reactions with piperidine. A comparison of curves K and L with that for chloroform B.P. (Fig. 4) suggests that chloroform contains about 0·25 per cent v/v chlorobromomethane, since the abrupt change in slopes of each of the curves occurs at approximately 9·0 (E); 17·0–18·0 (K); 26·0–28·0 (L) ml. of 0·02N silver nitrate solution.

Purification of Chloroform B.P.

At this stage it became apparent, that in addition to strychnine[7], both piperidine and pyrrolidine could be used to remove chlorobromomethane and possibly methylene dichloride from chloroform (Fig. 2). The method of Caws and Foster[7] was first attempted. Chloroform samples recovered from boiling under reflux with strychnine for various intervals of time were examined by reaction with piperidine, using the usual proportions of reactants at 60° (Fig. 3). It is clear that refluxing for 7 days at these concentrations is required to remove the impurity (or reduce its concentration to a minimum).

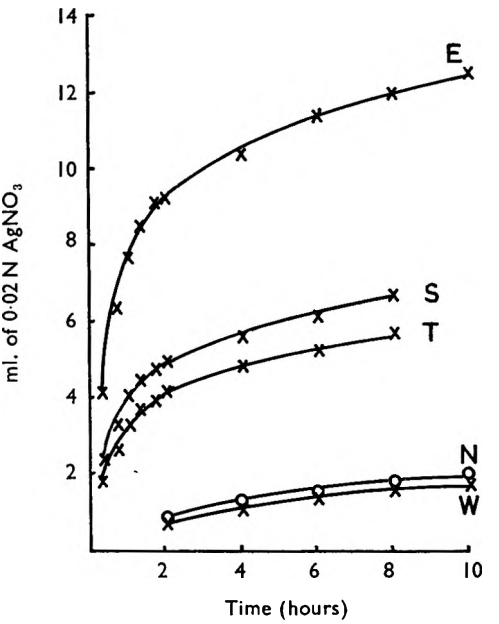


FIG. 3. Purification of Chloroform B.P. Temperature 60°.
Reaction between piperidine and recovered chloroform samples obtained by (i). refluxing 500 ml. chloroform B.P. with 10 g. of strychnine for (S) 10 hours (T) 22 hours, (W) 7 days, or (ii) refluxing 250 ml. chloroform B.P. with 7·1 g. of pyrrolidine for 72 hours. (Sample N.)
E = Reaction between piperidine and chloroform B.P.

Next, an attempt was made at employing pyrrolidine for this purpose. 7·1 g. of pyrrolidine (molar fraction) was boiled with 250 ml. chloroform under reflux for 72 hours. The separated chloroform was repeatedly acid washed followed by repeated water washing before standing over anhydrous calcium chloride for 24 hours and redistilling, 60·5 to 61·0°. The application of the piperidine reaction to the recovered chloroform (Fig. 3, curve N) indicates complete or almost complete removal of reactive halogeno-compounds.

An attempt was also made at removing the reactive impurities in chloroform by boiling under reflux with an ethanolic solution of potassium hydroxide. The piperidine reaction was applied to the chloroform before

treatment (this sample of chloroform B.P. was different from that used in the rest of the investigations) and also to the chloroform recovered (Table IV). It is clear that this method does not remove the impurities. Examination of the white solid separating during boiling showed it to be potassium chloride. No trace of bromide could be detected[33].

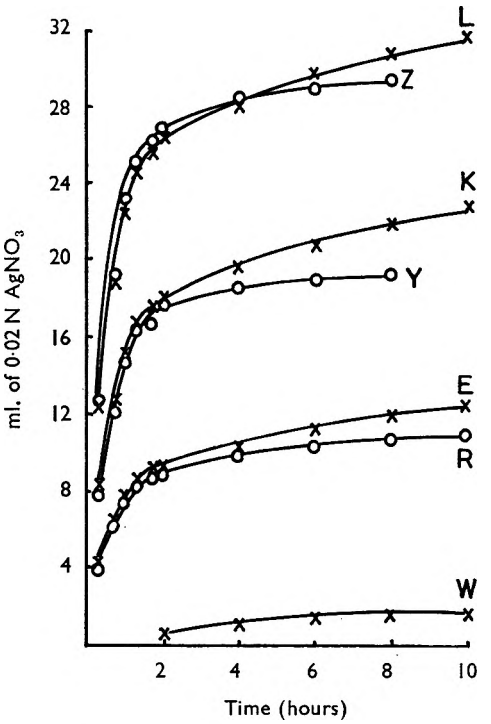


FIG. 4. Reaction between piperidine and chloroform. Temperature 60°.

×	W =	purified chloroform		
O	R =	" "	+ 0·25 per cent	CH_2ClBr
O	Y =	" "	+ 0·50 per cent	"
O	Z =	" "	+ 0·75 per cent	"
×	E =	chloroform B.P.		
×	K =	" "	+ 0·25 per cent	"
×	L =	" "	+ 0·50 per cent	"

Effect of Addition of Halogeno-methanes to Purified Chloroform

Having now prepared chloroform free from reactive impurities it seemed of interest to examine the action of piperidine on samples prepared from purified chloroform so as to contain 0·25 (R); 0·5 (Y); 0·75 (Z) per cent v/v chlorobromomethane, respectively. It can be seen (Fig. 4) that the abrupt change of slope in the curves occur at approximately the same corresponding volumes of 0·02N silver nitrate solution as with chloroform B.P. (E) and samples of chloroform B.P. containing 0·25 (K); 0·50 (L) per cent v/v chlorobromomethane.

Further examination shows, however, that after the first two-hour interval the following pairs of curves (E,R), (K,Y) and (L,Z) deviate from one another. The explanation for this may well be that curves R, Y and Z represent the action of piperidine on pure chloroform after

TABLE IV

ATTEMPTED PURIFICATION OF CHLOROFORM B.P.*

REACTION BETWEEN PIPERIDINE AND (*a*) CHLOROFORM B.P. (*b*) CHLOROFORM RECOVERED

Time		ml 0·02 N $AgNO_3$ required	
Hours	Min.	Chloroform B.P.	Chloroform recovered
	20	3·61	3·29
	40	5·35	5·31
1	00	6·21	6·15
1	20	6·90	6·82
1	40	7·27	6·77
2	00	7·72	7·41
4		8·66	8·40
6		9·54	9·14
8		10·55	9·78
10		11·42	—

* 7 g. potassium hydroxide, 100 ml. 95 per cent ethanol, 200 ml. chloroform B.P. refluxed for 44 hours Temperature 60°.

first reacting with the chlorobromomethane whilst curves E, K and L represent the action of piperidine on firstly, chlorobromomethane and then on chloroform plus another less reactive impurity, possibly methylene dichloride. Consequently, a sample was prepared from purified chloroform to contain 0·50 per cent v/v methylene dichloride. The piperidine

TABLE V

REACTIONS OF BASES WITH DIFFERENT SAMPLES* OF PURIFIED CHLOROFORM

	ml. 0·02N $AgNO_3$ soln. required					
	1		2		3	
Base	2 hours	16 hours	2 hours	16 hours	2 hours	16 hours
2-Methylpiperidine	0·63	—	0·29	—	0·41	1·10
n-Butylamine	0·63	—	0·24	0·84	0·34	0·93
Benzylamine	0·83	—	0·39	0·88	0·45	1·45

*The samples were prepared from chloroform B.P. after refluxing respectively with (1) pyrrolidine for 72 hours, (2) pyrrolidine for 24 hours, recovered and refluxed with additional base for further 4 days, (3) strychnine for 9 days.

reaction on this sample showed methylene dichloride to be considerably less reactive than chlorobromomethane.

The "Preparation" of Chloroform B.P. from Purified Chloroform

This was attained by adding to purified chloroform (*a*) 0·25 per cent v/v chlorobromomethane and (*b*) 0·10 per cent v/v methylene dichloride. Subjection to the piperidine reaction (Fig. 5) indicates that the addition of 0·10 per cent methylene dichloride virtually superimposes curve R (purified chloroform + 0·25 per cent v/v CH_2ClBr) onto curve E (Chloroform B.P.). This result appears to confirm that strychnine and pyrrolidine

remove chlorobromomethane and methylene dichloride from chloroform B.P. In fact, it was also shown that refluxing with pyrrolidine, at the concentrations previously indicated, for only 24 hours removed the reactive impurities almost entirely, as indicated by the piperidine reaction.

Since chloroform B.P. contains ethanol, the effect of adding controlled amounts of this to chloroform B.P. (already containing 1 to 2 per cent)

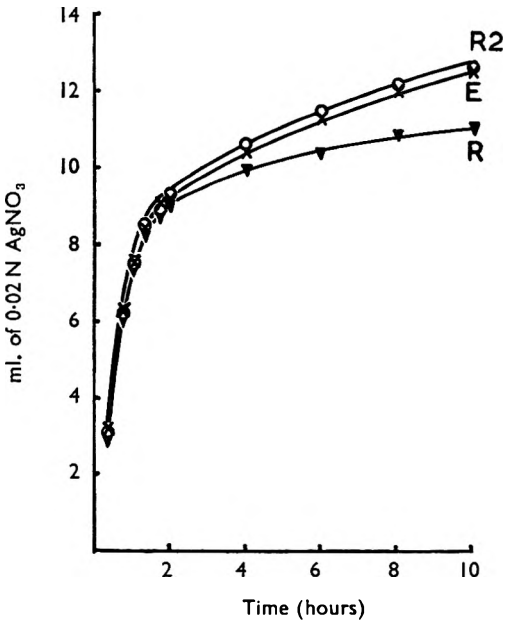


FIG. 5. Reaction between piperidine and purified chloroform containing added volumes of chlorobromomethane and methylene dichloride. Temperature 60°.

E = chloroform B.P.
R = purified chloroform + 0·25 per cent v/v CH_2ClBr
R2 = purified chloroform + 0·25 per cent v/v CH_2ClBr + 0·10 per cent v/v CH_2Cl_2

and to purified chloroform was investigated. No significant differences in extent of reaction with piperidine were noticed, when small amounts of ethanol were present.

Reaction of Bases with Purified Chloroform

At this stage, the action of organic bases, e.g. *n*-butylamine, benzylamine and 2-methylpiperidine on purified chloroform was examined (Table V). The reaction of piperidine with chloroform purified by both the strychnine and pyrrolidine methods are shown in Figure 3 (curves W and N). The reaction of pyrrolidine with chloroform purified by strychnine is shown in Figure 6 where it is compared with that of piperidine. Thus the decomposition affected by each of these bases is considerably less than that recorded in their reactions with chloroform B.P. It is difficult to decide whether or not the bases are attacking pure chloroform

(Table V) as the reactions may be due to small quantities of reactive impurities which have escaped removal. It does seem, however, that pyrrolidine and possibly piperidine react with pure chloroform.

Discussion

Though this paper does not offer any direct evidence on the mechanism of reaction of the bases studied with chlorobromomethane, dichloromethane and possibly chloroform itself, it may well be that quaternisation is involved. It is interesting to note that in the reaction of piperidine with purified chloroform containing 0·25 per cent v/v chlorobromomethane (Fig. 4, curve R) the change of slope in the curve, taken to signify the completion of reaction with chlorobromomethane, occurs between corresponding readings of approximately 8·0 and 9·5 ml. 0·02N silver nitrate solution. It can be calculated that this volume would be required provided only that both the chlorine and bromine content of the added chlorobromomethane were completely ionized. To account for this a quaternary ion of the following type may be formed, requiring two molecules of base per molecule of chlorobromomethane:

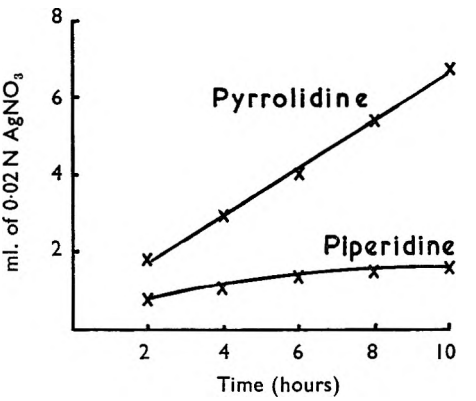
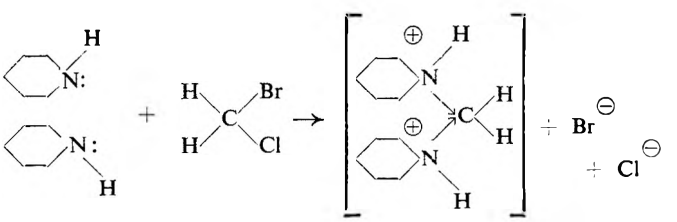


Fig. 6. Reaction between base and purified chloroform.
Temperature 60°.



The fact that strychnine, piperidine and pyrrolidine react preferentially with the chlorobromomethane in chloroform B.P. whilst a strong base such as potassium hydroxide does not, suggests that a particular structural feature embracing the basic nitrogen atom may be a necessary requirement. This structural feature is believed to be closely associated with the "tying back" of other groups attached to the nitrogen atom. This is effectively obtained in such molecules as those of strychnine, piperidine and pyrrolidine.

It has been suggested[7] that the origin of the chlorobromomethane impurity in chloroform B.P. is the commercial chlorine used in its manufacture. A survey of the technical literature by the present author confirms this view. It is reasonable to suppose that methylene dichloride is also present especially as some chloroform may be manufactured by the chlorination of methane.

The particular sample of chloroform B.P. used throughout this investigation appears to contain approximately 0·25 per cent v/v chlorobromomethane and 0·10 per cent v/v methylene dichloride. It has, however, been stressed[7] that chloroform B.P. can be expected to be a variable product.

This paper also indicates a method for assessing the quality of a sample of chloroform B.P., by treating it under the conditions prescribed with piperidine, following the rate of reaction and observing the position where the change of slope of the curve takes place.

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THE ISOLATION AND DETECTION OF ERGOMETRINE IN TOXICOLOGICAL ANALYSIS

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The isolation of ergometrine from viscera is accomplished by extraction with ether from an ammoniacal aqueous solution saturated with ammonium sulphate. Purification is achieved by paper chromatography and detection of 0·1 μg. by ultra-violet fluorescence and the *p*-dimethylaminobenzaldehyde reaction. The detection of ergometrine and procaine in urine samples after administration of therapeutic doses is described.

Ergot is one of the classical abortifacients and has been a drug of toxicological interest for many years. Most workers, however, in discussing its detection in viscera refer to sclererythrin and usually avoid specific reference to isolation and detection of the alkaloids.

In recent years ergometrine has replaced the crude drug in obstetric practice and, although this particular alkaloid has little oxytocic effect on the pregnant uterus before term, it has been used in an effort to procure abortion.

Koppe and Dille[1] added ergometrine to muscle, blood, and liver and using continuous extraction with chloroform of the sodium sulphate dried tissue were able to recover a substantial proportion. The same workers injected guinea pigs in doses of 25 mg./kg. and were just able to detect the alkaloid in the viscera 5 hours after. Notwithstanding these encouraging results no other work has been published. Recently, in a case of insulin poisoning, it was necessary to investigate an allegation that a woman had received 0·5 mg. injection of ergometrine maleate into her buttock some hours before her death[2] and because in the circumstances the method of Koppe and Dille was unsuitable the problem was re-investigated.

Experimental

Extraction Method

Before considering the process which could be used to extract the alkaloid from buttock tissue it was necessary to know the stability of the alkaloid in acid and alkali, and also its partition coefficients between an aqueous phase and organic solvents. The rate of decomposition of the alkaloid was followed by observations on the ultra-violet absorption spectra and by examination of paper chromatograms.

The E(1 per cent, 1 cm.) values for ergometrine maleate were as follows:—

Solvent	λ max (mμ)	λ min (mμ)	E(1 per cent, 1 cm.) (λ max)
0·1N NaOH ..	310	269	225
0·1N H_2SO_4 ..	312	269	221

Although at room temperature the alkaloid was stable in 0·1N sodium hydroxide or 0·1N sulphuric acid for several hours elevation of temperature or increase in acidity or alkalinity resulted in rapid loss of the alkaloid. In 5 per cent acetic acid the alkaloid survived heating in a boiling water bath for 30 minutes. If ether was used as the solvent for extraction the proportion of alkaloid extracted from ammoniacal aqueous solution could be significantly increased if the aqueous phase was saturated with ammonium sulphate. In this way 80 per cent of the alkaloid was recovered by extracting the ammoniacal solution twice with equal volumes of ether, whereas three extractions in the absence of the ammonium sulphate recovered only 36 per cent. These results led to experiments on the use of the Daubney and Nickolls method[3] for the extraction of the alkaloid from tissue and urine. The method of Stas and Otto was not used because of the high proportion of fat in the buttock tissue. The method used to extract the alkaloid was as follows. The sample of tissue, 60–200 g., was macerated with 350 ml. of 5 per cent v/v aqueous acetic acid and sufficient ammonium sulphate was added to make a saturated solution. After heating in a boiling water bath for 30 minutes the macerated tissue was filtered through a paper pulp pad on a sintered glass Buchner funnel. The tissue on the pad was washed twice with 250 ml. aliquots of hot 5 per cent acetic acid. It is important not to reduce the pressure in the filtration to such a degree that the molten fat is sucked through the pad. After cooling, the combined aqueous filtrate and washings were extracted with an equal volume of ether which was examined in this particular case for the phenolic preservatives which are added to commercial insulin preparations. The aqueous phase was then made alkaline with ammonia and resaturated with ammonium sulphate. Two extractions with equal volumes of ether followed by evaporation of the ether gave the crude alkaloid extract. Experiment showed that this method of extraction gave a recovery of approximately 40 per cent when 1, 10 or 100 μg. were added to 200 g. samples of buttock tissue. Urine was extracted in the same way after the addition of ammonium sulphate.

Purification

Paper chromatography was used as the method of purification. It was found possible to load up to about 60 per cent of the total alkaloid extract from 200 g. of tissue on to one spot. Because the limit of detection was approximately 0·1 μg. and the recovery 40 per cent, this implies that quantities above 0·5 μg. of ergometrine maleate in 200 g. quantities of tissue should be detectable. Experiment showed that 1 μg. added to tissue could be detected and further that storage of the tissue at 0° for several weeks did not lead to a loss of the alkaloid. There is a large volume of literature on the separation of the ergot alkaloids but none of it refers to the separation of ergometrine from body constituents. In this laboratory the *n*-butanol : citric acid solvent system of Curry and Powell[4] using Whatman No. 1 paper buffered with 5 per cent sodium dihydrogen citrate has been extensively used for the separation of alkaloids. Using this system it was found that ergometrine ran at R_F 0·23, a position unoccupied

by any of the constituents found in a normal buttock. Very good spots were obtained. Detection was by observation of the ultra-violet fluorescence and by the blue-violet colour obtained when the paper was gently heated after spraying with 0·5 per cent solution of *p*-dimethylaminobenzaldehyde in ethanol containing 10 per cent of concentrated sulphuric acid. 0·1 μg. of ergometrine could be detected using either method. In buttock tissue faint fluorescence from fat slightly reduced the sensitivity of this method but no such difficulty was experienced in urine samples. Most of this work was done using an ultra-violet light fitted with a Wood's glass filter. It was subsequently found that when a lamp fitted with a Chance OX7 filter was used the sensitivity of the fluorescence method was substantially increased. This series of experiments investigated only the isolation of the alkaloid from buttock tissue or urine but Dr. E. G. C. Clarke tells me that the method works equally well in the extraction from liver tissue.

The Urinary Excretion of Ergometrine and Procaine

The urinary excretion was followed using the methods described above. Urine was collected from women who had been given a single 0·5 mg. injection of ergometrine maleate during childbirth. 50 ml. samples were extracted once with ether from acid solution and then twice with ether from ammoniacal solution saturated with ammonium sulphate. 1 and 10 per cent aliquots of this alkaloid extract were examined by paper chromatography. It was found that the alkaloid could be detected using the *p*-dimethylaminobenzaldehyde reaction up to $7\frac{1}{2}$ hours after the injection. In one case where the time interval was $8\frac{1}{2}$ hours positive fluorescence spots were obtained using the Chance OX7 filtered light, although no chemical reaction could be obtained. The maximum concentration of ergometrine in the urine generally occurred 2–3 hours after the injection.

Procaine was the only common base that interfered with the reaction. This local anaesthetic was present in several urines usually in much higher concentration than the ergometrine after injections of procaine penicillin. It runs slightly faster than ergometrine ($R_F = 0{\cdot}25$) and gives a yellow colour immediately on spraying with the *p*-dimethylaminobenzaldehyde reagent.

Procaine is known to be hydrolysed in tissue by procaine esterase and doubts have been expressed about the chance of its successful detection by forensic toxicologists[5]. No difficulty was experienced in its detection in this series of experiments even after the urines had been stored for several weeks.

Discussion

In toxicological analysis emphasis is usually placed on the detection of toxic quantities of poisonous compounds. Occasionally, however, it is necessary to analyse viscera for non-toxic drugs alleged to have been given in therapeutic doses. Such an occasion arose recently when a murderer who used injections of insulin as the means to kill alleged that the hypodermic marks were caused by injections of ergometrine maleate. Despite

the extremely small doseage of this drug it was found possible to demonstrate that had such an injection been given its detection would have been ensured.

Acknowledgements. I am most grateful to Dr. D. E. Price for the supply of the samples of buttock tissue and urine, to Dr. F. G. Tryhorn for his encouragement and to Mr. E. R. Rutter for his skilled technical assistance.

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ANTAGONISM BETWEEN ALKYLATED AND NORCOMPOUNDS OF THE MORPHINE GROUP INJECTED INTRACISTERNALLY INTO MICE

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Received March 3, 1959

The analgesia which resulted from the intracisternal injection of mixtures of morphine and normorphine in mice developed more slowly than, and finally equalled but did not exceed that of the more active component of the mixture. Pethidine and morphine antagonised normorphine similarly. However, the analgesia which followed the intracisternal injection of mixtures of pethidine and norpethidine developed as rapidly but was less intense than that due to either single component of the mixtures.

BECKETT, Casey and Harper[1] advanced the tentative hypothesis that drugs of the morphine group may require to be dealkylated before they can exert their analgesic effect. The finding by Lockett and Davis[2] that morphine and normorphine had equal analgesic action when injected intracisternally into mice lent some support to this view since the action of normorphine developed a little more rapidly than that of morphine. But, it will be shown by the experiments reported below that normorphine proves less active than morphine when the exposure to ether for intracisternal injection is reduced to less than 30 seconds, although the effect of normorphine on the pain threshold still develops the more quickly. Lockett and Davis also observed that *N*-allylnormorphine antagonised the analgesic actions of intracisternal injections of normorphine and morphine equally and appeared to compete with these two drugs at receptor sites in the central nervous system.

The object of the present work has therefore been to decide, if possible, whether alkylated compounds of the morphine group can produce analgesia by direct combination with receptors, without preliminary dealkylation. This problem was attacked by making comparison in mice of the analgesic actions of alkylated and nor compounds, administered intracisternally, separately and in mixtures. Evidence of initial competition between the alkylated and nor compounds was expected to be followed by evidence of an additive action if the alkylated compounds are active only after dealkylation. Evidence of lasting competition between the alkylated and the norcompounds was expected if both the alkylated and the norcompounds are active analgesic agents. It was a necessary precaution in these experiments to avoid maximum drug effects. Therefore weights of drug were used throughout which produced changes in the pain thresholds less than 50 per cent of those readily induced by these same drugs in mice still showing brisk righting reflexes.

METHODS

Male white mice of weight range not exceeding 3 g. in any experiment were divided at random into groups of eight or more. Each mouse was

marked with a dye and the initial pain threshold measured. The intracisternal injection appropriate to the animal's group was made at the individual zero time, and pain thresholds were redetermined 5, 10 and 20 minutes later. The effect of an injection was measured for each mouse as the percentage change in the individual pain threshold. Mean percentage changes were compared by *t* tests; no correction was made for coarse grouping.

Intracisternal injections were made as described by Lockett and Davis[2] except that exposure to ether was reduced to 30 seconds and that neck flexion was minimised. *Pain thresholds* were measured by a modification of the previous method. The earlier tin electrodes were replaced by

TABLE I

ANTAGONISM BETWEEN THE ANALGESIC ACTIONS OF ALKYLATED AND NOR-COMPOUNDS INJECTED INTRACISTERNALLY INTO MICE

Compounds	μg./kg.	No. mice	Percentage increase in pain threshold min. after intracisternal injection		
			5	10	20
Nor−M	15	30	23·7 ± 1·86**	25·2 ± 3·36*	19·6 ± 4·51
M	5	28	15·4 ± 2·49**	26·2 ± 1·96*	22·8 ± 2·03
Nor−M+M	—	31	9·3 ± 2·60	17·1 ± 3·33	19·5 ± 4·69
Nor−M	15	11	14·9 ± 3·58*	15·3 ± 4·40	12·8 ± 5·09
M	2·5	13	6·5 ± 1·86	10·7 ± 2·92*	7·2 ± 2·56**
Nor−M+M	—	12	8·7 ± 1·65	17·3 ± 1·29	17·5 ± 2·51
Nor−M	15	11	15·7 ± 3·19*	17·4 ± 4·25	15·4 ± 5·57
M	1	10	2·1 ± 1·89	1·9 ± 3·07**	0·9 ± 3·03**
Nor−M+M	—	12	6·8 ± 2·06	11·2 ± 1·94	17·0 ± 3·49
Nor−M	15	39	18·4 ± 1·75**	22·2 ± 2·20*	22·7 ± 2·34
P	50	22	4·2 ± 1·74	5·2 ± 2·58*	5·3 ± 3·00**
Nor−M+P	—	39	6·0 ± 1·62	14·4 ± 2·57	22·4 ± 3·31
Nor−P	300	23	12·0 ± 2·33	7·6 ± 1·87	5·2 ± 2·54
P	100	24	14·5 ± 2·13*	14·6 ± 2·01*	11·7 ± 2·45**
Nor−P+P	—	23	7·8 ± 2·07	6·3 ± 2·17	2·8 ± 1·54
Nor−P	300	39	12·1 ± 1·75**	10·5 ± 1·67*	8·7 ± 1·80*
P	50	32	4·7 ± 1·93*	4·8 ± 1·99	4·4 ± 2·39
Nor−P+P	—	38	0·6 ± 0·86	1·5 ± 1·45	2·3 ± 1·73

Mean values are followed by the standard error of the mean. M, P, and Nor- represent morphine, pethidine, and the nor- compounds respectively. When two drugs were given together, the doses used were those immediately above. Significances of differences between the effects of two drugs and single drugs were determined by *t* test and are indicated by asterisks, one, $P = < 0{\cdot}05$, two, $P = < 0{\cdot}001$.

nickel-silver plates and the original very high resistance microammeter was exchanged for one of lower resistance. The modified apparatus had the greater durability and was used as previously described.[2] The values given for the pain thresholds by the modified apparatus were higher than the true values previously recorded because the resistance of the new milliammeter was no longer sufficient to prevent current from passing through it when the key was depressed. Mice with initial 'pain thresholds' outside the range 8–12 micro-amps were rejected. Since background noises could not be entirely eradicated and were found to raise the pain thresholds of mice, control groups of animals were injected with 0·9 per cent NaCl and were examined in parallel with those injected with drug. Corrections were made for changes in the mean pain threshold of control groups when these exceeded 5 per cent; experiments in which this change exceeded 10 per cent were discarded.

RESULTS

Antagonism of the Analgesic Action of Normorphine by Morphine and by Pethidine

In the first experiments, groups of mice received intracisternal injections either of 15 μg. /kg. of normorphine, or of 5 μg./kg. of morphine, or of the two together. The combined results of the three experiments made are shown at the top of Table I. The morphine and the normorphine raised the pain threshold similarly 10 minutes after injection, but the

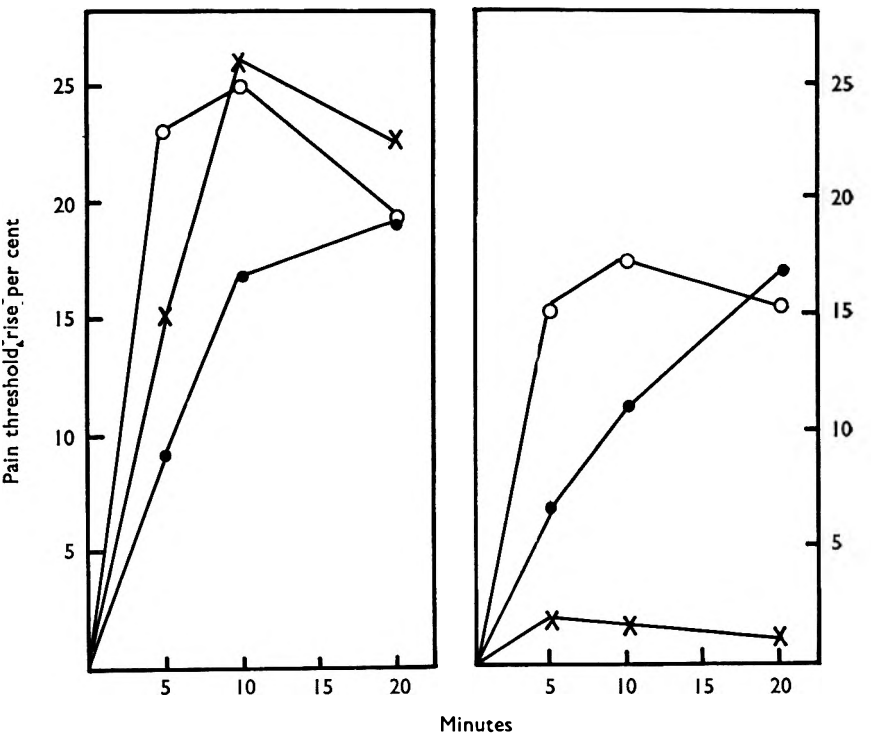

FIG. 1. Antagonism between morphine and normorphine on intracisternal injection into mice. Mean percentage increases in pain threshold are plotted as ordinates against time in minutes as abscissae. See Table I.

O——O 15 μg. kg. of normorphine.
×——× 5 mg./kg. of morphine (L.H. curve).
1 μg./kg. of morphine (R.H. curve).
●——● Combined effect.

effect of the normorphine developed the more rapidly, and waned the sooner. There was highly significant delay in the onset of analgesia when the previous amounts of morphine and normorphine were injected together, for the mean pain thresholds had increased by 23·7, 15·4 and 9·3 per cent respectively, 5 minutes after intracisternal injections of normorphine, morphine and the mixture had been made. The analgesia caused by the two drugs together was still less than that due to either alone

10 minutes after injection, but did not differ from that of either drug in 20 minutes (Fig. 1).

Variation was made in the amount of morphine used in the next experiments. When either 2·5 μg./kg. or 1·0 μg./kg. of morphine was employed and the weight of normorphine remained unchanged, there was again delay in the onset of the analgesic action of the mixture, but then followed a time-effect curve for the mixture not significantly different from that of the normorphine in the absence of morphine. (Table I and Fig. 1.)

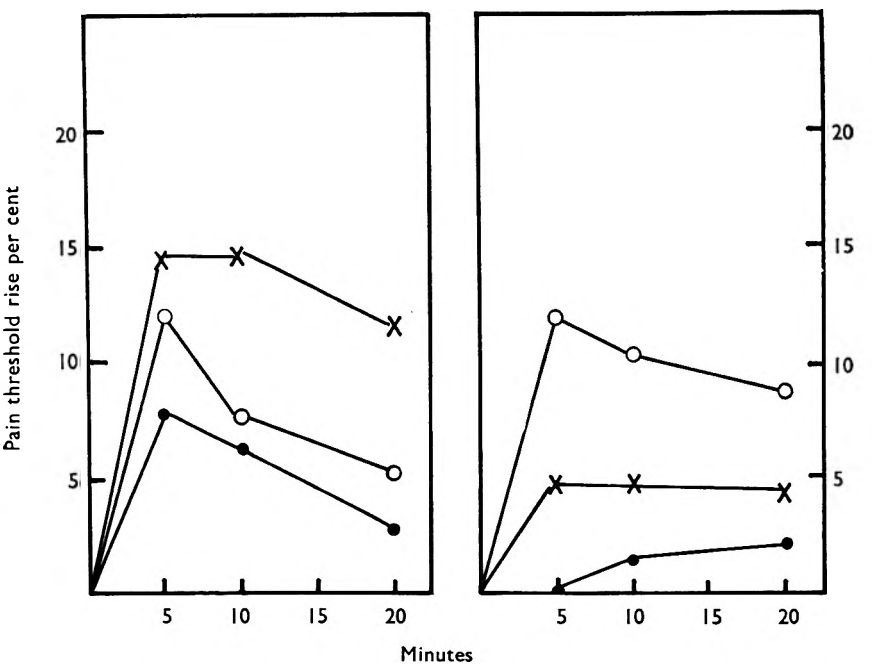

FIG. 2. Antagonism between pethidine and norpethidine on intra-cisternal injection into mice. Mean percentage increases in pain threshold are plotted as ordinates against time in minutes as abscissae. See Table I.

O——O 300 μg./kg. of norpethidine.
×——× 100 μg./kg. of pethidine (L.H. curve).
50 μg./kg. of pethidine (R.H. curve).
●——● Combined effect.

Pethidine 50 μg./kg. also antagonised the onset of analgesia caused by normorphine 15 μg./kg. This effect was evident at 5 minutes, less so at 10 minutes, and had disappeared 20 minutes after injection (Table I).

Antagonism between Pethidine and Norpethidine

Comparable increase in the mean pain threshold was caused by 300 μg./kg. of norpethidine and 100 μg./mg. of pethidine 5 minutes after intracisternal injection into mice (Table I) but the action of the nor-compound disappeared the more rapidly. When these doses of pethidine and norpethidine were given together the analgesia which developed was

delayed in onset, of less intensity than that given by either drug alone, and more like that of norpethidine than pethidine in duration (Table I, Fig. 2).

Pethidine, 50 μg./kg. produced a very small but significant increase in the pain threshold when injected intracisternally into mice. This dose of pethidine completely antagonised the analgesic action of 300 μg./kg. of norpethidine when the two drugs were injected together intracisternally (Table I, Fig. 2).

Discussion

The analgesic actions of intracisternal mixtures of morphine and normorphine developed only slowly to a maximum intensity which equalled that of whichever drug was, by reason of the doses used, the more active component of the mixture. Additive effects from the two components of the mixtures were never seen despite the fact that the changes induced in pain thresholds in the course of these experiments were submaximal and observations were continued for 40 minutes after intracisternal injection. Pethidine and morphine antagonised a more effective dose of normorphine similarly. There was, by contrast no delay in the onset of analgesia when a mixture of pethidine and norpethidine was used and the analgesic action of the mixtures were less than that of either component alone throughout the 40 minutes of observation. There appears therefore to be a difference between the actions of normorphine and norpethidine when these are injected intracisternally. This hypothesis is supported by the fact that the maximum change in pain threshold that can be induced by the intracisternal injection of these two drugs in mice is markedly the greater for normorphine. To postulate that simple competition is occurring between alkylated and nor compounds at common uniform receptor sites in the central nervous system is inadequate to explain these observations. An alternative hypothesis, based on the assumption that the alkylated compounds must be dealkylated before they develop analgesic action is unattractive in the absence of any demonstration of such a dealkylating system in the nervous tissue. The hypothesis could explain the delay in the onset of analgesia after the intracisternal injection of a mixture of morphine and normorphine. There was however no similar delay when a mixture of pethidine and norpethidine was injected. The failure of morphine to antagonise or to sum with normorphine in analgesic effect from the twentieth to the fortieth minute of observation is also in conflict with this hypothesis. It is however conceivable that the concentration of normorphine injected was sufficient to inhibit the proposed dealkylating system If this explanation be accepted, then the rate of dealkylation in the central nervous system must be governed, in large part by the concentration of the dealkylated compound; there may also exist another pathway for the removal of morphine from the sites of analgesic action. This last postulate is made necessary to account for the disappearance of the antagonism between morphine and normorphine by the twentieth minute after intracisternal injection. The prolonged antagonism of norpethidine by pethidine could be explained

similarly if there were no equally effective second pathway for the removal of pethidine from the sites of analgesic action.

It is however evident that neither hypothesis is an adequate explanation of the results of these experiments.

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THE INTERACTION OF PHENOLIC COMPOUNDS WITH BACTERIA

PART III. EVALUATION OF THE ANTIBACTERIAL ACTIVITY OF HEXYLRESORCINOL AGAINST *ESCHERICHIA COLI*

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Evaluation of the antibacterial activity of solutions containing hexylresorcinol in the presence and absence of cetomacrogol and sodium chloride is described. The relation between the extent of drug binding, light-scattering changes and the release of cell exudate in *E. coli* suspensions on addition of hexylresorcinol with the bactericidal activity is examined. Potentiation of the bactericidal activity of hexylresorcinol by sodium chloride and the inactivation by cetomacrogol is discussed with respect to drug binding, light-scattering changes in the bacterial suspensions and the release of bacterial cell exudate in the presence of these agents.

THE work described in previous papers in this series[1,2] has now been extended to include the antibacterial evaluation of solutions of hexylresorcinol against suspensions of *Escherichia coli*. The choice of method was influenced by a desire to determine whether any relation exists between antibacterial activity and a change in some physical property occurring during the interaction of the phenol and bacteria. The correlation of drug binding, light-scattering changes and cell exudate release in suspensions of *E. coli* observed on addition of hexylresorcinol with the antibacterial activity of the phenol under similar conditions is discussed in the present paper.

EXPERIMENTAL

The experimental techniques described previously[1,2] were used with slight modification; aseptic precautions were observed throughout to prevent chance contamination. Suspensions of *E. coli* were prepared in sterile distilled water instead of phosphate buffer. Solutions of hexylresorcinol were filtered through sintered glass (5/3) and the concentration in the filtrate was checked spectrophotometrically before use. All other solutions were sterilised by autoclaving.

The culture medium employed contained 1·0 per cent Oxoid peptone and 0·5 per cent sodium chloride at pH 7·3.

Bacteriostatic Evaluation

5 ml. volumes of filtered hexylresorcinol solutions (120–500 μg./ml.) were added to 5·0 ml. portions of sterile broth. Each solution was inoculated with one drop of a 24 hour broth culture of *E. coli* delivered from a standard dropping pipette. Ten tubes were set up at each concentration of the drug. The tubes were incubated at 37° for 48 hours and examined for the presence or absence of visible growth. The

minimum inhibitory concentration was that concentration of hexylresorcinol which just inhibited visible growth.

Solutions of hexylresorcinol (50, 125 and 250 μg./ml.) containing cetomacrogol (at molar ratios of 0·5, 1·0 and 2·0 of cetomacrogol to hexylresorcinol) were tested similarly.

Bactericidal Evaluation

The mean single survivor times of suspensions of *E. coli* in solutions of hexylresorcinol were determined under the same conditions as those used for the uptake measurements[1].

General method. Calibrated standard dropping pipettes[3] were used to to transfer 5 drops of a suspension of *E. coli* in water, containing 55×10^9 organisms/ml., to 10 ml. portions of hexylresorcinol solutions; the final bacterial concentration was 5×10^8 organisms/ml. The temperature of the contact suspension was maintained at 25 ± 1° until the addition of 5 ml. of sterile broth after suitable time intervals. The tubes were immediately transferred to an incubator at 37° for 24 hours. The concentration of hexylresorcinol present after dilution was less than the bacteriostatic concentration.

Hexylresorcinol solutions. The death time of *E. coli* in solutions of hexylresorcinol was determined using solutions containing 100–400 μg./ml. of the phenol and 5 tubes at each concentration level. One aliquot at each concentration was quenched at 10 minute intervals from 20–80 minutes after inoculation.

The mean single survivor time of *E. coli* in solutions containing 250 μg./ml. of hexylresorcinol was determined using 20 contact suspensions. One tube of each series was quenched at 7 minute intervals from 29–70 minutes after inoculation. The results were analysed by the method given by Cook and Wills[4].

Hexylresorcinol solutions containing cetomacrogol. Determination of the death time of *E. coli* in a solution containing 500 μg./ml. of hexylresorcinol and 1660 μg./ml. of cetomacrogol (molar ratio of cetomacrogol to hexylresorcinol of 0·5 : 1·0) was as described above for solutions containing hexylresorcinol.

Hexylresorcinol solutions and sodium chloride. 10 ml. portions of the bacterial suspensions, containing 2×10^8 organisms/ml. in distilled water, were added to 10 ml. of 0·4 M sodium chloride solutions and allowed to stand 10 minutes at 25°; 5 ml. of this suspension was then added to 5 ml. of hexylresorcinol solution and the test completed as in the general method. This furnished a test suspension containing 0·1 M sodium chloride and 5×10^8 organisms/ml. together with hexylresorcinol at any pre-determined concentration.

The death time of sodium chloride-treated suspensions of *E. coli* in solutions of hexylresorcinol (100 and 250 μg./ml.) were determined as above for hexylresorcinol alone.

The mean single survivor time of *E. coli*, pre-treated with sodium chloride, in solutions of hexylresorcinol containing 250 μg./ml. of the latter was determined using 20 contact suspensions. One aliquot of

each mixture was quenched at 7 minute intervals from 14 to 56 minutes after inoculation.

Results

Bacteriostatic Evaluation

The minimum inhibitory concentration of hexylresorcinol against *E. coli* in nutrient broth was found to be 120 μg./ml. after 48 hours at 37°. These results established that the quantity of hexylresorcinol carried over into the growth medium in the bactericidal tests was insufficient to inhibit the growth of intact bacteria. The inhibition of the bacteriostatic activity of hexylresorcinol against *E. coli* by cetomacrogol was also demonstrated even for solutions containing the minimum proportion of the nonionic substance and the highest practicable concentration of the phenol (250 μg./ml.).

Bactericidal Evaluation

All results quoted are for reactions at 25°.

Hexylresorcinol solutions. At 100 μg./ml. of hexylresorcinol, surviving organisms were still present after 80 minutes contact time. The approximate death time of *E. coli* in a solution containing 250 μg./ml. hexylresorcinol was 50 minutes. No survivors were apparent after 20 minutes in solutions containing 300 and 400 μg./ml. of the phenol.

The results for the determination of the mean single survivor time of *E. coli* in a hexylresorcinol solution containing 250 μg./ml. are represented by curve 1 of Figure 1. The mean single survivor time was 47 minutes.

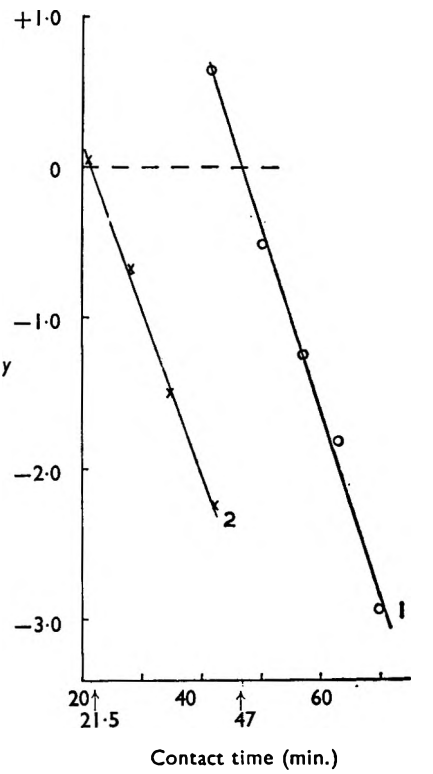

FIG. 1. The relation between y (log [$-$ log p], where p = the proportion of sterile tubes after incubation) and contact times for the exposure of *E. coli* to a solution of hexylresorcinol containing 250 μg./ml. (curve 1) and for the exposure of sodium chloride-treated *E. coli* to a solution containing the same concentration of hexylresorcinol (curve 2). The reaction temperature was 25° and the initial bacterial concentration was 5×10^8 organisms/ml.

Hexylresorcinol solutions containing cetomacrogol. Viable organisms were still present after 80 minutes contact time.

Hexylresorcinol solutions and sodium chloride. The approximate death times of sodium chloride-treated *E. coli* in solutions containing 100 and 250 μg./ml. of hexylresorcinol were 75 and 35 minutes respectively. The

results for the determination of the mean single survivor time in the latter solution are represented by curve 2 of Figure 1. The mean single survivor time was 21·5 minutes.

Discussion

The potentiating effect of sodium chloride and the inhibitory effect of cetomacrogol on the bactericidal activity of solutions of hexylresorcinol against *E. coli* will now be considered in conjunction with the information presented in previous papers[1,2].

Bactericidal Activity of Solutions of Hexylresorcinol

The amount of hexylresorcinol bound by *E. coli* suspensions from solutions initially containing 250 μg./ml. of hexylresorcinol of mean single survivor time of 47 minutes was 20 μg./5 $\times$ 10^8 organisms/ml., in the presence and absence of phosphate buffer. In Figure 2, this

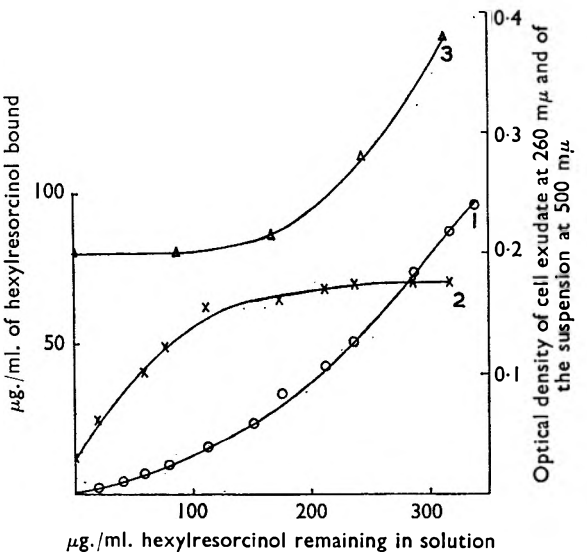

FIG. 2. Uptake of hexylresorcinol (1), release of cell exudate (2) and change of turbidity (3) of suspensions of *E. coli* (10^9 organisms/ml.) on treatment with hexylresorcinol.

hexylresorcinol concentration corresponds to approximately 210 μg./ml. on the abscissa, since the results are presented for a suspension containing 10^9 organisms/ml. At this level, the limiting value of cell exudate released (curve 2) has been achieved and there is a marked increase in the turbidity of the bacterial suspensions (curve 3). Possibly the bactericidal effects become apparent when the drug concentration is sufficient to initiate turbidity changes and, at the same time, effect maximum release of cell exudate. Thus, it is postulated that the bactericidal effects are linked with the interaction and consequent disorganisation of the cytoplasm by the phenol rather than reactions involving the cytoplasmic membrane and its associated osmotic barrier.

Since no turbidity changes could be detected until the hexylresorcinol concentration was sufficient to release the maximum amount, for hexylresorcinol, of cell exudate, no major physical change in the cytoplasmic contents is envisaged during this reaction. But, on addition of more hexylresorcinol to the system, the phenolic molecules probably penetrate into the cytoplasm with consequent effect on turbidity. This is supported by the observation that the addition of a solution of hexylresorcinol to a preparation containing the cytoplasmic constituents of *E. coli* (a disrupted suspension from which the whole cells and the cell walls had been removed) caused turbidity to develop in that preparation. Changes in the refractive index of the cytoplasm are indicated which could be associated with interference with the hydrogen bonding characteristics of nucleic acids, and particularly of deoxyribonucleic acid.

Approximately 10^8 molecules of hexylresorcinol are bound per bacterium under the bactericidal conditions defined above. Assuming that each organism is a smooth cylinder 3 μ in length and 0·6 μ in diameter, its apparent surface area, if equivalent to its geometrical surface area, is $5{\cdot}6 \times 10^8$ Å^2. The minimum surface area occupied by 10^8 molecules of hexylresorcinol, not allowing for intermolecular packing space, would be 26×10^8 Å^2 if the molecules were packed with the phenyl ring perpendicular to the surface, 38×10^8 Å^2 if the molecules were packed with the phenyl ring parallel to the surface and the *n*-hexyl chain protruding from it or 66×10^8 Å^2 if the phenyl ring and the alkyl chain were in the plane of the surface. Thus, accommodation of all the bound molecules as a uni- or bi-molecular layer is obviously impossible and partial penetration is again indicated.

Inactivation of Hexylresorcinol by Cetomacrogol

The simplest explanation of this effect is that the amount of hexylresorcinol bound by the organism is insufficient to cause the biological effect. However, by increasing the concentration of hexylresorcinol present and keeping the cetomacrogol concentration to the lowest practicable level, the amount of hexylresorcinol bound by the organisms (24 μg./5×10^8 organisms/ml. which is equivalent to $1{\cdot}5 \times 10^8$ molecules of hexylresorcinol/organism) may exceed that required to produce the bactericidal effect and light-scattering changes in suspensions of *E. coli* in the absence of the nonionic substance (20 μg./5×10^8 organisms/ml. which is equivalent to $1{\cdot}25 \times 10^8$ molecules of hexylresorcinol/organism, cf. Part II[2]). At this level, the entire amount of the phenol bound obviously cannot be accommodated on the surface of the bacterial cell especially since some cetomacrogol molecules must also be associated with it. Possibly, hexylresorcinol may become bound to the bacteria in the presence of excess cetomacrogol as a monomolecular layer of the phenol-nonionic complex with the nonionic associated with the other surface of the film, thus blocking the building of multilayers of the phenol and also preventing the phenolic molecules from penetrating the bacteria and causing the turbidity changes. If, however, the hexylresorcinol molecules, penetrate the bacteria in association with cetomacrogol molecules

the tendency to hydrogen bond to the oxygen atoms of the ether chains of the nonionic may be greater than the tendency to disrupt and replace the natural hydrogen bonding structure of the nucleic acids.

Potentiation of Hexylresorcinol Activity by Sodium Chloride

It was noted previously[2] that sodium chloride enhanced the effect of hexylresorcinol in producing turbidity changes in bacterial suspensions and the amount of cell exudate released whilst having a negligible effect on the extent of drug binding, but had no effect on these properties in the absence of this phenol. The potentiating effect of sodium chloride on the bactericidal action of hexylresorcinol against *E. coli* is attributed, therefore, to facilitated disorganisation of the cytoplasm and leakage of vital cellular constituents from the organisms.

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STUDIES ON *LEONTICE LEONTOPETALUM* LINN

PART III. THE MICROSCOPY OF THE ROOT TUBER OF *L. leontopetalum*

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Samples of the root tuber of *Leontice leontopetalum*, Linn. (Berberidaceae) have been examined histologically. An illustrated description of the distribution of tissues, the cells and cell contents is given.

IN a previous communication[1] the macroscopical characters of the root tuber of *L. leontopetalum*, in the fresh and dried condition, have been described. In this paper the detailed histological structure of the dried tuber is described and illustrated, reference being made to preparations of the fresh tuber in order to elucidate the formation of the lignified parenchyma which forms protuberances on the surface.

EXPERIMENTAL

Materials. The materials used consisted of samples sent by Dr. W. M. Ford-Robertson of the Lebanon Hospital for Mental and Nervous Disorders, Asfuriyeh, Beirut, and of specimens collected by one of us (P.F.N.) in Lebanon.

Sections of dormant tubers were examined and a line drawing made to show the distribution of tissues as seen in cross section (Fig. 1). Detailed drawings to show the microscopical characters of tissues seen in the whole and powdered drug were made (Figs. 2, 4–7). To determine the structure of the numerous protuberances, each of which develops at the base of a fine rootlet in the growing tuber, serial sections from a fresh tuber were cut through a protuberance and the base of its attached rootlet. The structure at the central part of the protuberance is illustrated (Fig. 3).

Histology of the root tuber of L. leontopetalum

EXTERNAL LAYERS (Figs. 2, 3). The thick outer layer consists of 1 to 6 bands of lignified, suberised cork cells alternating with bands of phellogen. Each layer of cork consists of 2 to 8 rows of regular tangentially elongated cells which are polygonal in surface view with straight anticlinal walls. The outermost layer is often crushed and distorted appearing in section as a granular, brown mass with the lumens of some of the cork cells visible as irregular cavities. In surface view the cork cells are 38–**130**–230 μ long and 24–**70**–136 μ wide; in section, 12–**33**–62 μ deep.* The layers of phellogen consist of 2 to 6 rows of cells similar

* Because of the irregular shape of the tuber and the ramification of the vascular tissue in every direction, the abbreviations for cell dimensions suggested by Moll and Janssonius[2] could not properly be used.

in shape and size to those of the cork but it is most frequently crushed, making it difficult to distinguish individual cells.

There are frequent protuberances on the surface and it appears that in these regions the innermost layer of phellogen produces parenchyma to the outside instead of cork. Some vascular strands from the inner part

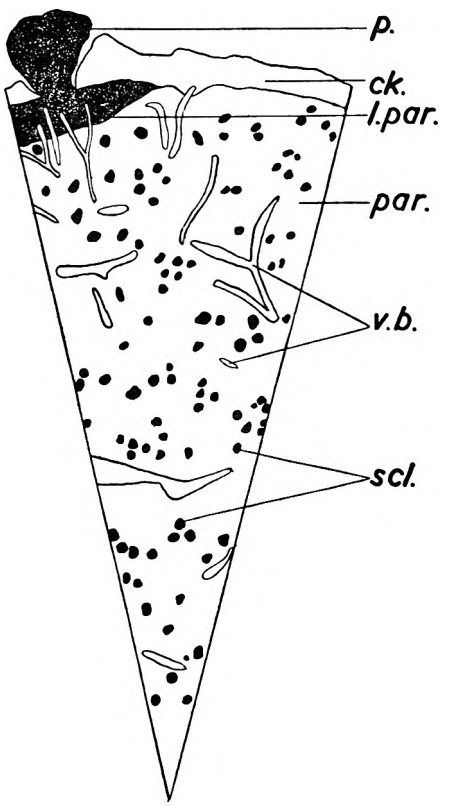

FIG. 1. *Leontice leontopetalum* tuber in radial section: distribution of tissues × 2½; *ck*, cork; *l. par*, lignified parenchyma; *p*, protuberance: *par*, parenchymatous ground tissue; *scl.* groups of sclereids; *v.b*, vascular bundles.

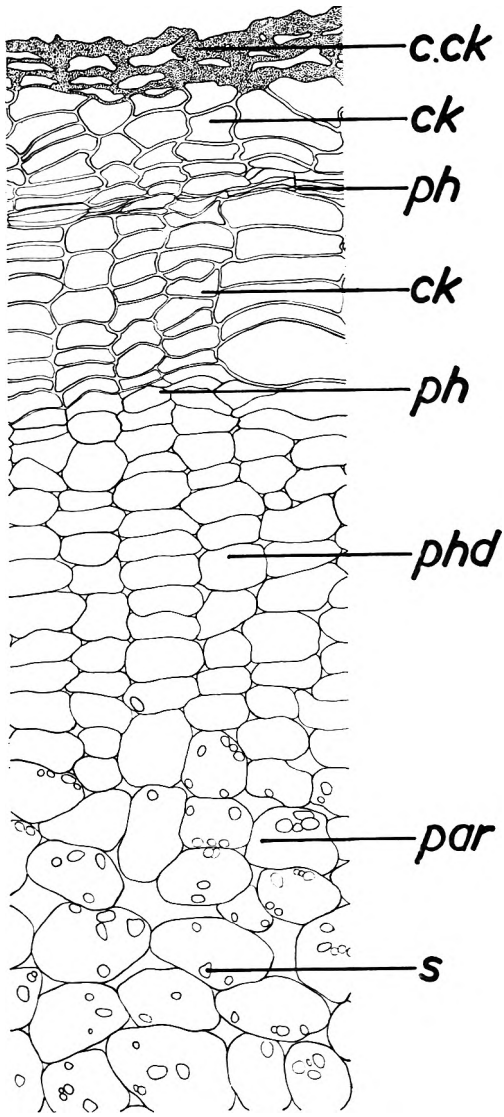

FIG. 2 (Right). *Leontice leontopetalum* tuber in section: outer layers × 125; *c.ck*, crushed cork; *ck*, cork; *par*, parenchyma of ground tissue; *ph*, phellogen; *phd*, phelloderm; *s*, starch granules.

of the tuber join below the phellogen and pass through both it and the externally developing parenchyma into the rootlet which is surrounded at the proximal end by an irregular ring of cork through which the external parenchyma grows to produce the protuberance. The slightly

lacunose parenchyma in the protuberances, which persist when the rootlets die off, becomes lignified and consists of light brown, fairly thin walled cells measuring 34–**80**–144 μ long and 18–**51**–88 μ broad. Groups of

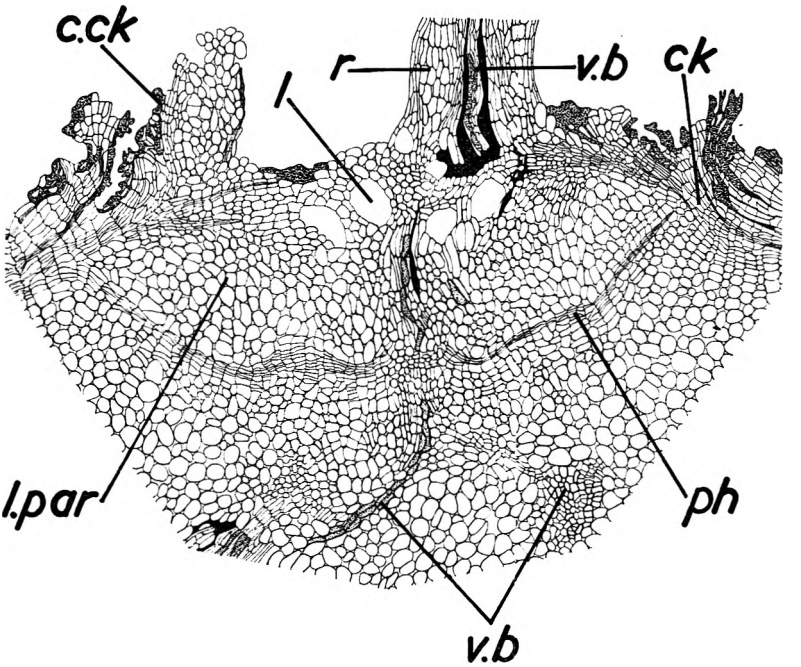

FIG. 3. *Leontice leontopetalum* tuber: radial section through central portion of protuberance and rootlet base × 30; *c.ck*, crushed cork; *ck*, cork; *l*, lacunae; *l.par*, lignified parenchyma; *ph*, phellogen; *r*, rootlet base; *v.b*, vascular bundles.

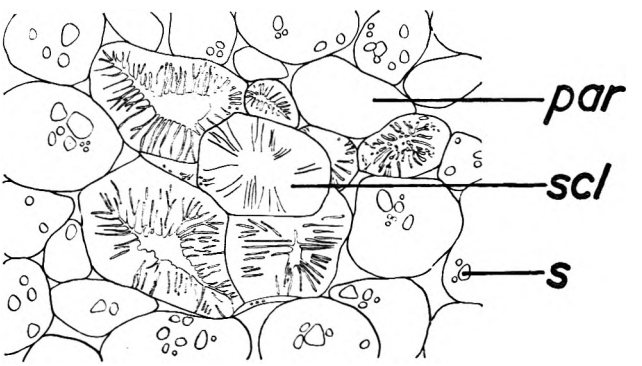

FIG. 4. *Leontice leontopetalum* tuber in section: sclereid group in parenchymatous ground tissue × 125; *par*, parenchyma of ground tissue; *s*, starch granules; *scl*, sclereids.

similar lignified cells are occasionally found internal to the innermost layer of cork.

CORTEX AND STELE. Next to the innermost layer of phellogen is a phelloderm (Fig. 2) which consists of up to 10 rows of cellulosic parenchyma, the cells of which are devoid of starch and are 32–**86**–158 μ

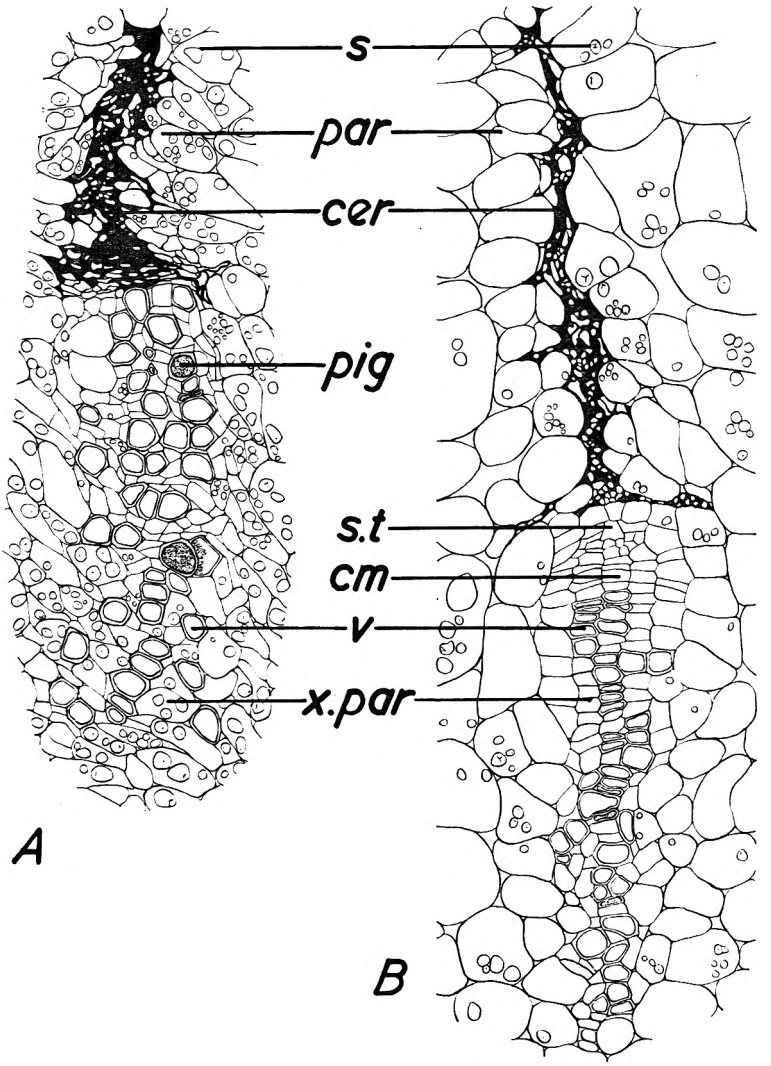

FIG. 5. *Leontice leontopetalum* tuber in section: vascular bundles cut transversely × 125: *A*, with xylem parenchyma and vessels irregularly disposed; *B*, with xylem parenchyma and vessels in regular rows; *cer*, ceratenchyma; *cm*, cambium; *par*, parenchyma of ground tissue; *pig*, dark brown pigment; *s*, starch granules; *s.t.* sieve tissue; *v*, vessels; *x.par*, xylem parenchyma.

long and 12–**31**–62 μ deep. The remainder of the tuber consists of ground tissue in which there are groups of sclereids and numerous vascular bundles. The ground tissue consists of rounded to slightly elongated

parenchymatous cells containing abundant starch. The cells are 32–**102**–184 μ long and 30–**70**–120 μ broad. The starch consists mainly of simple granules with very occasional compound granules of 2 to 3 components. The individual granules are rounded to irregularly ovoid in shape, 6–**24**–63 μ long and 6–**20**–49 μ broad, some having small tuberosities and some being truncate on one side. Striations are absent and the hilum, which is not always visible, consists of a point, split, or occasionally

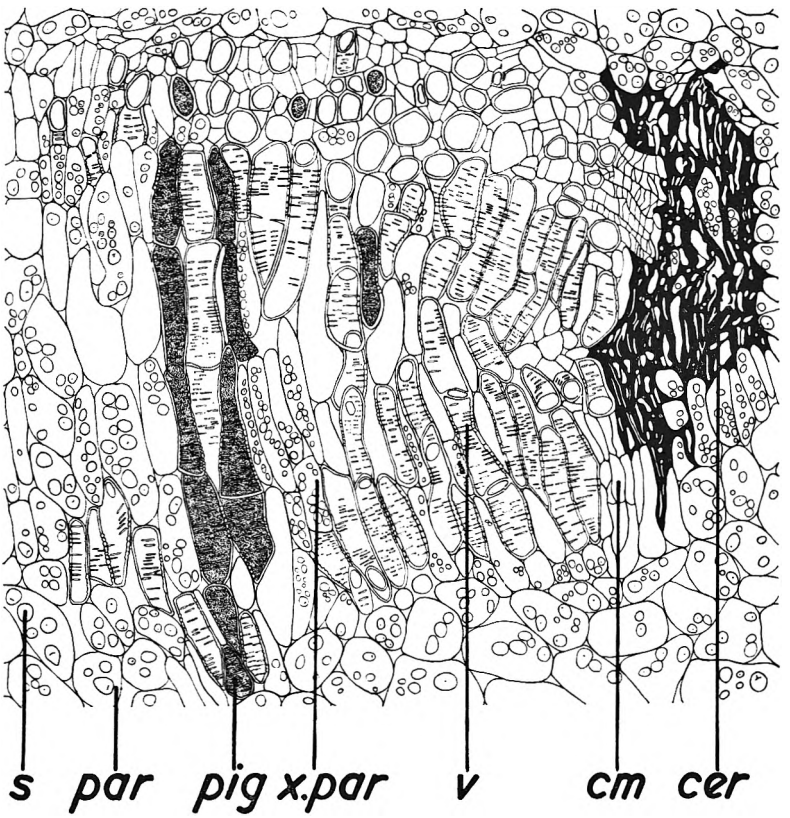

FIG. 6. *Leontice leontopetalum* tuber in section: vascular bundle cut longitudinally × 125; *cer*, ceratenchyma; *cm*, cambium; *par*, parenchyma of ground tissue; *pig*, dark brown pigment; *s*, starch granules; *v*, vessel; *x. par*, xylem parenchyma.

a 2–5 rayed cleft, sometimes central but more frequently somewhat eccentric. Scattered throughout the ground tissue are irregular groups of large, yellow sclereids, the groups varying considerably in size, being up to 9 cells in diameter (Fig. 4). The individual sclereids may be isodiametric or elongated with a length 92–**168**–383 μ, breadth 46–**106**–183 μ, and wall thickness 21–**41**–67 μ. The walls are heavily lignified and deeply pitted, the pits being simple or branched, appearing as circular pores in surface view; the walls shows striations only after

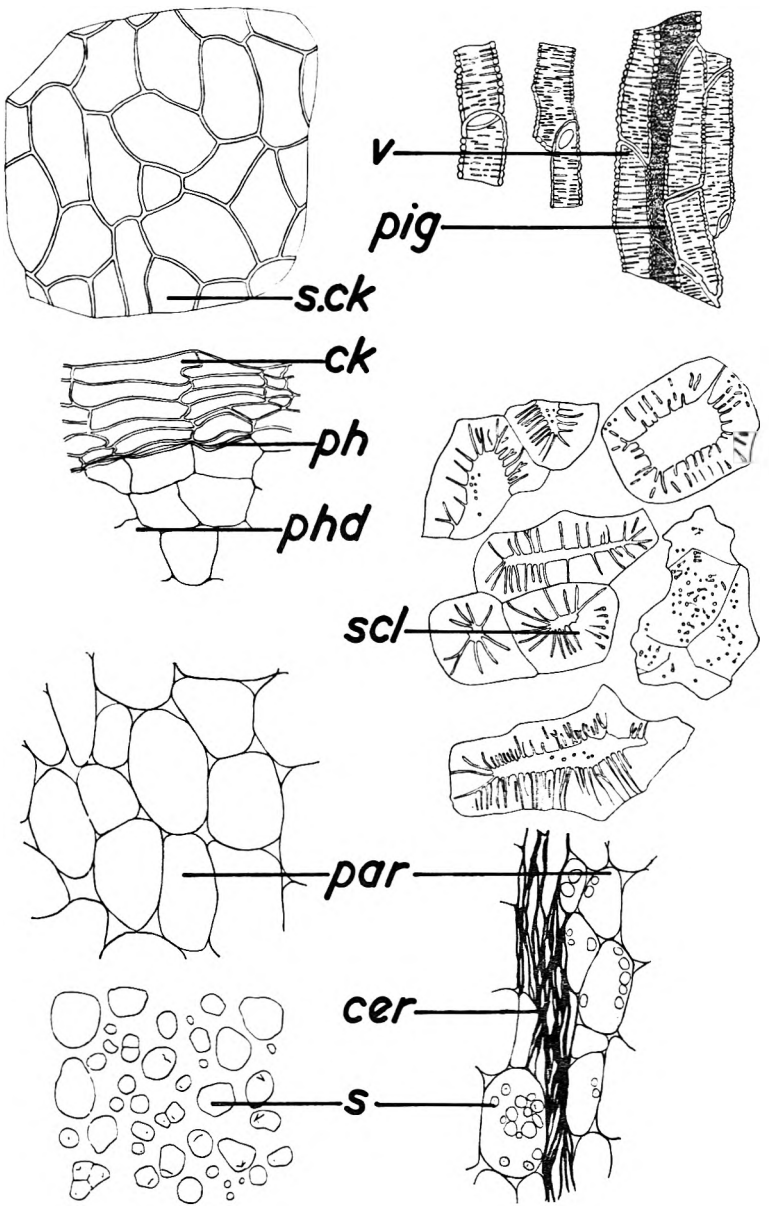

FIG. 7. *Leontice leontopetalum* tuber in No. 60 powder: all × 125; *cer*, ceratenchyma; *ck*, cork; *par*, parenchyma of ground tissue; *ph*, phellogen; *phd.* phelloderm; *pig*, dark brown pigment; *s*, starch granules; *s.ck*, cork in surface view; *scl*, sclereids; *v*, vessels.

prolonged heating with potassium hydroxide solution. The lumen is usually large but occasionally appears as a mere line.

The collateral vascular bundles (Figs. 5, 6) ramify throughout the tuber and in any section it is possible to find bundles cut transversely, longitudinally or obliquely. The phloem consists of a mass of ceratenchyma in which it is seldom possible to differentiate individual cells. The cambium in the bundles is usually not discernible but sometimes 2 to 9 rows of flattened tabular parenchymatous cells, 12–**29**–98 μ by 2–**13**–68 μ, are seen; in longitudinal view the cambial cells measure 30–**91**–190 μ. The xylem consists of parenchyma and vessels which in transverse section are sometimes irregularly disposed (Fig. 5A) but are usually arranged in regular rows (Fig. 5B). The cells of the parenchyma, which often contain starch, are thin walled and elongated along the axis of the bundle; in transverse view the cells are 16–**35**–74 μ long by 4–**19**–58 μ broad and in longitudinal view the cells measure 50–**129**–268 μ. The vessels consist of short tracheid-like segments 60–**141**–262 μ long by 10–**27**–64 μ wide and each segment has a circular to oval pore at each end. The walls are slightly lignified and irregularly pitted giving a reticulate appearance. A few of the vessels are filled with dark brown material which appears as branching strands running from one vessel to another.

POWDER (Fig. 7). The yellowish-brown powder has a pleasant odour but has a sternutatory effect and a bitter taste.

A number 60 powder was found to have the following microscopical characters, the details of which have been described above:

1. Brown cork in surface view and in section. In section the lignified cork is sometimes seen alternating with bands of phellogen; cells of the phelloderm may also be attached.

2. Small groups of light brown lignified parenchyma.

3. Small groups of large cellulosic parenchymatous cells, many of which contain starch granules, some of which also occur loose.

4. Large, yellow sclereids singly or in small groups.

5. Slightly lignified vessel-segments occurring singly or in groups. The majority are yellowish but some contain a dark brown material.

6. Small, flat area of colourless ceratenchyma.

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THE STABILITY OF THIOGLYCOLLATE SOLUTIONS

PART II. MISCELLANEOUS FACTORS ASSOCIATED WITH THE OXIDATION AND STABILITY

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The rate of oxidation of thioglycollate in aqueous solution increases with increasing pH, but when dextrose is present, neutral solutions are oxidised more rapidly than alkaline solutions. The effect of sodium metabisulphite on the oxidation was indecisive. Boiling thioglycollate solutions evolved hydrogen sulphide. Heating old solutions of thioglycollate caused regeneration of the oxidised material but the extent of this regeneration was less than might have been expected. A yellow colour in some thioglycollate solutions is believed to be due to the action of alkali on the thioglycollic acid. The tetracarboxy acid occurring in samples of the acid was isolated. It is recommended that thioglycollate media be stored in air-tight containers at as low a temperature as possible.

IN Part I of this paper[1] the oxidation of thioglycollate in aqueous solution was shown to be dependent upon whether heat was employed or not in preparation, the pH of the solution and the temperature at which the solution was stored. In this second part, the effects of added dextrose and of sodium metabisulphite are reported. The effect of heat on thioglycollate solutions has been studied together with other miscellaneous factors associated with thioglycollic acid and its solutions. An attempt has been made to correlate the results in parts I and II with their application to thioglycollate media for bacteriological use.

EXPERIMENTAL AND RESULTS

Effect of Dextrose on the Oxidation of Thioglycollate

Solutions containing 1 per cent of dextrose and 0·1 per cent of thioglycollic acid with 10·7, 10·85 or 11·0 ml. of 0·1N sodium hydroxide per 100 ml. of solution were prepared with the aid of heat. These solutions are referred to as acid, neutral or alkaline, respectively, with pH values of 5·6, 7·2 and 7·8, respectively. Similar solutions without dextrose were prepared at the same time.

Samples of each solution were stored in glass-stoppered bottles in the dark at 4, 20 and 37°. At varying intervals, portions were withdrawn and their thioglycollate content determined by titration with potassium iodate[2]. Dextrose was found not to interfere with the assay.

In all cases, the rate of oxidation of the thioglycollate was reduced when dextrose was present in the solution. Figure 1 shows the oxidation of the acid solutions on storage at the different temperatures; essentially similar graphs are obtained with the neutral and alkaline solutions. Solutions containing dextrose prepared to have an approximately neutral

reaction were oxidised more rapidly than alkaline solutions which, in turn, were oxidised more rapidly than acid solutions. This is shown in Figure 2. The phenomenon was noted at all three storage temperatures.

Effect of Sodium Metabisulphite on the Oxidation of Thioglycollate

Solutions containing 0·1 per cent of thioglycollic acid, 0·05 per cent of sodium metabisulphite and varying quantities of 0·1N sodium hydroxide as before, were prepared and autoclaved. This concentration of sodium metabisulphite was chosen as it had previously been shown to be below the bacteriostatic concentration for all of the nine different bacterial species used by Cook, Steel and Wills[3]. The solutions were stored as before.

As sodium metabisulphite reacts with potassium iodate, it was not possible to estimate the thioglycollate content of the stored solutions in the usual manner. Attempts were made, however, to follow the oxidation qualitatively by the colorimetric reaction with ammoniacal sodium nitroprusside. This reaction was found to have a sensitivity of 20 μg. of thioglycollate and concentration limits of 1 in 5×10^5. Akiba and Ishii[4] reported the sensitivity to be 1 μg. of sodium thioglycollate per ml. Sodium metabisulphite and its decomposition products interfere with this colorimetric reaction, producing an amber to orange colouration with the reactants.

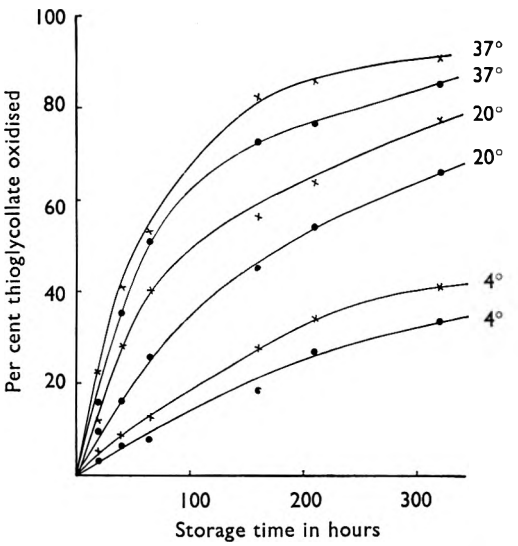


FIG. 1. The oxidation of thioglycollate in a 0·1 per cent. solution, with and without 1 per cent of dextrose, on storage at 4°, 20° and 37°.
×—× Aqueous solution.
●—● Solution with dextrose.

After up to 3 months storage, all the solutions containing the antoxidant still gave a positive reaction for thioglycollate. Only those adjusted to an acid pH and stored at 37° failed to give a strong positive reaction. The reactions obtained were, in all cases, stronger than those produced by simple aqueous thioglycollate solutions with or without dextrose after storage under comparable conditions.

Effect of Heat on Thioglycollate Solutions

Aqueous solutions and peptone water mixtures containing varying concentrations of thioglycollic acid or its sodium salt were boiled and the vapours tested for the presence of hydrogen sulphide by means of

lead acetate paper. The odour of hydrogen sulphide was appreciable from preparations containing 0·5 per cent or more of thioglycollate and the gas was detected, although its odour was not discernible, from solutions containing 0·1 per cent of thioglycollate but not less. There was no difference in the behaviour of the aqueous solutions or the broth mixtures towards boiling.

Solutions containing 1·0 or 0·1 per cent of thioglycollic acid, adjusted to an acid, neutral or alkaline reaction and with or without 1·0 per cent of dextrose were assayed for their thioglycollate content after storage for between 17 and 50 days. Samples of the solutions were then heated at 98–100° for 30 minutes and, after cooling, were re-assayed.

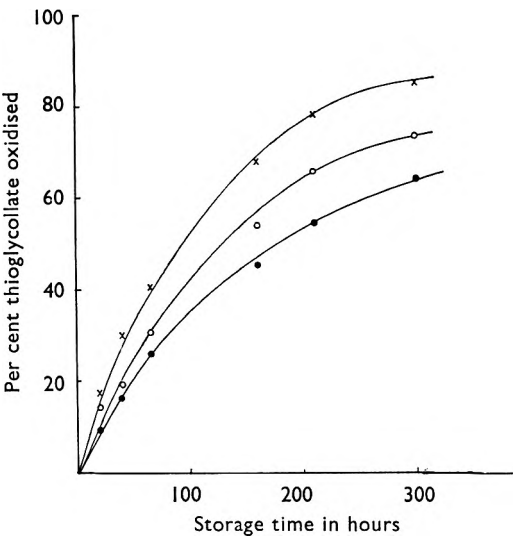


FIG. 2. The oxidation of thioglycollate in a 0·1 per cent solution containing 1 per cent of dextrose, on storage at 20°.

●—● Acid solution.
×—× Neutral solution.
○—○ Alkaline.

A comparison of the thioglycollate contents of the solutions before and after heat treatment by means of a paired *t* test suggested that a hypothesis that there was no difference between the two sets of results was in reasonable accord with the experimental data. Closer inspection of the results showed that the original unheated solutions could be roughly classified into two groups; the first consisting of solutions which still contained about 5–10 per cent or more of their original thioglycollate content, and the second of solutions which had undergone further oxidation so that their thioglycollate content had been reduced to about 5 per cent or less of the original value. Heating solutions of the first group caused no change or further loss in their thioglycollate content, whilst solutions of the second group showed an increase in their thioglycollate content after heating.

The Yellow Colour Appearing in Thioglycollate Solutions

During the work, some 1 per cent thioglycollate solutions were observed to develop a distinctly yellow colour on storage. This colouration was seen in heated solutions having an alkaline reaction; unheated solutions developed the colouration on storage at 4 or 20°, but at 37° only those containing an excess of alkali showed this yellow colour. A freshly

prepared 2 per cent solution of a commercial sample of sodium thioglycollate was also yellowish in colour.

The Storage of Thioglycollic Acid

Two samples of thioglycollic acid (nominally 97–98 per cent w/w $HS{\cdot}CH_2{\cdot}COOH$) were examined. One had been stored at room temperature for 17 months and was hazy in appearance with some sedimentation of white material at the base of the container, whilst the other had been stored at 4° for 29 months and was perfectly clear. Both bottles had been opened and some of their contents used, thus permitting contact with air and possibly water vapour. Assay of the two samples showed thioglycollic acid contents of 83·9 and 86·2 per cent w/w respectively.

To the first sample, containing the sediment, the addition of water or dilute hydrochloric acid produced no change in appearance whereas the addition of N sodium hydroxide solution caused an almost immediate solution of the precipitated material resulting in a clear solution. The mean results for the thioglycollic acid content of aliquots of the first sample assayed by the potassium iodate method immediately or after 10 minutes alkali treatment were 83·87 and 83·85 per cent w/w respectively. Thus alkali does not produce any –SH when added to the precipitate.

The sample containing the white precipitate was centrifuged to collect the sediment which was re-suspended in purified water and washed by re-centrifuging. This washing and centrifugation was repeated until the supernatant was free from sulphydryl. The white material obtained was insoluble in water but readily soluble in sodium hydroxide solution and in sodium bicarbonate solution with effervescence. It was soluble in concentrated sulphuric acid producing an amber coloured solution which on standing became yellowish. When treated with a mixture of concentrated sulphuric acid and perchloric acid (60 per cent $HClO_4$) an orange-red colour was produced which passed to yellow on standing and on the addition of water became colourless.

Discussion

Dextrose in a concentration of 1 per cent is included in Brewer's medium[5] both as a fermentable carbohydrate and as a reducing agent to keep the thioglycollate in the reduced state and to maintain the oxidation-reduction potential of the medium at a low level. Skerman[6], however, reported that dextrose does not aid the establishment of anaerobic conditions. From our results it appears that the presence of dextrose in thioglycollate solutions does reduce the rate of oxidation compared with plain aqueous solutions. The rate of oxidation of thioglycollate in aqueous solution has been shown to increase with increasing pH[1] but when dextrose is present in the solutions, neutral solutions were found to be oxidised more rapidly than alkaline solutions which, in turn, became oxidised more rapidly than acid solutions.

Dilute acids have little or no effect upon dextrose (α-glucose), and in the solutions adjusted to an acid or neutral reaction the mutarotation

occurring is expected to produce an equilibrium between the α- and β-forms involving no overall change in the cyclic pyran structure[7]. The action of dilute alkali upon dextrose is to cause re-arrangement without scission of the carbon chain, tending to result in the formation of an equilibrium mixture of glucose, fructose and mannose. The fructose in such a mixture will itself undergo mutarotation which, in this case, involves a furanose to pyranose shift and vice versa[7]. In such a mixture of the three monosaccharides the existence of the acyclic hydrated aldehyde (aldehydrol) and the enolic (1 : 2-enediol) forms of glucose are postulated[8]. It is possible that this phenomenon may occur in the thioglycollate solution adjusted to an alkaline pH, the overall effect being to enhance the reducing activity of the dextrose. This would explain the decreased oxidation occurring in the alkaline solution. The possible effect of dextrose upon the oxidation-reduction potential of the systems has not been investigated.

The reactions obtained for the presence of thioglycollate in originally dilute solutions containing 0·05 per cent of sodium metabisulphite point to the efficacy of this material as an antoxidant for thioglycollate. However, only quantitative determinations will prove whether sodium metabisulphite is of value for this purpose, and it is proposed to continue this investigation using a quantitative colorimetric reaction in which sodium metabisulphite does not interfere. No explanation of the apparent value of sodium metabisulphite as an antoxidant for thioglycollate is as yet advanced.

The evolution of hydrogen sulphide when thioglycollate solutions and broth mixtures are boiled has been confirmed in a personal communication to the authors by Sykes, who believes that this decomposition is normal and not caused catalytically by constituents of the broth. He states that the sterilisation of media containing 0·4 per cent of sodium thioglycollate is accompanied by evolution of hydrogen sulphide and this is responsible for the dark colour which sometimes appears in thioglycollate media after sterilisation.

Brewer's medium, before use, is usually heated for half an hour in a boiling water bath to remove dissolved air, the removal being accompanied by a discharge of the green colour of the oxidation : reduction potential indicator (methylene blue) added to the medium. Whilst this shows that dissolved air has been removed and that the oxidation : reduction potential has been reduced, it does not show whether the thioglycollate is in the reduced or oxidised state, although it is believed that removal of dissolved air is accompanied by reduction of any disulphide present to the sulphydryl form. Practically, it is well known that a sample of thiocollate medium which has become oxidised and gives no reaction for sulphydryl with ammoniacal sodium nitroprusside will, after heating, give a positive reaction for sulphydryl, support the growth of anaerobic organisms and antagonise the effects of mercurial bacteriostats.

Thus it appears that the effect of heating thioglycollate solutions which have deteriorated is to cause some regeneration of their sulphydryl content. The greatest reversal was obtained with those solutions which had

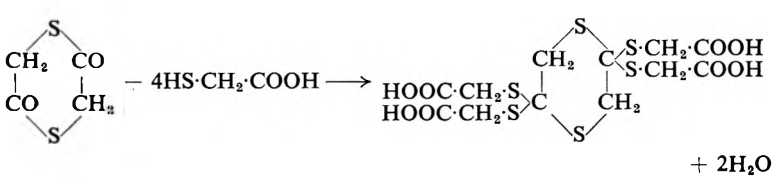
become oxidised to the greatest extent. These results lend support to the work of Skerman[6], who demonstrated that the disulphide formed on oxidation was hydrolysed to the extent of about 20 per cent to the sulphydryl compound on heating, the actual amount of this hydrolysis depending upon the temperature and the time of heating. Skerman believes this regeneration is accelerated by the other constituents of thioglycollate media.

The production of a yellow colour in thioglycollate solutions, apart from the solution made with the sodium salt, was not observed until they had been stored for at least 14 days, and from our present and previous results[1] seems to be associated with the action of sodium hydroxide upon thioglycollic acid.

The effect of storage conditions upon thioglycollic acid are well shown by the appearance and $HS{\cdot}CH_2{\cdot}COOH$ content of the two samples examined. The older sample stored at a lower temperature had undergone less decomposition than a fresher sample kept at room temperature. These results are in agreement with the conclusions reached in Part I of this paper[1] where the oxidation of thioglycollic acid was found to increase with dilution and temperature rise. A World Health Organisation report[9] recommends that thioglycollic acid for the preparation of sterility test media be periodically assayed and rejected if its content falls below 75 per cent. It further advises storage of thioglycollic acid in tightly stoppered bottles which prevent the access of moisture, protected from light and in a cool dry place. Leussing and Kolthoff[10] reported that the oxidation of the acid could be reduced to a very low level if the container was flushed with nitrogen during and after removal of samples.

Since no significant difference existed between the $HS{\cdot}CH_2{\cdot}COOH$ content of the sample of thioglycollic acid containing a white sediment before or after alkali treatment it appears that the sodium hydroxide merely reacts to form a sodium salt with the precipitated material, which is water-soluble, but produces no other change. The amount of alkali required to clarify the sample of thioglycollic acid was less than 5 per cent of that required for complete neutralisation of the acid, and it seems likely that the precipitated material is a stronger acid.

Under certain conditions, dithioglycollide will react with more thioglycollic acid to form a tetracarboxylic acid[11]:



This compound (2:2:5:5-tetracarboxymethylmercapto-1:4-dithiane) has been shown to be identical with the white crystalline material which separates from thioglycollic acid on prolonged storage. Some properties and reactions of this compound have been reported[12], of which the colour reaction with perchloric and sulphuric acids is one.

Whilst insufficient of the white material occurring in the old sample of thioglycollic acid was obtained for melting point determination, elemental analysis and other tests, it is believed that it was the tetracarboxy acid which Schöberl and Wiehler[12] have isolated.

Application of the Results to Thioglycollate Media

Brewer[5], in his original paper, stated that his medium remained anaerobic for a month and did not require heating before use. He also reported that bubbling air through the medium had no adverse effects upon the growth of strict anaerobes afterwards inoculated into it. The deterioration of thioglycollate media has been noted by several workers[13–15], who have pointed out that this may be accompanied by an increased toxicity. Whilst suggested modifications of the formula for thioglycollate media have been studied[16], comparatively little attention has been paid to the oxidation of thioglycollate in such media.

Using a polarographic method for the determination of thioglycollate, Skerman[6] showed that 0·1 per cent of thioglycollate in a medium 7 cm. deep exposed to air at 37° was completely oxidised in 80 hours. He showed the rate of oxidation to be unaffected by the presence of agar in the medium. Sykes, in a personal communication, reports that by the use of the colorimetric method of Schöberl and Ludwig[17] he has shown that of 0·1 per cent thioglycollate in a meat medium, approximately half is decomposed on autoclaving whilst the remainder (in plugged tubes) has decomposed after one week. Sykes, Royce and Hugo[18] consider there is always some slight loss of thioglycollate on autoclaving and this loss usually represents about 0·05 per cent in thioglycollate concentration. Hence in a medium containing 0·1 per cent of thioglycollate there may be a serious deficiency. Apart from this initial loss during sterilisation, they report that media containing 0·4 per cent of sodium thioglycollate stored in screw-capped bottles shows negligible loss by oxidation up to one month. To overcome the deterioration of thioglycollate media on storage, Australian workers[19] prepared their media daily by the addition of sufficient of a sterile 10 per cent thioglycollic acid solution to the otherwise complete medium; this addition caused a reduction in the pH of the medium from 8·1 to 7·1. Sufficient of a 10 per cent thioglycollic acid solution was prepared for one to three weeks use and it was stored in small sterile air-tight containers.

Brewer[5] recommended that his medium be stored at room temperature as low temperature storage increased the amount of air dissolving in it. Skerman[6] suggests the use of screw-capped containers in place of cotton wool-plugged tubes. Sykes (personal communication) concludes that plugged tubes are unsuitable for thioglycollate media unless they are to be used immediately or have a paraffin seal, and reports bottled media is only suitable for about a week unless a rubber liner is incorporated to make an air-tight seal.

Conclusions

From a consideration of the results obtained in this investigation, the following conclusions are made. Whilst the value of dextrose as a

reducing agent in thioglycollate solutions has been shown, it is possible that the addition of an antoxidant may minimise the rate of oxidation of the thioglycollate. Thioglycollate media should be adjusted to as low a pH value as is compatible with bacterial growth. Provided thioglycollate media is contained in air-tight containers it should be stored at as low a temperature as possible. Storage at 37° to check on the sterility of the media is obviously detrimental. The preparation of thioglycollate media in small batches will provide for more rapid turnover of the media. During sterilisation, the caps of the containers should be loosened to permit the escape of hydrogen sulphide, which otherwise results in a darkening of the media, but must be screwed down tight immediately on removal from the autoclave.

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THE DETERMINATION OF INORGANIC SULPHATE IN SODIUM LAURYL SULPHATE

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The official method for the determination of inorganic sulphate in sodium lauryl sulphate is time-consuming. Separation of organic and inorganic sulphate via the lead salts followed by complexometry leads to a rapid and convenient method with readily reproducible results.

Sodium lauryl sulphate first became the subject of an official monograph in the British Pharmacopoeia, 1948. The specified method[1] for determination of inorganic sulphate requires its separation by dissolving organic sulphate by ethanol followed by a classical gravimetric determination as barium sulphate. A determination commenced on one day cannot be completed before noon of the following day.

An alternative method[2] converts both forms of sulphate to the corresponding benzidine salts. These are then separated by means of their differing solubilities in ethanol, and titrated with alkali. Fischer and Chen[3] have, however, pointed out certain inherent errors in this approach to the determination of (inorganic) sulphate.

It has previously been shown that barium lauryl sulphate is readily soluble in hot water and it was suggested that it might be possible to separate inorganic and certain organic sulphates by this means[4].

Experimental

Barium chloride was added to boiling aqueous solutions of synthetic mixtures of sodium sulphate and sodium lauryl sulphate under various conditions of pH, and the resultant precipitates collected and thoroughly washed with hot water. They were then dissolved in excess of ammoniacal EDTA[5] and the excess of the latter titrated with magnesium chloride to Eriochrome Black T.[6] Poor separations and variable recoveries were obtained, possibly due, in part at least, to insufficient ageing of the precipitates.

Barium lauryl sulphate is also soluble in certain organic solvents, for example, formamide and dimethyl formamide. But, attempts to separate it quantitatively from barium sulphate by this means were also unsuccessful. Other cations forming insoluble or sparingly soluble sulphates were then investigated as precipitants and lead (*-ic*) proved to be suitable. Chloride is usually present in significant quantities in sodium lauryl sulphate, but both lead chloride and lead lauryl sulphate are freely soluble in hot 50 per cent ethanol, whereas the solubility of lead sulphate is greatly reduced under these conditions[7,8]. Separations were thus readily effected.

Lead sulphate is conveniently determined by complexometry[9] and Sporek[8] has recently described the determination of sulphate in ores by

this route. He prefers to depress the solubility of lead sulphate by addition of *iso*propanol and dissolves the precipitate in EDTA by the addition of ammonia; excess EDTA is then titrated with zinc chloride. In a re-appraisal of this approach, we have found that use of an ammonia: ammonium chloride buffer with the EDTA solution is less likely to lead to frothing during the final titration. Furthermore, zinc sulphate has proved to be of greater convenience as the final titrant.

METHOD

Reagents

Lead nitrate solution 0·1 M. Dissolve 33·1 g. of lead nitrate in water and dilute to 1 litre.

EDTA solution 0·1 M. Dissolve 37·225 g. of the purified[10] disodium salt of ethylenediaminetetra-acetic acid in water and dilute to 1 litre. While this is regarded as a primary standard[10], it may if necessary be checked against dried A.R. sodium sulphate by the proposed method.

Ammoniacal buffer solution. Dissolve 8·25 g. of ammonium chloride in water, add 113 ml. of 0·88 ammonia solution and dilute to 1 litre with water.

0·05 *M zinc sulphate solution.* Dissolve 14·4 g. of A.R. zinc sulphate ($ZnSO_4.7H_2O$) in water, dilute to 1 litre and standardise against the above 0·1 M EDTA solution.

Procedure

Weigh accurately about 1 g. of sodium lauryl sulphate into a 250-ml. beaker. Add 35 ml. of distilled water and warm to dissolve. Add 2·0 $\pm$ 0·2 ml. of 5 per cent nitric acid, mix, and then add 50 ml. of ethanol. Heat to boiling on a hot plate and slowly add 10 ml. of 0·1 M lead nitrate solution, with stirring. Cover with a clock glass and simmer for 5 minutes. Allow to settle. If the supernatant liquid is hazy, stand for 10 minutes, re-heat to boiling, and again allow to settle.

While the solution is still almost boiling, decant as much liquid as possible through a 9 cm. no. 41 Whatman paper. Wash four times by decantation, each time using 50 ml. of 50 per cent ethanol and bringing the mixture to the boil. Finally transfer the filter paper to the original beaker, and immediately add 40 ml. of water, 10·0 ml. of 0·1 M EDTA and 5 ml. of ammoniacal buffer solution. Warm to dissolve the precipitate and titrate with 0·05 M zinc sulphate solution to Eriochrome Black T.

$$\text{Then } Na_2SO_4 = (10f - tb) \times \frac{14{\cdot}2}{w} \text{ per cent}$$

where f = molarity of EDTA solution
b = molarity of zinc sulphate solution
t = ml. zinc sulphate solution required
and w = g. of sample taken.

RESULTS

The recovery of lead sulphate was first checked in the absence of organic sulphate. Results were very satisfactory and typical figures are given in Table I.

TABLE I

RECOVERY OF SODIUM SULPHATE BY PRECIPITATION AS LEAD SULPHATE AND DETERMINATION BY EDTA AND ZINC SULPHATE

mg. Na_2SO_4 taken	25·3	50·6	63·2	75·9
mg. Na_2SO_4 found	25·7	50·7	63·0	76·0

For a more realistic check, a sample of sodium lauryl sulphate known to be already of very low inorganic sulphate content[4] was repeatedly extracted into absolute ethanol to free it from inorganic salts[1]. It was estimated finally to contain 0·00012 per cent sodium sulphate. From this highly purified material, synthetic mixtures with A.R. sodium sulphate and of known composition were prepared. The proposed method was then applied to these and typical results are shown in Table II; results by the official method[1] are included for comparison.

DISCUSSION

Using the proposed method, the (inorganic) sodium sulphate content of sodium lauryl sulphate B.P. can be determined conveniently in 30–40 minutes. This compares favourably with 18–24 hours actual elapsed time by the official method[1], of which about 1·5 hours represent actual manipulative time. Additionally, in our laboratories the new method has yielded more readily reproducible and more precise results (cf. Table II).

TABLE II

RECOVERY OF SODIUM SULPHATE FROM SYNTHETIC MIXTURES CONTAINING PURIFIED SODIUM LAURYL SULPHATE

(Total mixture per determination: 0·95–1·05 g., weighed accurately)

Na_2SO_4 per cent in mixture	mg. Na_2SO_4 taken	Proposed method		Official method	
		mg. Na_2SO_4 found	error per cent	mg. Na_2SO_4 found	error per cent
Nil	Nil	Nil	–	Trace	–
1·63	15·7	14·9	−5·1	14·6	−7·0
3·17	31·5	32·0	+1·6	33·2	+5·4
4·68	47·2	47·6	+0·8	45·6	−3·4
6·15	63·0	64·0	+1·6	65·9·	+4·6
7·52	78·8	80·0	+1·5	79·8	+1·3
9·04	94·4	94·0	−0·4	97·8	+3·6

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BOOK REVIEW

ION EXCHANGERS IN ORGANIC AND BIOCHEMISTRY. Edited by Calvin Calmon and T. R. E. Kressman. Pp. xii + 761 (including Index). Interscience Publishers, 1957. London, 124s.; New York, $15.00.

This is a comprehensive survey of the field indicated by the title, in which 37 authors, mostly from Great Britain and the United States, have written 37 chapters which, together with their bibliographies, give a complete account of developments in the ion exchange field, up to the date of publication. The editors have succeeded in correlating the diverse material so as to form a reasonably connected story.

The first nine chapters forming Parts I and II, contain a general survey of ion exchange materials and techniques, giving a good introduction to the subject for a reader who has had no previous experience with these reagents. The remaining chapters, Part III of the book, deal with specific applications. These cover the ion exchange properties of bone. of cells and of tissues, the use of ion exchange methods in virology and bacteriology, the separation of amino acids, peptides, proteins, nucleic acids, hormones, carbohydrates and the components of urine. Other sections deal with the use of ion exchange techniques in studies of enzymes and of blood, and their application in plant physiology and chemistry. There are chapters of particular interest to pharmacists, concerned with the applications of these versatile materials to pharmacy and medicine in general, to the purification and analysis of antibiotics, alkaloids and vitamins. In addition there are reviews of the purification of water and sugars and of the treatment of alcoholic beverages, fruit juices, milk and milk products.

The subject-matter is well presented in a readable form and each chapter forms a review of its subject, complete with copious references to the literature. This book should be included in the library of anyone who is concerned with the use of ion exchange resins.

LETTERS TO THE EDITOR

The Dietary Toxicity of Glycerol Formal in the Rat

SIR,—As an extension to recent studies on the acute toxicity of glycerol formal before its adoption as a solvent for use in toxicity testing[1], a short test on the dietary toxicity to the male rat has now been completed.

Groups of ten male rats of Wistar strain, of average weight 90 g., were maintained for sixteen weeks on a diet of an aqueous paste of MRC diet 41B meal to which respectively 0, 316, 1000, 3162 and 10,000 p.p.m. by weight of glycerol formal had been added. In addition to observations for death and toxic effects, daily food consumptions and weekly body weight changes were measured. During this period, the control, 316 and 1000 p.p.m. groups showed no significant differences of any kind. The 3162 p.p.m. group showed a slight but significant reduced rate of weight gain (90–94 per cent of that of the controls), but no other apparent effects, though there was one death in this group in the fourteenth week, probably due to a kidney infection. The 10,000 p.p.m. group showed a reduction of rate of weight gain, falling to 70–80 per cent of the controls after 4 weeks. The animals were obviously weak and undernourished, but showed no decreased food consumption or specific toxic effects. Six rats in this group died between the fifth and the thirteenth weeks, apparently due to gastrointestinal infections possibly induced by lowered resistance. The survivors of this group were killed after thirteen weeks, and showed no specific macroscopic pathology other than that associated with undernourishment; relative liver and kidney weights were higher than normal, probably due to malnutrition.

After sixteen weeks, it was decided to examine the remaining groups for derangement of drug-metabolising functions (e.g., liver-enzyme balance). This was done by adding to the diets of all groups the same quantities of the organophosphorus insecticide rogor (*OO*-dimethyl-*N*-methylcarbamoylmethylphosphorodithioate), whose *in vivo* anticholinesterase properties are dependent on the balance of a number of activating and detoxifying enzyme systems, mainly in the liver. It was thought that similar rates of cholinesterase inhibition between groups when this substance was administered would be a good index of metabolic normality. Accordingly, while the glycerol formal treatment continued, the diets of all groups were further supplemented by 25 p.p.m. of pure rogor for four weeks, and then 50 p.p.m. for three weeks, while weekly heart blood samples were taken for plasma and erythrocyte cholinesterase activity determinations by a standard manometric method[2]. During this period, the previous pattern of weight gain and absence of toxic effects continued, and there was no difference between cholinesterase activities of the groups, except for a marginally greater degree of erythrocyte cholinesterase inhibition in the 3162 p.p.m. group.

All animals were then killed, after a total of 23 weeks, and showed no macroscopic pathology or significant group variation of liver or kidney weights, or of brain cholinesterase and no liver or kidney histopathology at 1000 p.p.m..

Hence it may be concluded that incorporation of up to 1000 p.p.m. of glycerol formal in the diet of male rats (equivalent to 75–150 mg./kg./day, according to body weight), caused no significant toxic effects or metabolic changes. This amount is therefore considered satisfactory for use as solvent in dietary toxicity testing of solid materials insoluble in other less toxic solvents; inclusion of a

solvent control is advised. Higher amounts caused reduced weight gain, disturbance of drug metabolism, and increased susceptibility to infection.

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Mast Cell Response in Aseptic Inflammation

SIR,—It has been reported previously that when rats are infected with *Staphylococcus aureus*, there is widespread degranulation of tissue mast cells.[1] The histamine content of the skin is increased after remote surgical injury,[2] and as much of the histamine in the skin is contained in the mast cells,[3] it was of interest to study the mast cell changes in the subcutaneous tissue of the rat during aseptic inflammation.

Groups of 6 rats of approximately 150 g. body weight, of either sex, were employed for each observation. Four small cotton wool pellets soaked in spirit of turpentine were implanted under the dorsal skin under ether anaesthesia. Groups of animals were killed at intervals and the resulting changes were studied.

There were no mast cells in the inflamed area, and degranulation and swelling of the cells was seen in the adjacent areas after 6 hours. The maximum changes were noted 24 hours after injury, when almost all the cells in the adjacent areas were ruptured, and metachromatic materials were seen lying free in the tissue. After a further 24 hours, free metachromatic material had disappeared, presumably having been washed out, or phagocytosed[4] by the inflammatory exudate. The inflammatory response was also very much reduced at this time. At the end of 72 hours there was considerable proliferation of new mast cells along side the blood vessels in the adjacent areas. In the next 24 hours, the cells were seen to have migrated away from the blood vessels towards the inflamed area and form cell clumps. In many cell clumps the outline of the individual cells could not be distinguished and it is possible that this was the result of rapid cell division without complete separation of the cytoplasm. The cells were also partly degranulated. By the end of 7 days the cells, though still partly degranulated had separated. At this time there was development of fibrous tissue round the implanted pellets. The fibrosis was very much marked in 10–14 days, when the mast cells presented a nearly normal appearance.

After intraperitoneal injections of turpentine, it has been previously noted[4] that there is rupture of mast cells, inflammation and necrosis, but not much of cell proliferation. The disappearance of mast cells in the inflamed area, may possibly be due to the effects of turpentine, but the changes noted in the adjacent areas are most likely to be related to the inflammatory changes. The earlier disruptions of mast cells may cause liberation of histamine, and thereby increase vasodilatation and capillary permeability, and thus help in forming the inflammatory exudate. The later proliferation of mast cells and invasion of the

inflamed area is followed by development of fibrous tissue. In view of the fact that mast cells may secrete hyaluronic acid,[5] it is possible that these two changes may be related. A full report of these findings will appear elsewhere.

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The Influence of Chlorpromazine on Vascular Effects of Vasopressin and some other Pressor Agents in Dogs

SIR,—Chlorpromazine has been found to potentiate the antidiuretic action of endogenous as well as exogenous vasopressin in rats[1,2]. It is assumed that this potentiation might be due to the inhibition of the enzymes which normally break down vasopressin in the body. If it were so, the other effects of vasopressin should be potentiated by chlorpromazine too. Therefore the vascular effects of vasopressin after chlorpromazine were studied. Some other pressor substances were also investigated.

Normotensive dogs were anaesthetised with a mixture of chloralose (70 mg./kg.) and urethane (1 g./kg.). Carotid blood pressure was recorded by means of a mercury manometer. All injections were made by the intravenous route. A submaximal dose of the pressor agents was given before and after chlorpromazine.

Chlorpromazine in doses of 1-5 mg./kg. markedly augments the vascular effects of vasopressin (Tonephin: Hoechst), angiotonin and ergotamine. This potentiating effect manifests itself about 10 minutes after the injection of chlorpromazine and persists for several hours. Barium chloride as a nonspecific pressor agent was used as control in all experiments. It was found that pressor effects of barium chloride are not augmented by chlorpromazine. The same holds true for 5-hydroxytryptomine and lysergic acid diethylamide. According to Kopera[3] neither the vascular effects of sympathomimetic drugs and central nervous system stimulants are potentiated by chlorpromazine. It seems therefore that chlorpromazine potentiates only those pressor agents which contain peptide linkages. Since the pressor peptides used in this study are rather chemically different substances, the chlorpromazine induced potentiation is likely to be ascribed to the inhibition of a nonspecific peptidase.

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