REVIEW ARTICLES

THE PHARMACOLOGY OF MEMBRANES

THE PASSAGE OF SUBSTANCES ACROSS BIOLOGICAL MEMBRANES*

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THE meaning of the term "membrane" in biology is not well defined. In the widest sense of the word any structure or complex of structures through which substances or ions pass at rates significantly different from those at which they would pass through a similar layer of water may be considered to be a membrane.

The Variety of Membrane Structures

The cell membrane should be considered first, or perhaps we should rather say the plasma membrane. Secondly the term is applied to sheets of cells separating two different phases, as for instance in the capillary wall, the intestinal epithelium or the acini and ducts of glands. Finally the term is applied to various intracellular structures, such as the nuclear membrane and the mitochondrial membrane. Probably we should also include the walls of certain vacuoles and the cytoplasmatic reticulum. I wish to restrict my field to the processes which may be involved in the passage of substances across biological membranes and to select a few examples for illustration. For this latter purpose I have chosen to confine myself to a single group of non-electrolytes, namely the monosaccharides.

The Structure of the Cell Membrane

As far as the structure of the cell membrane is concerned it is only in recent years that methods for rational investigation have become available, and we have to admit that our knowledge is still superficial.

The prevalent view to-day is that the cell membrane consists of a bimolecular leaflet of lipids covered on the outside and on the inside by a layer of unfolded protein or mucoprotein.

The main evidence for a structure of this kind has come from British studies on the X-ray diffraction patterns of the myelin sheath of nerves. From electron micrographs this sheath is known to be built up by concentric layers and from the embryological studies of Geren (1954) it appears that each of these layers must be formed from two layers of the cell membrane of the Schwann cell, which become wrapped around the axon after its invagination into the Schwann cell. From studies on the radial repeat periods of fresh as well as dried and lipid extracted myelin sheaths, Finean came to the conclusion that two such Schwann cell membranes in apposition had a thickness of about 170 Å, and that each of these membranes consisted of a double layer of lipids with protein monolayers

* Based on a lecture at the joint meeting of the British Pharmacological Society and the Scandinavian Pharmacological Society, Copenhagen 1960.

attached on either side. Fig. 1 from Engström and Finean's book (1958) gives a schematic representation of these interpretations. The radial repeat period representing two membranes is indicated, and in each of these are seen the "hairpins" of the phospholipid and cholesterol molecules with their non-polar ends facing each other and with protein layers attached to the polar ends.

One major function of the cell membrane is evidently to prevent or at least greatly to impede the loss of essential components of the cytoplasm, but at the same time the metabolism demands that some substances can pass or be passed at fairly high speeds across the membrane. A highly discriminative handling of different substances is therefore a main feature of the function of the cell membrane. A membrane like the one suggested by Finean may well account for some of this discriminative handling but

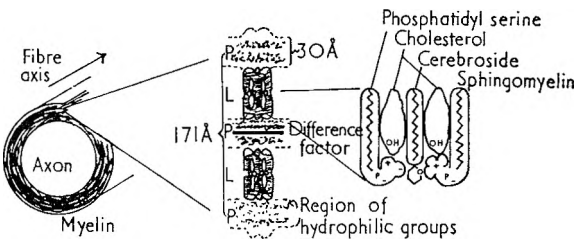

FIG 1. Drawing showing possible arrangement of molecules (including phospholipid-cholesterol complex) in the structural unit of the myelin sheath.

there are a number of observations which call for considerable modification of this crude model.

At the present stage of our knowledge it seems reasonable to distinguish between five main groups of mechanisms by which substances or ions may pass the cell membrane. These are set out in Table I.

If the cell were completely surrounded by a lipid double-layer with no crevices in it simple diffusion of substances into and out of the cell would be impossible. To diffuse through such a membrane a substance would first have to make a jump from a water phase into the lipid layer and later another jump from the lipid layer into a water phase. To make the first jump a strongly polar substance would require a considerable activation energy in order to break the hydrogen bonds to the water molecules, and since this energy would greatly exceed the average thermal agitation energy of the molecules such jumps would occur only on rare occasions. A non-polar substance—not being held by such strong ties to the water phase, could much more frequently obtain sufficient energy to make the jump into the lipid layer, but it would, on the other hand, require some energy to pass from the lipid layer into the cytoplasm.

Activated Diffusion

A theory for such activated diffusion, as it has been called, has been developed by Danielli (Davson and Danielli, 1952). Very briefly stated it predicts that the permeability of a cell membrane for various substances

is a function of their lipid-water partition coefficients and their molecular sizes. These predictions are, as we all know, in good agreement with the findings in a number of studies on the rates of penetration of a variety of substances into different cells, and there is therefore hardly any doubt that the penetration of a considerable number of substances through the cell membranes is a matter of activated diffusion.

Water-filled Pores

There are, however, a number of exceptions.

One of these is the penetration of some lipid-insoluble substances of very small molecular size, as for example water and methanol which pass many cell membranes much faster than one might expect from their oil-water partition coefficients. It is therefore generally accepted that they penetrate by simple diffusion through minute water-filled "pores".

The major evidence for the existence of water-filled channels through the cell membranes has come from studies of the passage of water across the membranes. Experiments on a variety of cells have shown that the

TABLE I

MECHANISMS OF PASSAGE THROUGH CELL MEMBRANES*

"Passive"	*Simple diffusion*	—through continuous water phase	Rate of passage proportional to concentration difference across membrane
	Activated diffusion	—through a non-water phase	
	Facilitated diffusion	—reversible binding to membrane carrier moving by thermal agitation	
"Active" (transportation immediately dependent upon cellular energy)	*Facilitated (propelled) penetration*	—reversible binding to membrane carrier, the movement of which is accelerated by cellular energy	Rate of passage showing upper limit ("saturable system")
	Unidirectional uphill transportation	—transportation mechanism undefined, carrier system possibly involved	

* Formation of vacuoles by pinocytosis may be important in some cases of cellular uptake but does not per se involve passage through the cell membrane.

permeability constants for diffusion of water across the membranes, as determined by isotopically labelled water, are generally considerably smaller than those determined from the net flux of water, produced by differences in the osmotic pressures on the two sides of the membrane. As emphasized by Ussing (Koefoed-Johnsen and Ussing, 1953), this fact that the membrane offers a smaller resistance to bulk flow of water than to diffusion of water molecules must mean that there are continuous water filled channels through the membranes. Our present means for evaluation of the equivalent diameter of these pores are beset with many uncertainties, but for what they are worth, they have led to figures of the magnitude of 7 Å (Paganelli and Solomon, 1957; Mullins, 1959). Such pores would obviously allow free diffusion of nothing but the smallest molecules. Even the diffusion of very small ions through the pores would be greatly restricted if the pore walls were occupied by fixed electric charges of the same sign.

There are a number of substances which should be unable to pass a lipid membrane with pores of this size at significant rates by either simple diffusion or activated diffusion because their molecules are too big to pass through the pores and too polar to become detached from the water molecules with any frequency worth mentioning. Among these we should expect to find the monosaccharides, and indeed a number of monosaccharides have been found to be unable to cross many cell membranes. It is, however, a fact that certain others, for example in some instances their stereoisomers may pass the same membranes at fairly high rates.

Mechanisms other than Simple Diffusion and Activated Diffusion

Evidently, some monosaccharides, and only some, are capable of passing the cell membranes by mechanisms other than simple diffusion and activated diffusion. To illustrate this point I shall discuss in a little more

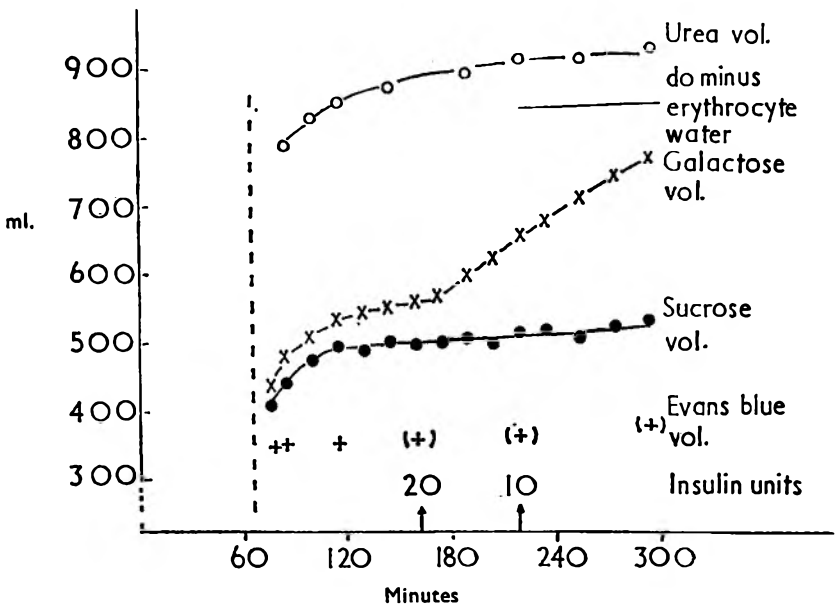

FIG. 2. Effect of insulin on the distribution of galactose; distribution volumes for sucrose, urea and Evans blue determined for comparison. Weight of hind limb preparation 1,050 g. Start of perfusion at zero time. 2·3 g. of galactose + 0·8 g. of sucrose + 1·8 g. of urea + 20 mg. of Evans blue dissolved in 10 ml. added at 65 min.; addition indicated by vertical broken line.

detail the passage of monosaccharides across the cell membrane of skeletal muscle and heart muscle and the effect of insulin on these passages.

My own limited experience in this field started in the early forties when, together with Professor Lundsgaard and Dr. Gammeltoft, I was engaged in some studies on isolated, perfused hind-limb preparations of the cat (Gammeltoft, Kruhøffer and Lundsgaard, 1944). As an indication of the marked steric specificity of the processes involved in passage of monosaccharides into muscle cells it was found in these studies that insulin is

without effect upon the cellular uptake of fructose, in contrast to its well-known uptake-promoting effect on glucose.

Some years later Levine and his colleagues (Levine, Goldstein, Huddlestun and Klein, 1950; Goldstein, Henry, Huddlestun and Levine, 1953) made the important discovery that the uptake-promoting effect of insulin is not confined to glucose. In eviscerated, nephrectomized dogs these investigators demonstrated that the rate of distribution in the body of certain sugars, such as D-galactose, D-xylose and L-arabinose, was greatly accelerated by insulin, whereas no such effect was exerted on the distribution of others, such as D-fructose.

Levine's publications gave rise to intensive studies on the passage of various monosaccharides across the muscle cell membrane. Personally I

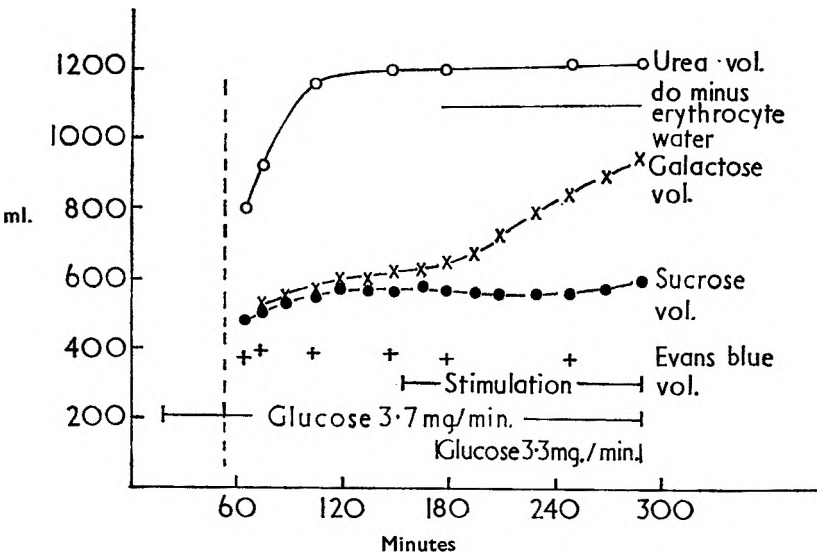

FIG. 3. Effect of muscular exercise on the distribution of galactose; distribution volume for sucrose, urea and Evans blue determined for comparison.

was able to confirm his findings in the somewhat simpler preparation of the isolated, perfused hindlimbs of the cat. Figs 2 and 3 show some results from these studies (Huycke and Kruhøffer, 1955).

Some time after the recirculation of blood through the hindlimb preparation had been established in these experiments a mixture of known amounts of D-galactose, Evans blue, sucrose and urea was added to the perfusion blood. The figures show the apparent distribution volumes of these substances as a function of the subsequent time. It will be noted that initially the galactose volume increases only slightly faster than the sucrose volume.

However, as seen in Fig. 2, shortly after the addition of insulin the galactose volume starts to increase much more rapidly and after some 2 hr. it has approached the urea distribution volume.

Fig. 3 shows the results from a similar experiment in which, however, a period of muscular exercise (induced by electric stimulation) was substituted for the addition of insulin. It is apparent that muscular exercise has the same effect on the rate of distribution of galactose as has insulin. It may be added that other experiments showed that muscular exercise, just like insulin, is without effect upon the rate of cellular uptake of fructose.

Later experiments, in particular those of Park, Reinwein, Henderson, Cadenas and Morgan (1959) on isolated perfused rat hearts, have added a great deal of further information which strongly supports the view that the passage of glucose and various other monosaccharides across the muscle cell membrane occurs by one of the processes commonly called facilitated diffusion and facilitated penetration.

Facilitated Diffusion and Facilitated Penetration

Before discussing these more recent results it seems appropriate to say a few words on these types of transport mechanisms.

Both of them are characterised by the fact that the substance under transport does not pass the membrane in the free state, but is attached to a carrier molecule or a carrier group. In facilitated diffusion the carrier is assumed to shuttle back and forth through the membrane merely by thermal agitation, whereas in facilitated penetration the back and forth movement of the carrier is speeded up at the expense of metabolic energy.

In both types of transportation the reaction between carrier and substance is assumed to be a simple reversible chemisorption, requiring no cellular energy and taking place in the same way at both sides of the membrane.

The blood haemoglobin in the body constitutes a good macro-scale model for the mechanism of facilitated penetration, with the propelled movement of the carrier between alveolar air and tissues being taken care of by the circulation.

A non-circulating haemoglobin solution separating two other phases serves as a good model for the mechanism of facilitated diffusion.

From our knowledge of such systems it is readily seen what the fundamental properties of the systems of facilitated diffusion and penetration must be. I shall propose then to enumerate these basic properties and for each of them to mention briefly the related findings from experiments on the passage of monosaccharides across the muscle cell membrane.

Increased transportation capacity. (1) As the first point it is obvious that the presence of the carrier will greatly increase the transportation capacity for those substances which can be attached to it. This tallies well with the fact that some monosaccharides may pass the cell membrane at fairly high rates, whereas others hardly pass at all.

Saturation of the transport system. (2) The transport system is saturable, that is, it is capable only of transportation up to a certain rate, but this and the saturation concentration may vary from one substance to another. Thus the haemoglobin system will be saturated with CO at a lower tension than with O_2, but the more ready release of O_2 from haemoglobin may

endow the Hb-system with a higher transporting capacity for O_2 than for CO, if only O_2 is offered at a sufficiently high tension.

The system of glucose uptake in muscle cells is also saturable, that is, the rate of glucose uptake increases only up to a certain limiting value with increasing extracellular glucose concentrations. It appears, furthermore, that this limiting rate is the same whether insulin is present or not. Much higher external glucose concentrations are, obviously, required to reach the limiting rate when insulin is absent (Lundsgaard, Nielsen and Ørskov, 1939; Park, Reinwein, Henderson, Cadenas and Morgan, 1959).

The release of the transported substance unchanged. (3) The substance transported should be released as such on the exit side, and if not subsequently converted it should be demonstrable there, and it should finally reach a similar concentration to that on the entrance side.

For non-metabolisable, penetrating sugars like D-galactose and 3-methyl-glucose it has been found that the intracellular concentration in muscle cells, after adequate time, approaches, but does not exceed a maintained extracellular concentration. Glucose is, however, converted chemically inside the muscle cells and it is therefore not surprising that it has only been possible to demonstrate free glucose there under special conditions, namely, with high extracellular concentrations and in the presence of insulin.

Ambi-directional operation. (4) The transport systems will work equally well in both directions.

It has been found that once heart cells have been loaded with a non-metabolisable, penetrating sugar it can be washed out of them at a comparable rate by switching to a perfusion fluid not containing the sugar.

Competition between transport substances. (5) When two substances are present which both combine with the carrier one will tend to depress the transportation of the other, and the strongest depressive effect will be exerted by the substance which has the higher affinity for the carrier.

Such mutual effects are well known for CO and O_2 and similar competitive effects have been demonstrated for the uptake of different sugars in muscle cells. It seems also likely that the depressive effect of phloridzin on such uptakes is due to a competition for a membrane carrier.

These examples will suffice to show, that there is substantial evidence for the view that the passage of various monosaccharides across the muscle cell membrane occurs by facilitated diffusion or facilitated penetration, and that the carrier involved has a definite specificity, but not at all a specificity which is entirely confined to glucose.

Monosaccharides in Other Cells

Similar systems are unquestionably engaged in the uptake of monosaccharides in other cells. Intensive studies, in particular by Le Fevre (1954) have strongly suggested that this may be so in the case of erythrocytes. There is, however, one major difference, namely that insulin has no effect upon the entrance of monosaccharides into erythrocytes.

At the moment we are without knowledge of the intimate nature of the membrane carriers involved in these transports. It seems possible,

however, that some clue to the chemical structure of the sorptive site of the carrier may be derived from a recent suggestion by Le Fevre and Marshall (1958). They pointed out that those monosaccharides which penetrate the erythrocyte membrane are apt to assume a particular bending of the ring, the so-called C–1 resting chair conformation, whereas those which do not penetrate are more apt to assume other conformations.

Another problem which remains unsolved is how insulin is capable of accelerating the influx and outflux of various sugars from muscle cells. From the promptness of this effect and from the size of the insulin molecule it seems likely that it is caused by an action on the outside of the membrane. In trying to visualise the type of action involved it would seem to be a fact of importance that the maximum rate of glucose uptake appears to be the same whether insulin is present or not. This, in my opinion suggests that insulin does not affect the carrier system directly, but that it rather acts by removing some diffusion hindrance located between the free extracellular fluid and that site in the membrane outside, where glucose becomes attached to the carrier.

The Passage of Monosaccharides across Sheets of Cells

Let us now consider a somewhat different subject namely the passage of monosaccharides across membranes made up by sheets of cells, and let us among these first consider the capillary wall.

It is a common view to-day that only two processes are involved in the passage of substances across the capillary wall. These are diffusion through pores for lipid-insoluble substances like monosaccharides, and activated diffusion through the entity of the endothelial cells for highly lipid-soluble substances.

That pores do exist in some capillaries has been demonstrated by electron-microscopy, but this method has failed to visualise any pores in muscle and connective tissue capillaries and in the capillaries of the central nervous system (Bennett, Luft and Hampton, 1959).

It appears that in the case of muscle and connective tissue capillaries we still have to believe in the existence of pores considerably larger than those traversing the common cell membrane since various small-molecular, lipid-insoluble substances appear to pass through these capillary walls at rates which are fairly proportional to their free diffusion rates.

In the case of the capillaries of the CNS the situation may be quite different. Some recent observations by Dr. Crone (Crone, 1960) in our department may serve to illustrate this point.

The technique in these experiments was as follows. A single brief injection was made into an artery supplying the organ under investigation. The solution injected contained known proportions of Evans blue and the substance, the transcapillary passage of which was to be studied. If the substance was already present in the blood, the substance was given in a labelled form. Over a subsequent period of some 10–15 sec. fractional collections were made of the venous blood leaving the organ—in the case of the brain these collections were made from the sagittal sinus. On the assumption that no Evans blue was lost during a single passage

through the capillaries the extraction percentage for the substance under study could be determined. For the first venous samples these extraction percentages should very nearly represent the true outflux of the substance through the capillary wall, since, with the rapidly rising arterial concentration, the back-flux would be negligible in these early periods.

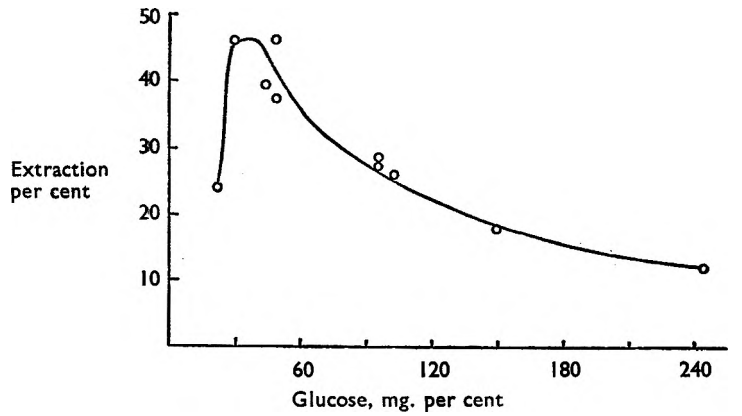

FIG. 4. Initial percentage loss of radioglucose from blood during a single passage through the brain vessels of dogs as determined at various blood glucose concentrations.

Now, in the case of the brain it was found that the initial extraction percentage for fructose was only a few per cent, and that up to plasma concentrations of some 200 mg. per cent there was no indication of a variation in its magnitude. These findings were interpreted to mean that

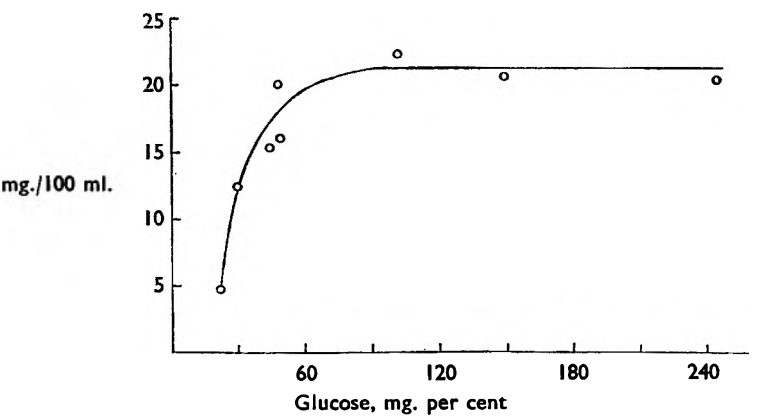

FIG. 5. Relationship between the blood glucose concentration (abscissa) and the outflux of glucose per 100 ml. of blood passing through the brain vessels (ordinate). Calculated from the data of Fig. 4 by the application of a small correction for simple diffusion outflux.

small amounts of fructose could be lost by simple diffusion through the capillary walls.

In the case of labelled glucose the findings were quite different. From Fig. 4 it will be seen that the initial extraction percentage for this substance was found to be smaller and smaller at increasing levels of the blood glucose concentration. These findings obviously indicate that a saturable process is involved in the blood to brain transport of glucose. A more striking illustration of this is seen in Fig. 5. Here the amounts of glucose lost per 100 ml. of blood have been calculated, and from these have been subtracted those amounts which, according to the fructose experiments, should have been lost by simple diffusion. It is seen that this excess amount of glucose lost per 100 ml. of blood (ordinate) rises with increasing blood glucose concentrations (as determined by a non-specific "reduction" method) until at a value of some 60 mg. per cent it reaches a limiting value.

Two important facts have thus been borne out by Crone's studies. Firstly, the very low extraction percentage for fructose as compared with figures of 40–60 per cent obtained in other capillaries, adds further evidence to the view that the blood-brain barrier is much more tight than other blood-organ barriers. Secondly, they show that there is a preferential penetration of glucose by a saturable process through the blood-brain barrier.

The nature of this important process, which provides for adequate supplies of glucose to the brain at even quite low blood glucose concentrations is not yet known. It seems quite reasonable that it could be a matter of facilitated diffusion or penetration across each of the cell membranes of the endothelial cells.

On the other hand, it cannot be excluded that it may be a matter of an uphill transportation. As we all know transportation of this type is involved in the passage of monohexoses across other cell sheets such as the renal proximal tubules and the intestinal epithelium.

The intimate nature of these directional mechanisms by which glucose can be taken in at a lower concentration at one end of the cell and delivered at the other end at a higher concentration remains obscure.

The phosphorylation-dephosphorylation hypothesis has been dismissed because it has turned out that sugars, which are not phosphorylated, may be transported uphill almost as fast as glucose. All present views are therefore based upon mere speculations.

If I should make a guess as to the nature of these mechanisms I would make it in accordance with the economy principle which seems to have a wide application in biology. In other words, I would suggest that the uphill transportations of monosaccharides make use of the same carriers as those involved in facilitated diffusion and penetration, but that at the exit side of the membrane the carrier is converted, somehow, into a form with reduced sorptive affinity for monosaccharides, and that upon return to the entrance side, it is reconverted into the original form.

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The Effect of Drugs on Cell Membranes with Special Reference to Local Anaesthetics*

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Increasing knowledge about the cell and its functions has made it clear that the cell membrane and the membranes inside the cell, in the mitochondria, the microsomes, the cell nucleus and in the cytoplasm are not only diffusion barriers which separate water phases but that a large number of very important processes takes place at these membranes. This knowledge has raised a number of questions.

The Structure of the Cell Membrane

First of all, what is the structure of the membranes?

The exact structure is unknown but it seems that lipids are an essential part of the membranes and that these lipids are arranged in a bimolecular layer where the lipophilic groups face each other while the hydrophilic groups turn to the water phases on the two sides of the membrane. On the lipids on both sides of the membrane there seems to be adsorbed a monolayer of partly unfolded proteins (Danielli, 1958) (Fig. 1).

The Relation between Structure and Function

Secondly, what is the significance of the physical structure of the membranes in relation to the processes which take place in them?

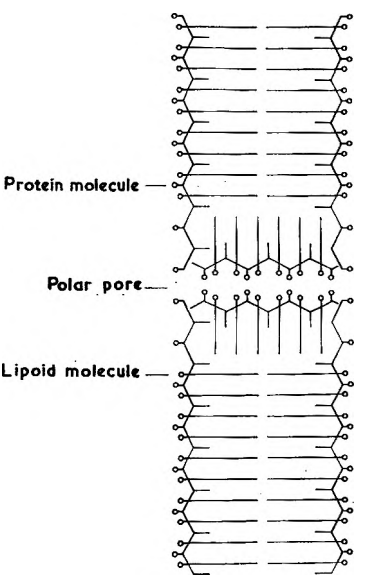

Fig. 1. Model of a cell membrane showing a polar pore (modified from Danielli, 1958).

* Based on a lecture at the joint meeting of the British Pharmacological Society and the Scandinavian Pharmacological Society, Copenhagen, 1960.

Are the membranes only the building stones on which the active elements are placed, or are the membrane elements part of the system? What is the difference between an enzymatic process which takes place at an interface and one which takes place in a solution? Are there other ways

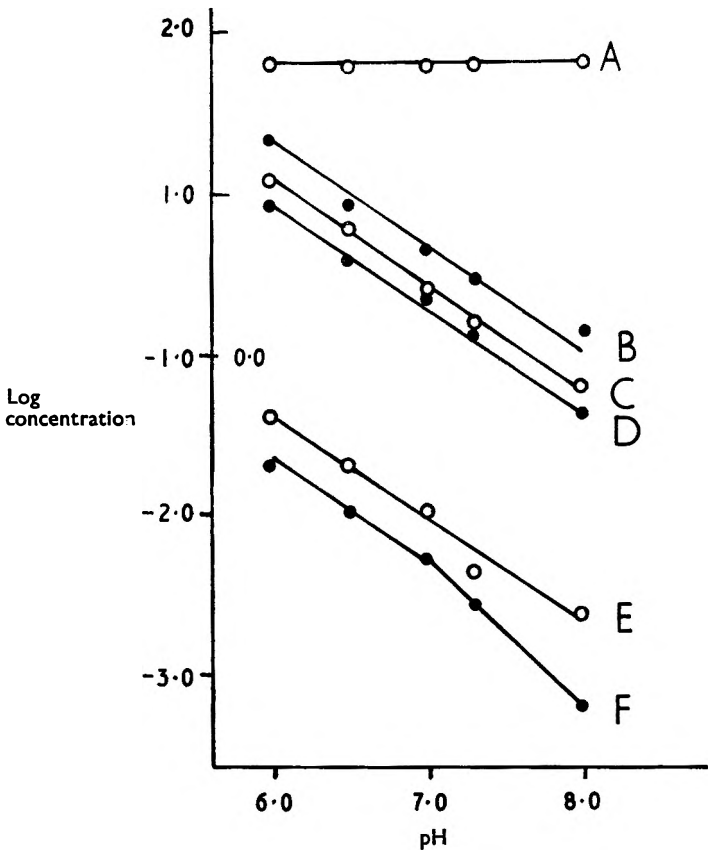

FIG. 2. The dependence of the minimum blocking concentrations on the pH (Skou, 1954a). Ordinate: the logarithms of the minimum blocking concentrations in mM. The lower scale applies to amethocaine (E) and cinchocaine (F), the upper scale to butanol (A), procaine (B), cocaine (C), tropacocaine (D).

at the interface by which the process can be influenced or controlled which differ from those in a solution? And if so, what are they?

Function and Drug Action

Thirdly, how must we interpret the effects that drugs have on the reactions taking place at an interface? Is the effect of a drug at an interface different from that found in a solution? Will a drug by altering the structure of a membrane be able to influence indirectly the chemical processes therein?

It is not possible at the present moment to give an answer to these questions, but in the following pages will be described some experiments

with local anaesthetics which show one of the ways by which it may be possible to get information about these problems.

The Effect of Local Anaesthetics

Most of the local anaesthetics in use are tertiary amines and their pK value is of such an order that the drugs at a physiological pH will be in the solution partly as the undissociated base and partly as the cation. The physicochemical properties of these two components are different. The cation is much more soluble in water than in lipids, while the undissociated base is much more lipid than water soluble. It is therefore reasonable to assume that the two components of the molecule have different pharmacological effects.

The Undissociated Base

For local anaesthetics which are tertiary amines the minimum concentration which can block nerve conduction decreases with increasing pH

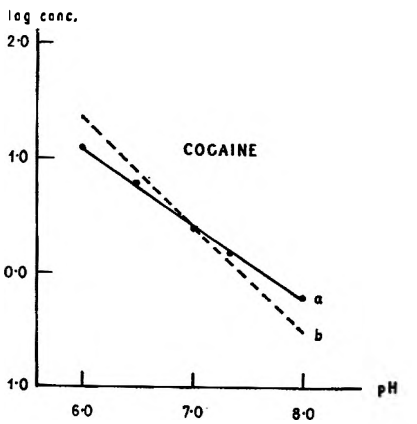

FIG. 3. Cocaine, the dependence of the minimum blocking concentration on pH. Ordinate: Curve 'a', logarithms of the minimum blocking concentrations in mM. Curve 'b' indicates the concentrations of cocaine which at the different values of pH give the same concentration of undissociated base in the solution as the minimum blocking concentration of cocaine at pH 7·0 (Skou, 1954b).

(Fig. 2). For butanol which also can block nerve conduction the minimum blocking concentration is independent of pH. Since butanol is undissociated this indicates that a change in the hydrogen ion concentration does not in itself influence the excitability of the nerve. The change in minimum blocking concentration of the tertiary amines must therefore be due to the change in the ratio between cation and undissociated base.

With an increase in pH the amount of undissociated base increases while the amount of cation decreases. The increase in blocking potency with increasing pH therefore makes it reasonable to assume that it is the undissociated base which is the blocking agent. If this is correct the minimum blocking concentration calculated as the concentration of undissociated base must be the same at different values of pH. But as seen from Fig. 3 this does not seem to be the case. The line 'b' indicates

the concentrations of cocaine which in solution give the same concentration of undissociated base at the different values of pH, while the line 'a' is the estimated minimum blocking concentration of cocaine at different values of pH (*cf.* Fig. 2). The slope of the two lines is similar but not identical, which means that the minimum blocking concentration of undissociated base varies with pH; it increases with increasing pH. If, however, as pointed out by Shanes (1958), an activity correction is applied to the estimated concentrations of the undissociated base, the minimum blocking concentrations at different pH correspond to the same activity of base in solution. That means that the slope of the corrected

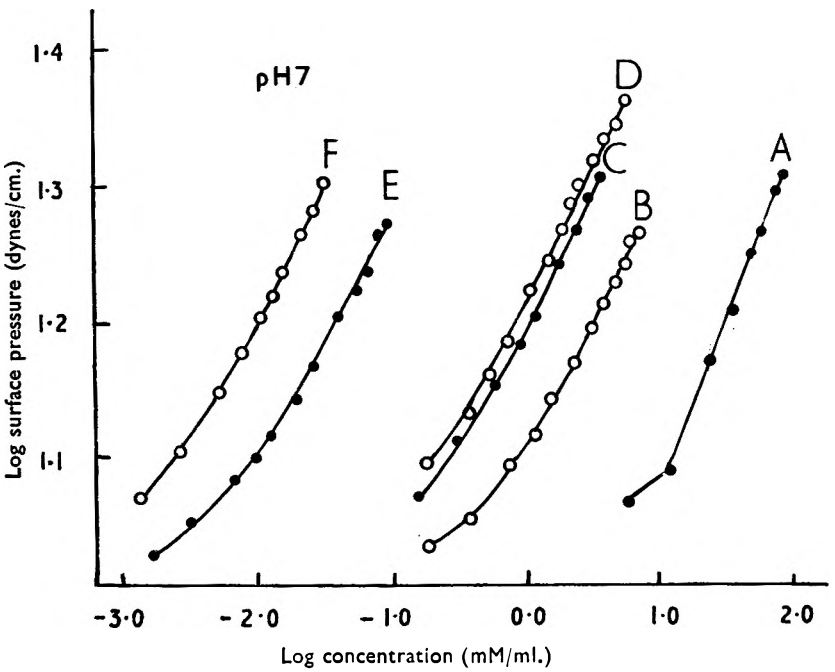

FIG. 4. Curves showing the relation of pressure to concentration for: butanol (A), procaine (B), cocaine (C), tropacocaine (D), amethocaine (E), cinchocaine (F). Monolayer of nerve-tissue lipids in a Ringer phosphate buffer pH 7·0 (Skou, 1954c).

curve for the estimated minimum blocking concentrations will be identical with the slope of 'b' and that it is the undissociated base which is the blocking agent.

The undissociated base of a local anaesthetic is more lipid than water soluble. It is therefore reasonable to assume that in the cell, it will be found in a higher concentration in the lipid phase in the cell membrane than in the water phase.

When an extract of lipids from peripheral nerves is spread on a water surface where the area of the surface is large enough compared to the amount of lipids, the lipid molecules will form a monomolecular layer. In this monolayer the molecules will be oriented in relation to the water

surface in the same way as it is assumed that they are in the cell membrane (*cf.* Fig. 1). Such a monolayer thus forms a model of the interface between water and lipids in the membrane and it may therefore be possible by the help of this model to gather information about the effect of the local anaesthetics on the cell membrane.

The area of the monolayer can be varied by a barrier which is placed across the trough in which the water surface is at the same level as the side of the trough. When the area is decreased the surface pressure is increased. The surface pressure is identical with the decrease of the surface tension of the water surface due to the monolayer.

In the investigations on the effect of local anaesthetics on this model of a cell, lipids extracted from peripheral nerves were spread on the surface

TABLE I

THE RATIOS BETWEEN THE MINIMUM BLOCKING CONCENTRATIONS AND BETWEEN THE INCREASES IN PRESSURE IN THE MONOLAYER, PH 7·0. IN COLUMNS 3 AND 5, PROCAINE HAS BEEN TAKEN AS 1 (SKOU, 1954 c).

	Minimum blocking concentration	Relative blocking potency	Increase in pressure in monolayer	Relative increase in pressure
	mM/l.		dynes/cm.	
Procaine	4·6	1	7·0	1
Cocaine	2·6	1·8	8·9	1·3
Tropacocaine	2·2	2·1	9·4	1·3
Amethocaine	0·01	460	2·5	0·4
Cinchocaine	0·005	920	3·8	0·5
Butanol	68	0·07	9·8	1·4

of a Ringer's solution, pH 7·0. The area of the monolayer was adjusted so the pressure in the monolayer was 10 dynes/cm. This value was arbitrarily chosen. The local anaesthetics were injected in the water-phase beneath the monolayer and after a thorough mixing the change in surface pressure due to the penetration of the local anaesthetic was measured at a constant area of the monolayer.

The results of these experiments are shown in Fig. 4, and in Table I is shown the pressure increases in the monolayer produced by a concentration in the water phase beneath the monolayer equal to the minimum blocking concentration of the local anaesthetic.

It is seen from Fig. 4 and Table I, Column 3, that the order in which the local anaesthetics increase the pressure in the monolayer is the same as the order of their blocking potency. As shown in Table I the minimum blocking concentrations of the local anaesthetics vary from 4·6 to 0·005 mM/l., that is, by a factor 1:920; if butanol is included the factor is 1:13,500. Considering this, the pressure increase produced by the minimum blocking concentrations, even if it varies with a factor of 1:3, seems to be of the same size for all the agents.

In Fig. 5 curve 'm' shows the concentrations of cocaine that at pH 6·0 and 7·5 produce the same increase in pressure in the lipid monolayer, 8·9 dynes/cm., as the minimum blocking concentration 2·6 mM/l. at pH 7·0. The curve 'a' indicates the slope of the curve for the dependence of the minimum blocking concentrations on the pH (*cf.* Fig. 2). The

similarity between the slope of these two lines indicates that the ability of the local anaesthetic to increase the pressure in the monolayer depends on pH in a manner which is similar to its ability to block nerve conduction.

In Table II is shown the results of the investigations of a number of drugs, which like the tertiary amines, are able to block the impulse conduction in peripheral nerves. They are all undissociated in aqueous solution. Also it was found that when these drugs were injected in the water phase below a monolayer of nerve lipids, they gave an increase in pressure in the monolayer. And as it is seen from the Table the minimum blocking concentrations of these drugs gave pressure increases in the monolayer which were of the same size.

The experiments on the monolayer thus show (1) that the order of the ability of the drugs to increase the pressure in a monolayer of nerve tissue

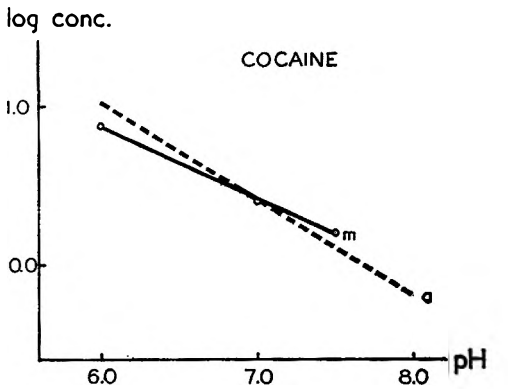

FIG. 5. Cocaine, the dependence on the pH of the penetration (Skou, 1954c). Curve 'm', logarithms of the concentrations which produce an increase in pressure of 8·9 dynes/cm. Curve 'a' indicates the slope of the curve for the dependence on the pH of the minimum blocking concentrations.

lipids follows that of their ability to block nerve conduction, (2) that the minimum blocking concentrations of the drugs give increases in the pressure in the monolayer which are of the same size, and (3), that the ability of the tertiary amines to increase the pressure in the monolayer depends on pH in a manner similar to their ability to block nerve conduction.

It seems therefore reasonable to conclude that there is a correlation between the ability of the drugs investigated to block nerve conduction and their ability to increase the pressure in a monolayer of nerve tissue lipids.

This indicates (1) that the site of the blocking action is a lipid containing membrane and (2) that the effect is due to the physical changes which are produced by the penetration of the drugs into the membrane.

This view has found strong support in the results of experiments by Shanes and Gershfeld (1960) on stearic acid films. It was found that labilisers such as some veratrum alkaloids and stabilisers such as procaine, which is known to have an antagonistic effect on the nerve membrane, also have antagonistic effects on the monolayer. Labilisers decreased the

spreading force while stabilisers, in accordance with the results from the experiments on the monolayer of lipids from nerve tissue, increased the spreading force.

The nerve impulse is caused by a transient increase in the permeability of the axon membrane to Na^+ (Hodgkin, 1951). It is therefore reasonable to assume that the membrane at which the local anaesthetics exert their blocking action is the axon membrane, and that it is the physical changes in the lipid part of the membrane due to the penetration of the drugs that prevents the increase in permeability to Na^+ and thereby block the impulse.

According to Danielli (1958), the ions move through pores in the membrane and he has suggested that the pore is the space between two protein lamellae which are placed with the polar groups facing each other

TABLE II*

THE PRESSURE INCREASE PRODUCED BY THE MINIMUM BLOCKING CONCENTRATIONS OF DRUGS IN A MONOLAYER OF NERVE TISSUE LIPIDS AT PH7

1	2 Minimum blocking concentrations mM/l.	3 Corresponding pressure increases in monolayer dynes/cm.	4 Concentrations giving a pressure increase of 9·3 dynes/cm. mM/l.	5 Relative blocking potencies	6 Relative penetrating potencies
Propanol	218	8·5	240	1·0	1·0
Isopropanol ..	351	8·0	438	0·6	0·6
Butanol	68	8·2†	80	3·2	3·0
Pentanol	21	10·0	19	10·4	12·6
t-Pentanol ..	81	8·9	85	2·7	2·8
Menthol	0·58	11·8	0·37	376	649
Thymol	0·22	9·8	0·20	991	1200
β-Naphthol ..	0·30	9·5	0·29	727	828
Average		9·3			

* Skou, 1958.

† In previous studies, see Table I, the corresponding figure was found to be equal to 9·7 dynes/cm. The explanation presumably is that the vapour pressure of butanol above the monolayer was too high in the earlier experiments, the atmosphere being saturated with butanol.

while the non-polar groups face the lipid part of the membrane (*cf.* Fig. 1).

What makes the pores in the membrane discriminate between Na^+ and K^+ is unknown as is also the mechanism that underlies the change in permeability of Na^+ during the impulse. But whatever the mechanisms it is reasonable to assume, as suggested by Danielli, that proteins play an important role.

How then can a drug which penetrates into the lipid part of the membrane influence this process, or, to put it in another way, how can a drug which penetrates into the lipid part of the membrane influence the proteins at the interface in a pore in the membrane?

At an interface between water and lipids a protein will be more or less unfolded and the degree of unfolding will depend on the interfacial tension. Furthermore, the side chains of the protein will be oriented relative to the interface, and the orientation will depend among other factors such as pH, temperature and ions in the water phase, on the

lipids; the orientation of the lipophilic side chains is influenced by their affinity for the lipophilic parts of the lipids and the orientation of the hydrophilic side chains is effected by their interaction with the hydrophilic groups of the lipids. A change in the structure of the lipids may therefore lead (1) to a change in the degree of unfolding of the protein and (2) to a change in the orientation of the side chains.

In order to see whether a change in one of these factors might influence the activity of a protein, a technique was elaborated (Skou, 1959) which made it possible to investigate how the activity of an enzyme at an interface depends on the degree of unfolding and on the orientation of the side chains of the protein.

When a protein is spread on aqueous surface, an unfolding of the molecule takes place (Bull, 1947). The rate of unfolding depends on a

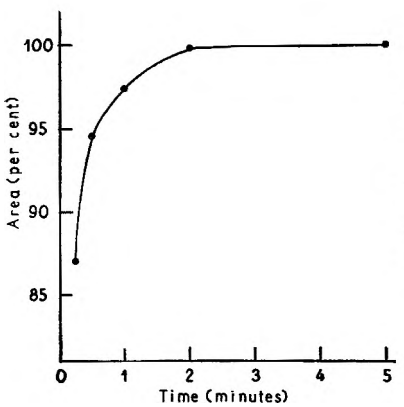

FIG. 6. Area of the surface-spread protein after the protein has been allowed to spread against zero film pressure for varying periods after the end of application. The area is expressed as a percentage of the area at complete unfolding (Skou, 1959).

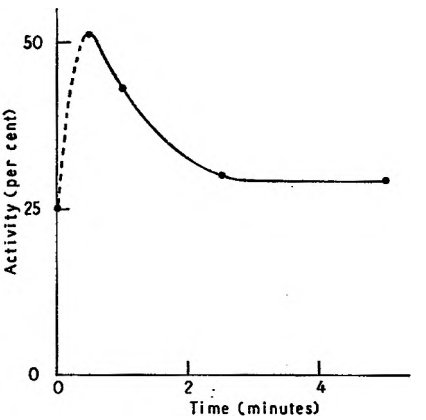

FIG. 7. Acetylcholinesterase activity of the surface-spread protein after it has been allowed to spread against zero film pressure for varying periods after the end of the application. The activity was determined at a surface pressure of 10 dynes/cm. and is expressed as a percentage of the activity of the same amount of acetylcholinesterase in solution (Skou, 1959).

number of factors such as the temperature, pH, and ionic composition of the fluid. In addition, it depends on the surface pressure; the higher the pressure the more difficult is the unfolding and at a sufficiently high surface pressure unfolding is prevented.

Complete unfolding takes a certain time; for the protein investigated, acetylcholinesterase, it took 2 min. (Fig. 6). At any degree of unfolding the unfolding could be stopped by applying a surface pressure of 7 dynes/cm. or greater. It was therefore possible to obtain the protein at any degree of unfolding by applying a surface pressure of 7 dynes/cm. at different time intervals after the application of the protein to the surface.

The orientation of the side chains of a protein on a water surface in relation to the surface varies with the surface pressure (Bull, 1947; Davies,

1953; Ellis and Pankhurst, 1954; Hughes and Rideal, 1932). At zero film pressure the side chains will lie flat in the surface. If the pressure is increased, the polar groups are orientated towards the water phase, while the non-polar groups will be raised in the air. It is therefore possible to vary the orientation of the side chains by applying surface pressures of varying magnitude.

The enzyme investigated was acetylcholinesterase purified from tissue from *Electrophorus electricus* (Rothenberg and Nachmansohn, 1947). The activity of this enzyme spread on the surface could be measured by measuring the breakdown of acetylcholine in the water phase beneath the

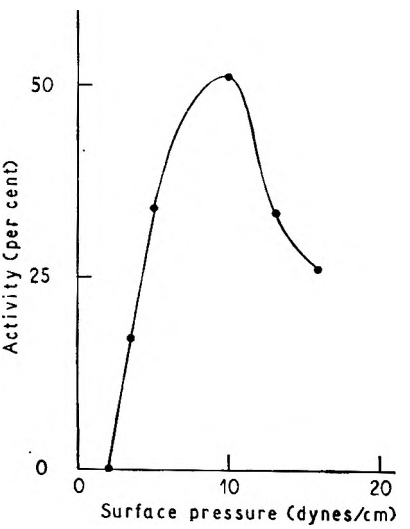

FIG. 8. Acetylcholinesterase activity of the surface-spread protein at varying surface pressures. In all experiments the protein was allowed to spread against zero film pressure for 30 sec. The activity is expressed as a percentage of the activity of the same amount of acetylcholinesterase in solution (Skou, 1959).

surface. The activity of the enzyme at the surface is expressed as the percentage of the activity of the same amount of acetylcholinesterase in solution.

As is seen from Fig. 7, the activity of the enzyme varies with the time the protein has been allowed to unfold at the surface, that is, with the degree of unfolding (*cf.* Fig. 6). At complete unfolding and at a surface pressure of 10 dynes/cm. the enzyme still has about 29 per cent of the activity of the enzyme in solution.

In Fig. 8 is shown how the activity of the enzyme at the surface varies with the surface pressure at a given degree of unfolding. In all these experiments the protein was allowed to spread for 30 sec. against zero film pressure. It appears that maximum activity, 51 per cent, is attained at a pressure of 10 dynes/cm. and that an increase of the pressure from 10 to 16 dynes/cm. gives a decrease in activity from 50 per cent to 26 per cent while a decrease in pressure to 2 dynes/cm. results in complete abolition of the activity. It should, however, be noted that unfolding at

pressures ranging from 2 to 5 dynes/cm. is somewhat greater than at higher pressure, since, as previously mentioned, some further unfolding will take place after adjustment at these pressures. The values found are accordingly a little too low.

Experiments similar to those shown in Fig. 8 were also performed with a spreading time of 5 min., that is, at complete unfolding at the surface (*cf.* Fig. 6). Under these conditions, maximum enzyme activity, 29 per cent, was also found at a pressure of 10 dynes/cm.; at 2 dynes/cm. the activity was zero.

It is seen from these experiments that both a change in the degree of unfolding and in the orientation of the side chains will give a change in activity, and of these two factors the variation in the orientation of the side chains give the greatest change in activity.

As mentioned above, the orientation of the side chains of a protein at an interface between water and lipid among other factors depends on the interactions between the side chains and the lipophilic and hydrophilic part of the lipids. It is therefore reasonable to assume that a drug which penetrates into the lipid part of a membrane may indirectly, due to its effect on the lipids, influence the orientation of the side chains of proteins at the interface. This may, according to the results of the experiments presented above, lead to a change in the activity of processes in which the proteins at the interface participate.

Eisenman Rudin and Casby (1957a) have been able to make glass electrodes which have different sensitivities for Na^+ and K^+ and they (1957b) (see also Rudin and Eisenman, 1959; Isard, 1959) have put forward a theory according to which the different sensitivities of a system, living or non-living, to alkali metal ions are due to differences in electrostatic field strengths of the negatively charged groups in the system. At low negative electrostatic field strength the sensitivity for K^+ is higher than for Na^+, at high negative electrostatic field strength the sensitivity for Na^+ is higher than for K^+.

In a membrane the electrostatic field strength of the fixed negatively charged groups at the interface is determined by the overlapping of the electric fields of the positive and negative charged groups on the lipids as well as on the side chains of the proteins. The negative electrostatic field strength of the charged groups on the proteins is furthermore determined by the distribution of the electrons along the protein chain.

In a pore in a membrane both the overlapping of the electric fields of the charged groups and the distribution of electrons along the protein chain may be influenced by the potential across the membrane. At the resting potential across the nerve membrane the overlapping of the charged groups and the distribution of the electrons may be such as to give a low negative field strength of the fixed anions and that means, according to the theory of Eisenman and others (1957b) a high permeability of the pore to K^+ and a low permeability to Na^+. At a lowering of the potential the overlapping of the charged groups and the distribution of electrons may be shifted so as to give an increase in the negative electrostatic field strengths, that is an increase in permeability to Na^+.

Local anaesthetics, due to their effect on the lipids, may indirectly influence the orientation of the side chains of the proteins in the interface. This may change the overlapping of the electric fields of the charged groups at the interface in such a way that the depolarisation of the membrane, even if it gives a change in the distribution of the electrons along the protein chain, cannot lead to that increase in negative electrostatic field strengths which is necessary to increase the permeability of the pore to Na^+; that is the nerve impulse is blocked.

The Cation

The cationic part of the molecule of a local anaesthetic also has an effect on a cell membrane and as will be seen from the following this effect seems to be to open a membrane to Na^+ (Skou and Zerahn, 1959).

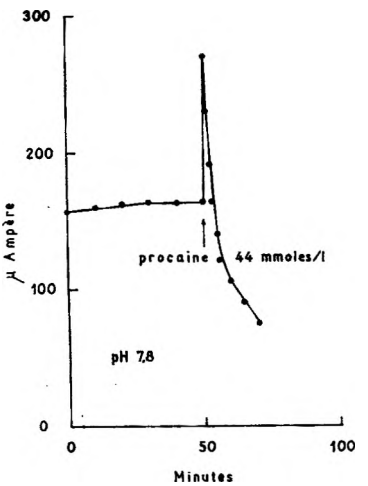

FIG. 9. The short circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 44 mM, pH 7·8 (Skou and Zerahn, 1959).

The frog skin is able to transport Na^+ from the outside of the skin to the inside. This transport can be measured by the current which is necessary to short-circuit the potential across the skin. (Koefoed-Johnsen and Ussing, 1958; Ussing and Zerahn, 1951).

When a local anaesthetic, for example, procaine, is added to the solution bathing the outside of the frog skin at pH 7·8 the short circuit current, that is the transport of Na^+, is first increased but shortly after again decreases to a value which is lower than before the addition (Fig. 9).

If the experiment is repeated at pH 6·0 where practically all the procaine is in the form of the cation it can be seen that the increase in current is not followed by a decrease (Fig. 10).

At pH 10·0, where nearly all the procaine is as the undissociated base addition of procaine to the outside gives no increase in current, only a decrease (Fig. 11).

If the tertiary procaine is converted to a quaternary drug by adding an extra CH_3 group on the amine, that is to change it to a drug which at

any pH is in the cationic form, this drug added to the outside of the skin always gives an increase in the short circuit current independent of the pH in the solution. In Fig. 12 is shown the effect at pH 7·8 (*cf.* Fig. 9).

Besides procaine, a number of other local anaesthetics have been tested. all were tertiary amines and for all of them it was found that in the cationic form they increased the active transport of Na^+ across the frog skin when

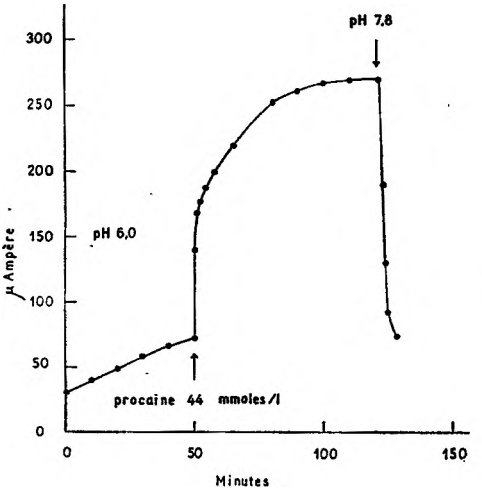

FIG. 10. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 44 mM, pH 6·0 (Skou and Zerahn, 1959).

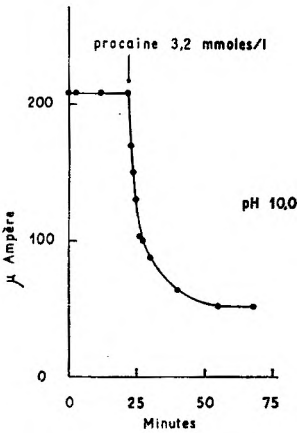

FIG. 11. The short-circuit current for an isolated frog skin before and after the addition of procaine to the Ringer solution bathing the outside of the skin. Procaine concentration 3·2 mM, pH 10·0 (Skou and Zerahn, 1959).

they were added to the outside solution. As undissociated bases they inhibited the active transport.

Added to the solution bathing the inside of the skin, the cationic form of the drugs have no effect while the undissociated base inhibits the active transport of Na^+.

The stimulating effect is not only limited to local anaesthetics, but a number of widely different drugs have the same effect. Common for all these drugs are (1) that they are amines, (2) that they must be added to the solution bathing the outside of the skin and (3) that they must be present in ionised form in the solution to exert the effect.

The stimulating effect seems to be independent of whether the drugs are primary, secondary, tertiary or quaternary amines. The effects of tertiary procaine and of quaternary "procaine" were of the same order of magnitude, and so were the effects of the primary amine, amphetamine, and the secondary amine, methamphetamine.

On the other hand, the effect seems to depend on the molecule to which the amino group is linked; it was found that the effect of the quaternary amine, acetylcholine, was much less than that of the quaternary "procaine".

Koefoed-Johnson and Ussing (1958) showed that the potential across the frog skin varied with the concentrations of Na^+ and K^+ on the two sides of the skin in such a manner that it must be assumed that the frog skin contains two functional membranes; an outer membrane, which is specifically permeable to Na^+ but not to the other cations, and which probably corresponds to the surface of the epithelial cells, and an inner membrane, which is specifically permeable to K^+, and which is probably situated at the inward-facing membrane of the stratum germinativum. These authors assume that the active transport of Na^+ is localised to the

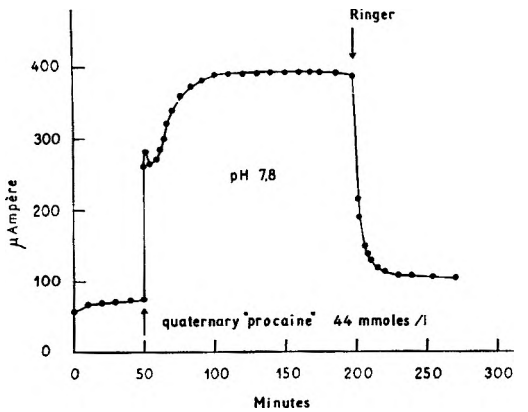

FIG. 12. The short-circuit current for an isolated frog skin before and after addition of quaternary "procaine" to the Ringer solution bathing the outside of the skin. Concentration of quaternary "procaine" 44 mM, pH 7·8 (Skou and Zerahn, 1959).

innermost of these two membranes, while no active transport occurs through the outer membrane. If transport through the skin is to take place, Na^+ must be transported passively through the outer membrane into the epithelial cells, from which active transport takes place through the inner membrane. This hypothesis has been supported by work by Engbæk and Hoshiko (1957) and by Schmidt (1960) who showed that the total potential across the epithelial layer is the sum of two potentials.

In experiments with giant axons, Hodgkin and Keynes (1956) showed that the active transport of Na^+ out of the nerve increased proportionally to the intra-axonal concentration of Na^+. If, similarly, the active transport of Na^+ from the epithelial cells of the frog skin to the fluid inside is proportional to the intracellular Na^+ concentration, an increase in the influx of Na^+ from the solution outside into the epithelial cells must result in an increase in active transport.

It is the ionized amines that are capable of increasing the active transport of Na^+, but only when they are added to the outside solution. Since the ionized amines do not pass through the skin, their point of attack is presumably the outermost of the two membranes in the frog skin, and their effect may consist in increasing the permeability of this membrane to Na^+. Since there is an electrochemical gradient for Na^+ from the solution outside to the epithelial cells when this solution is a Ringer

solution, an increase in the permeability of the membrane to Na^+ leads to an increase in the Na^+ influx and hence in the Na^+ concentration in the epithelial cells. As mentioned above this must be assumed to produce an increase in the active transport of Na^+ across the inner membrane.

This hypothesis about the effect of the ionized amines may be supported by the fact that quaternary amines are able to increase the permeability to Na^+ of the membranes of the muscle end-plate (*cf.* Riker, 1953) and of the electroplax from *Electrophorus electricus* (Altamirano, Coates, Grundfest and Nachmansohn, 1955; Schoffeniels and Nachmansohn, 1957).

We have made no experiments on the effect of the cationic form of the local anaesthetics on the nerve membrane.

The experiments with local anaesthetics, which are tertiary amines thus show that the effect of the undissociated base and of the cation are different but that both effects are due to an effect on a cell membrane.

The effect of the undissociated base is to block the increase in permeability of the nerve membrane to Na^+ and this effect seems to be due to a penetration of the molecule into the lipid part of the nerve membrane. The effect of the cation seems to be to increase the permeability of a membrane, the outer membrane of the frog skin, to Na^+.

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RESEARCH PAPERS

THE MECHANISM OF THE ANTI-INFLAMMATORY ACTIVITY OF SALICYLATE

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Salicylate reduced the leakage of circulating dye in the reversed passive cutaneous anaphylaxis reaction in the guinea pig. 2,4-Dinitrophenol, although a more powerful uncoupling reagent than salicylate, had no effect compared to corresponding control animals. In this test in the guinea-pig, salicylate inhibits the increased capillary permeability due to cutaneous anaphylaxis and to histamine, but not to Miles-Wilhelm permeability factor. It is concluded that the effect of salicylate in reducing the increased capillary permeability produced in these reactions does not result from an uncoupling action on oxidative phosphorylation processes. The results suggest that salicylates act by preventing antigen-antibody combinations from exerting their effects on the capillary wall.

SALICYLATE uncouples oxidative phosphorylation reactions in respiring mitochondrial preparations (Brody, 1956) and many of its metabolic and toxic effects are explicable in terms of this action (Smith, 1959). Adams and Cobb (1958) studied the effects of a series of non-hormonal anti-inflammatory drugs, including salicylate, on erythema induced in the guinea pig by ultra-violet light. They observed a general parallelism between uncoupling and anti-inflammatory activity. It was noted however, that 2,4-dinitrophenol, which is a more powerful uncoupling reagent than salicylate, failed to affect the erythema test. In the present work we have compared the actions of salicylate and 2,4-dinitrophenol on the increased capillary permeability in passive cutaneous anaphylaxis in the guinea-pig. In addition, the effects of salicylate on the cutaneous lesions induced in the guinea-pig by the permeability factor of Miles and Wilhelm (1955) and by histamine, have been studied. A preliminary account of part of the work has already been published (Marks and Smith, 1960).

EXPERIMENTAL

Animals. Albino guinea-pigs (wt. 450–500 g.) all of which were bred from the same stock, were depilated by applying barium sulphide paste to the previously clipped hair of the back and flanks.

Materials. Specific pneumococcal polysaccharide type III (SIII) and antibodies to SIII prepared in rabbits (anti-SIII) were obtained from the National Institute for Medical Research. Guinea-pig permeability

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factor (PF) was obtained from the Lister Institute of Preventive Medicine. The histamine was used as a solution of its acid phosphate in 0·9 per cent (w/v) saline. Pontamine Sky Blue 6 BX (G. T. Gurr) was prepared as a 5 per cent solution in sterile 0·9 per cent (w/v) saline and given by intravenous injection in a dose of 6 mg./kg. body weight. The salicylate solution contained 125 mg. of salicylate ion per ml. and was prepared by dissolving sodium salicylate B.P. in 0·9 per cent (w/v) saline and adjusting to pH 7·5 with 0·1 N HCl. It was given by intraperitoneal or intravenous injection in a dose of either 500 or 100 mg./kg. body weight. 2,4-Dinitrophenol (DNP) solution, containing 5 mg./ml. in 0·9 per cent (w/v) saline adjusted to pH 7·5 with 0·1 N KOH, was administered by intraperitoneal injection in a dose of 20 mg./kg. body weight.

Passive cutaneous anaphylaxis. The following two methods involving either intradermal sensitisation with antiserum followed by a systemic challenge with the antigen after 24 hr. or systemic sensitisation with antiserum and an intradermal challenge with antigen after 48 hr. were used.

Method 1. The animals were given intradermal injections of 0·1 ml. saline containing amounts of anti-SIII ranging from 0·5 to 50 μg. of antibody N per ml. at random sites on the back and flanks. After 24 hr. the control animals received intraperitoneal injections of 2 ml. of saline and the test animals received either salicylate or DNP solution by the same route. 2 hr. later each animal received 200 μg. of SIII in 0·5 ml. saline, together with Pontamine Blue solution by intravenous injection. The diameters of the cutaneous lesions produced were measured after 20 min. and the degree of "blueing" of the lesions assessed visually in terms of arbitary units. Additional experiments were made in which the time interval between the intraperitoneal injection of 500 mg. of salicylate per kg. and the intravenous injection of the antigen plus dye was reduced from 2 hr. to between 15 and 30 min. Salicylate in a dose of 100 mg./kg. was also injected intravenously immediately before challenging with the antigen in a further group of 4 animals.

Method 2. The guinea-pigs received an intraperitoneal injection of anti-SIII in a dose of 0·8 mg./kg. contained in 1 ml. of saline, followed after 48 hr. by the intravenous injection of the pontamine blue solution. Each animal then received a total of 17 intradermal injections distributed at random. These injections, each of 0·1 ml., comprised one of saline, four of saline containing 30 μg. of SIII, four of a saline solution of PF, 20 μg./ml., and the remainder consisting of saline solutions of histamine, four containing 6·7 μg./ml., four containing 20 μg./ml. The diameters and intensity of "blueing" of the lesions were measured after 20 min. Each animal then received an intraperitoneal injection of either saline or salicylate 500 or 100 mg./kg. Intradermal injections of each of the four test solutions were then repeated at intervals of 15, 30 and 60 min. after the saline or salicylate had been given and their size and intensity of colour assessed after a further 20 min. The intraperitoneal injections of saline were given to determine the effects of the "blueing" of the cutaneous lesion due to the time intervals which had elapsed since the intravenous administration of the dye. Blood glucose determinations were made by the

glucose oxidase method on venous samples obtained before and at one and two hours after the intraperitoneal injections.

RESULTS

The results given in Table I show that the intraperitoneal injection of 500 mg./kg. of salicylate given 2 hr. before the administration of antigen and dye caused a significant reduction in the colour intensity of the cutaneous lesions produced by the intradermal injections of 10 to 50 μg./ml. concentrations of the antibody. The effects of the similar administration of 20 mg./kg. of DNP did not differ from that produced in corresponding control animals and neither of the uncoupling reagents altered the size of the areas in which extravasation of circulating dye had occurred.

In the control and DNP-treated animals, the cutaneous lesions were sharply circumscribed and the "blueing" was uniform throughout. However, in the animals injected with salicylate, the lesions were irregular in outline and also showed a mottled appearance. This "mottling" was the

TABLE I

EFFECT OF SALICYLATE (500 MG./KG. BODY WT.) AND DNP (20 MG./KG. BODY WT.) ON THE SIZE AND INTENSITY OF STAINING OF SKIN LESIONS PRODUCED IN THE REVERSED PCA TEST IN THE GUINEA-PIG

Antibody concentration (μg. antibody N./ml.)	No. of lesions per animal	Control (10)		Salicylate (10)		DNP (10)	
		Size (mm.)	Intensity	Size (mm.)	Intensity	Size (mm.)	Intensity
50	2	17·7 ± 2·6	2·8 ± 0·52	18·4 ± 2·0	2·0 ± 0·58	18·2 ± 2·5	2·5 ± 1·05
25	4	16·1 ± 2·3	2·6 ± 0·59	17·0 ± 2·0	1·7 ± 0·67	16·9 ± 2·5	2·7 ± 0·63
10	4	14·1 ± 2·2	2·4 ± 0·71	15·3 ± 2·7	1·7 ± 0·65	14·7 ± 2·4	2·4 ± 0·52
5	2	10·8 ± 2·4	1·4 ± 0·26	12·2 ± 2·9	1·0 ± 0·75	12·1 ± 1·8	1·8 ± 0·60
2·5	2	9·9 ± 1·8	0·8 ± 0·14	10·3 ± 2·6	0·8 ± 0·53	9·9 ± 3·2	1·1 ± 0·75
0·5	2	0	0	0	0	0	0

The salicylate and DNP were administered 2 hr. before the intradermal injection of the antigen. The number of animals in each group is given in parentheses and the intensity is expressed as arbitrary units. The results are given as means together with the standard deviations. Comparison of the results by the *t*-test showed a significant difference ($P < 0.05$) between the colour intensities of the skin lesions produced by 10 to 50 μg./ml. of antibody in the control and salicylate groups only.

most characteristic change produced by salicylate and was observed to occur in every animal tested. The application of intermittently weak negative pressure to the lesions, 5 to 10 mm. of Hg., failed to produce a uniform distribution of colour in the mottled areas showing that a reduced arteriolar pressure was not responsible for the mottling.

When the time interval between the intraperitoneal injection of salicylate and the intravenous injection of the antigen was reduced to between 15 and 30 min., these changes were less evident. There was no significant reduction in the intensity of blueing of the lesions and only one of the four salicylate-treated animals showed mottling. A similar result was obtained in 4 guinea-pigs which received 100 mg./kg. body weight of salicylate by intravenous injection immediately before challenging with the antigen. Mottling of the cutaneous lesions occurred in one animal but the extravasation of the circulating dye, as assessed by the colour intensity of the lesions, was not significantly affected.

The results, represented graphically in Fig. 1, show that both 500 and 100 mg./kg. of salicylate caused a marked reduction in the colour intensity of the lesions produced by passive cutaneous anaphylaxis to SIII. Mottling was observed in about three-quarters of the lesions in the animals given the larger dose of salicylate and in about half of those receiving the smaller dose. 500 mg./kg. of salicylate caused a slight reduction in the colour intensity of the lesions produced by PF but the smaller dose of salicylate was without effect. Mottling was not seen in any of the lesions produced by PF in salicylate-treated animals. 100 mg. salicylate caused reductions of more than 50 per cent in the colour intensities of the lesions

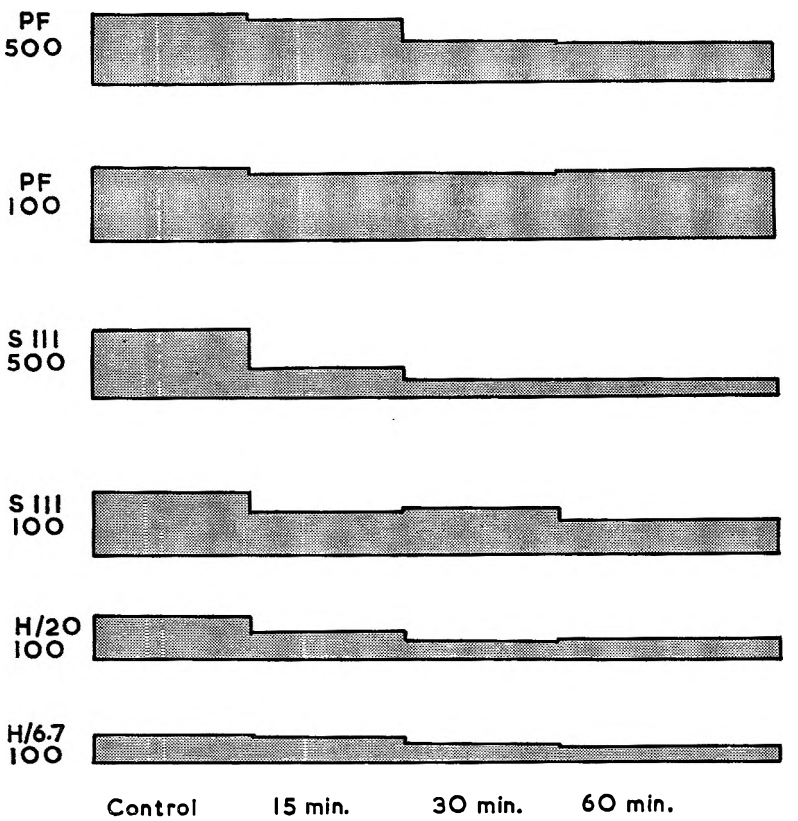

FIG. 1. The effects on the colour intensity of lesions produced by PF, SIII, and histamine (H) before, and 15, 30, and 60 min. after the intraperitoneal injection of salicylate in doses of 500 or 100 mg./kg. The thickness of the columns indicates the intensity of blueing in arbitary units. The sizes of the lesions were unaffected and are not shown.

produced by both doses of histamine. However, mottling occurred in about half of the lesions produced by histamine, both in control animals receiving saline and in test animals before the salicylate had been given. The administration of salicylate did not increase the frequency of mottling in the histamine-induced lesions.

Discussion

The results show that salicylate, but not DNP, affects anaphylactic capillary permeability in the guinea-pig. The inhibitory action of salicylate on these allergic lesions as well as in the erythema reaction (Adams and Cobb, 1958) must therefore be mediated by a mechanism other than an uncoupling action on oxidative phosphorylation processes. It was evident (Fig. 1) that an interval of 15 min. between the salicylate administration and the challenging dose of antigen sufficed to produce a reduced leakage of the circulating dye. The sizes of the lesions were not altered by the salicylate showing that the drug did not affect the sensitisation of the tissue by the antibody.

The present work has attempted to define the mode of action of salicylate more closely by using guinea-pig permeability factor and histamine in conjunction with the reversed passive cutaneous anaphylaxis reaction. The essential lesion in the cutaneous anaphylaxis reactions is an increased capillary permeability. Salicylate could reduce increased capillary permeability by a number of mechanisms. A suppression of the formation of antibody to the antigen is an unacceptable explanation because salicylate is effective both in the passive Arthus reaction (Smith and Humphrey, 1949) and in the present work, where preformed antibodies are injected in the animal. A dissociation of the antigen-antibody combination is unlikely since, although this has been reported to occur under *in vitro* conditions (Friend, 1953), it necessitated relatively large concentrations of salicylate and may have involved denaturation of the proteins. A further possibility is that salicylate stimulates the adrenal cortex and that adrenal corticosteroids are the effective agents in reducing capillary permeability but the balance of the evidence is against this interpretation (Spector, 1958). Adrenal medullary stimulation by salicylate leading to an increased secretion of adrenaline must also be considered as a possible mechanism. However it is not certain that adrenaline would diminish increased capillary permeability (Spector, 1958) and a determination of blood glucose levels of the salicylate-treated animals in the present work did not detect a hyperglycaemic response characteristic of adrenal medullary stimulation. There remain the possibilities that salicylate either prevents the antigen-antibody combination from exerting its effects on the capillary wall, whether this be direct or indirect, as by releasing chemical mediators, or that salicylate depresses the reactivity of the capillary wall to stimuli which increase permeability.

The present results would appear to support the penultimate hypothesis in that the effects of the antigen (SIII) and histamine were reduced by salicylate whereas that of guinea-pig permeability factor was not. If salicylate caused a general depression of reactivity of the capillary wall to stimuli capable of increasing permeability, then it would also be expected to inhibit the action of PF. However, as suggested by Spector (1958) for corticoids, it may be that salicylate may affect the reactivity of the capillary wall to some but not all stimuli which increase permeability. Mill and others (1958) have also reported that the effectiveness of human PF factor was reduced by previous admixture with salicylate. However, a direct

in vitro combination between the two materials cannot be excluded and their conditions and salicylate concentrations are not comparable with those used in the present *in vivo* experiments.

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THE PHARMACOGNOSY OF THE ROOT OF *RAUWOLFIA LIGUSTRINA* ROEM. AND SCHULT.

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The morphological and anatomical characters of *Rauwolfia ligustrina* roots are described and compared with those of other *Rauwolfia* species. The characters of the stem, particularly in so far as they concern its detection when mixed with root, are recorded. Analytical studies on individual plants indicate that reserpine is confined almost entirely to the root-bark, the concentration rapidly decreasing towards the crown and becoming negligible in the stems. Samples of root from different localities may vary greatly in reserpine content.

Recent investigations (Woodson, 1957; Rao, 1956) have suggested that some 34 species of *Rauwolfia* are indigenous to tropical and sub-tropical America ranging from *R. linearifolia* Brit. and Wils., a small plant of up to 50 cm. in height to *R. praecox* K.Sch., a tree of up to 30 metres. Two species are of widespread distribution, *R. tetraphylla* L. which is found in the Antilles, Central America, Colombia, Ecuador, Peru and Venezuela and *R. ligustrina* R. and S. having a similar geographical distribution. They differ in the number of leaves at each node and the length of the inflorescence relative to the associated large leaf (Rao, 1956). *R. tetraphylla* L. (synonyms: *R. canescens* L., *R. heterophylla* R. and S., *R. hirsuta* Jacq.) (Woodson, 1957; Rao, 1956) is a commercial source of the rauwolfia alkaloids. The pharmacognosy of the roots has been studied by Youngken (1954, 1955) (*R. heterophylla* and *R. canescens*), Esdorn and Nolde (1955) and Esdorn and Schmitz (1956) (*R. heterophylla* and *R. canescens*), and Dillemann and Paris (1958) (*R. tetraphylla*). *R. ligustrina* R. and S. is considered by Rao (1956) to include the species *R. ternifolia* H.B.K., *R. parvifolia* Bert. ex Spreng., *R. parvifolia* var. *cubana* A.DC., *R. parvifolia* var. *tomentella* Muell.-Arg., *R. alphonsiana* Muell.-Arg. and *R. indecora* Woodson, the differences in leaf characteristics originally used as the basis for the establishment of a new species being unreliable. The native names for *R. ligustrina* include Brazil–Paratudo, Mamao de Sapo; El Salvador—San Jose; Mexico—Chirillo, Veneno; Colombia–Contra, Venenito. The plant's natural habitat is chiefly moist situations, near the seashore in coastal thickets, on the plains near rivers, in savannas and wet meadows, with an altitude range of from sea-level to about 1,000 metres. In common with other species of the genus, *R. ligustrina* has been the subject of considerable research and at least 21 different alkaloids have been reported in roots described as *R. ternifolia*, *R. indecora* or *R. ligustrina* (Ishidate, Okada and Saito, 1955; Cardoso and Venâncio, 1956; Korzun, St. André and Ulshafer, 1957; Müller, 1957). Fernandez (1958) estimated the total alkaloidal content of fresh roots of *R. ligustrina* as 2·6 per cent and the reserpine content as 0·03 per cent.

As *R. ligustrina* contains reserpine in the roots, the preparation of a systematic description of the roots seemed desirable, together with an investigation of the distribution of reserpine in the main axis and in samples from different sources. A more limited study of the stem was undertaken when it was observed that its total alkaloidal content was very different to that of the root and that its addition to commercial material would be undesirable.

Plant Material

The following specimens of *R. ligustrina* were utilised in this investigation:

1. Dried plants consisting of roots, rootstock and the attached aerial stems, collected in Trinidad by Dr. F. J. Simmonds, Imperial College of Tropical Agriculture, Trinidad.

2. Large roots and rootstocks with attached stem-bases, collected in N.E. Brazil, identified by Dr. Hürlimann and donated by Ciba Ltd., Basle.

3. Roots of plants collected in Brazil, identified by Dr. A. Ducke and presented by Professor Francisco José de Abreu Matos of the University of Céara, Brazil.

4. Powdered root from Professor H. T. Cardoso, Instituto Oswaldo Cruz, Rio de Janeiro.

5. A small root segment, authenticated by Dr. A. Ducke and donated by Professor R. E. Woodson, Jnr.

Macroscopy

Large roots may be up to 20 cm. long and taper from 10 cm. diameter at the crown to 1·5 cm. in the lower portions of the root system; they bear numerous lateral roots, circular scars and stumps which vary from about 1 mm. to 3 cm. in diameter. The small wiry roots are brittle and the large ones tough and difficult to fracture, eventually breaking with a fracture short in the bark and splintery in the wood. The outer yellowish-brown cork, slightly darker in colour in Brazilian than Trinidad samples is marked by short longitudinal discontinuous ridges and furrows; in old samples it may be scaly and occasionally exfoliates to reveal the orange-brown phloem or yellowish wood. A smoothed transverse surface of a root exhibits a yellow-brown bark rarely exceeding 1·5 mm. in thickness and a yellowish finely radiate, porous wood, showing in the larger pieces faint growth rings. Large roots may show a distinctly darker heartwood or may occasionally be hollow resulting from decay or insect attack. When examined under screened ultra-violet light the cork appears a velvet-brown colour, the phloem fluoresces a yellowish-brown and the xylem pale yellow. The odour is slight and the taste, particularly of the bark, bitter.

Microscopy

Variation in the general appearance of transverse sections of roots is due mainly to the presence or absence of sclereids in the bark and of

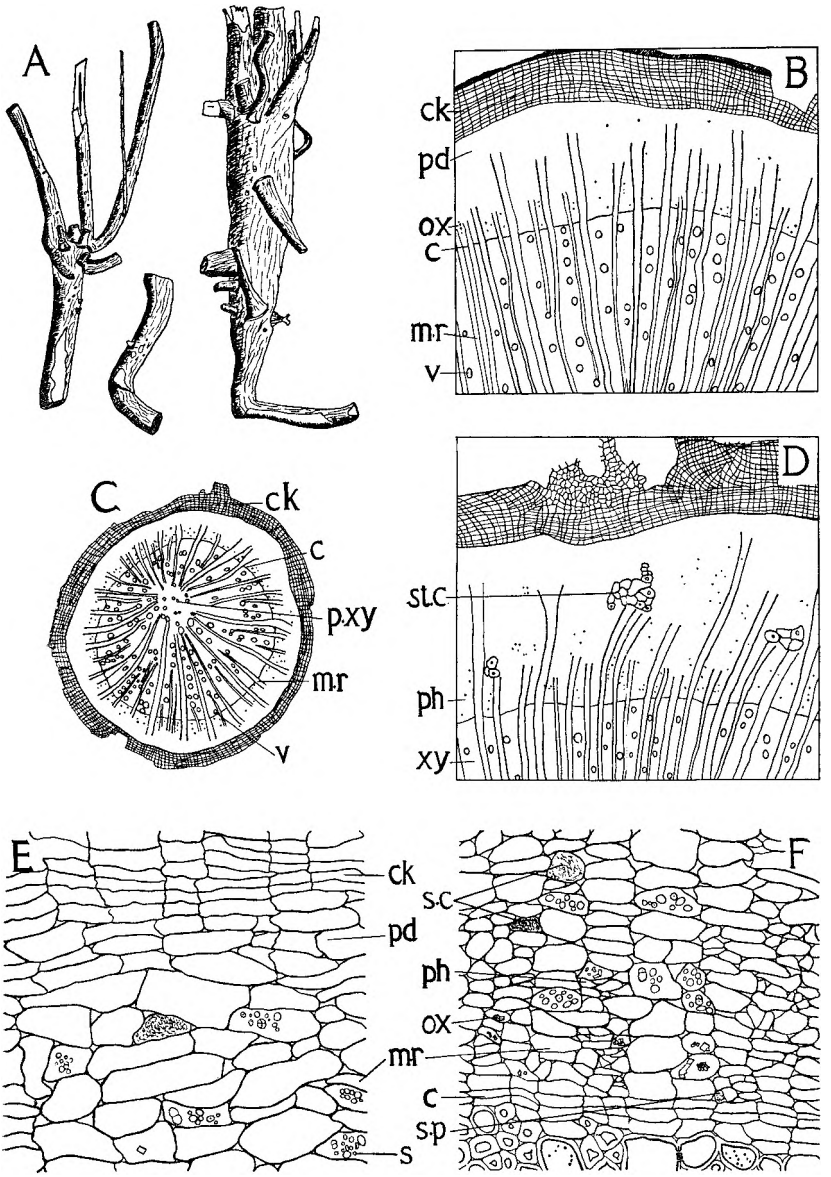

FIG. 1. *Rauwolfia ligustrina* Roem. and Schult. Root. A, roots and attached stems × ¼. B–D, general diagrams of transverse sections of roots. B, 5 mm. diameter × 25; C, 2 mm. diameter × 15; D, 1·8 cm. diameter × 25. E, F, transverse sections of bark of root of diameter 1·5 cm., both × 200. E, outer tissues; F, inner phloem. c, cambium; ck, cork; m.r, medullary rays; ox, calcium oxalate crystal; pd, phelloderm; ph, phloem; p.xy, primary xylem; s, starch; s.c, secretion cell; s.p, sieve plate; st.c, stone cell; v, vessel; xy, xylem.

a rhytidoma. Sclereids occur often in the larger roots but are commonly absent in roots of diameter less than 8 mm. Externally there is frequently a brown layer of collapsed cells and the periphery of the cork layer may show radial clefts containing small areas of ovoid or polygonal, lignified cells. Large roots may possess a rhytidoma-like structure, consisting of oval, tangentially elongated groups of brown lignified ovoid cells and occasional well formed sclereids, surrounded by the cork layer. The cork cells, which usually occur in a single layer of up to about 40 radial cells are suberised and usually, although not invariably unlignified; in surface view they are polygonal, R = 8 to **12** to **20** to 28 μ, T = 12 to **24** to **44** to 68 μ and L = 12 to **20** to **48** to 60 μ (Fig. 1, E; 2, A; 4, ck).

The phellogen consists of a few layers of radially compressed cells (Fig. 2, A). The phelloderm may possess up to about 15 radial rows of cells, the inner layers of tangentially elongated, oval cells being usually displaced by sliding growth; the walls are sometimes collenchymatous. R = 16 to **20** to **32** to 48 μ, T = 16 to **32** to **56** to 80 μ and L = 20 to **36** to **60** to 80 μ (Fig. 1, E; 2, A). Calcium oxalate, usually as twinned prisms, and starch, as single or compound granules of up to four components, occur in variable amounts. Single starch granules are spherical or ovoid, 3 to **4** to **12** to 20 μ in diameter with central hila as points or stellate clefts.

Internal to the phelloderm is a relatively wide zone of secondary phloem composed of radial groups of sieve tubes and companion cells separated by phloem parenchyma, medullary rays, secretory cells and sclereids. The sieve tubes have thin cellulosic walls, a wide lumen and possess sieve plates on the end walls (Fig. 1, F; 2, B; 5,C), the companion cells are narrower and the phloem parenchyma cells are of variable proportions, being up to five times as long as they are wide. Two types of cell are present in the medullary rays. There is a central core of relatively small isodiametric, or slightly radially elongated procumbent cells, 3 to 5 cells wide and up to 15 cells high, with upper and lower extensions of larger erect cells which may be continuous with the cells of the medullary rays immediately above or below (Fig. 1, F; 2, A,B,D; 5, J). For the procumbent cells R = 16 to **20** to **32** to 40 μ, T = 12 to **20** to **28** to 40 μ, L = 12 to **16** to **28** to 32 μ and for the erect cells R = 12 to **16** to **28** to 32 μ, T = 20 to **28** to **44** to 52 μ, L = 28 to **36** to **56** to 60μ. Sclereids may occur in the outer phloem, either as solitary cells or as groups of up to about 4, 7 and 10 cells in the radial, tangential and vertical planes respectively (Fig. 2, C). In longitudinal sections the apices of the groups may be prolonged by narrow elongated sclereids with ill-defined cross walls. In outline, isolated cells may be circular, polygonal or elongated with a sinuous margin; some have solid projections at the apices. Length = 36–**60**–**160** to 280 μ, breadth = 20 to **32** to **72** to 116 μ. Well developed sclereids possess irregular, sometimes branched lumina, with funnel-shaped pits and stratified, lignified walls (Fig. 4, st.c; 5, M). Secretory cells occur with variable frequency in the phelloderm and phloem, being completely absent in some roots and very frequent in others. The contents stain orange-brown with a solution of iodine and

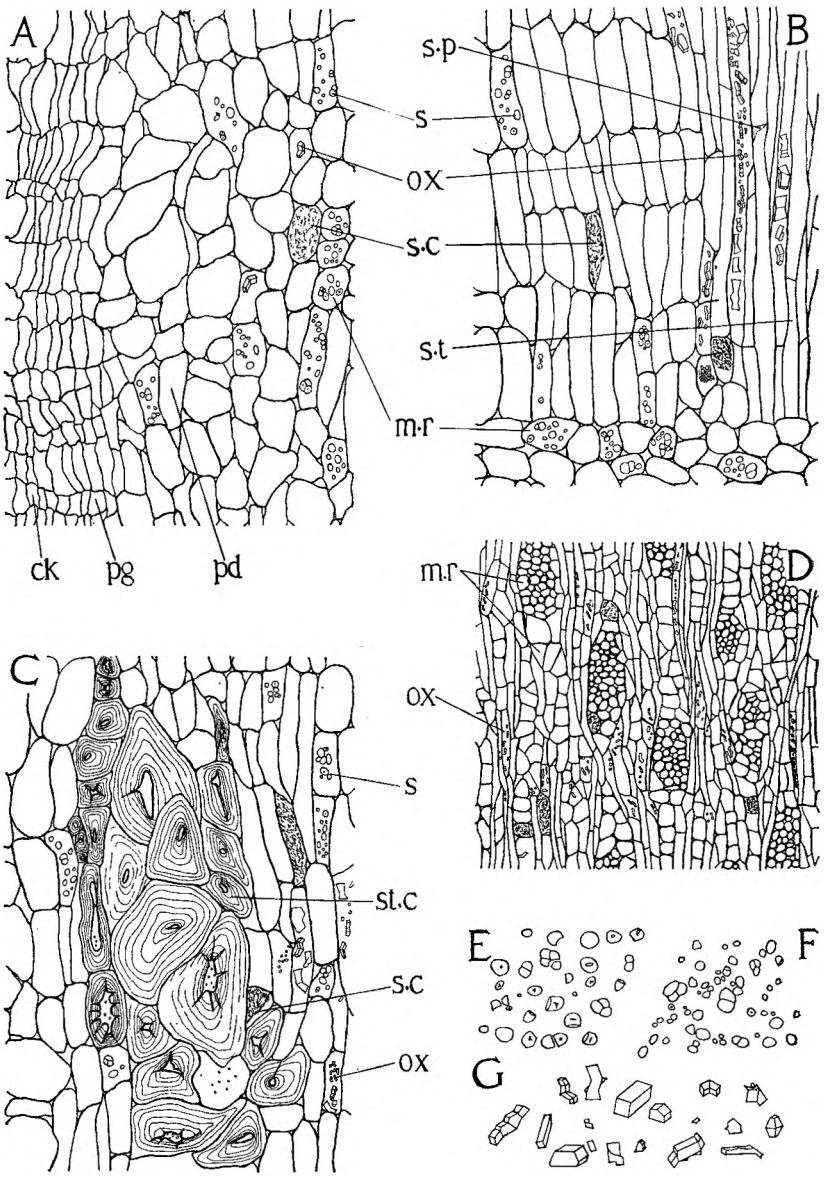

FIG. 2. *Rauwolfia ligustrina* Roem. and Schult. Root bark. A, radial longitudinal section of the outer tissues, root diameter 1 cm. B, ditto inner tissues. C, ditto phelloderm and outer phloem, root diameter 1·8 cm. All × 200. D, tangential longitudinal section of inner phloem, root diameter 1 cm. × 50. E, F, starch grains of wood and bark respectively. G, calcium oxalate crystals from the bark, × 200. ck, cork; m.r, medullary ray; ox, calcium oxalate crystal; pd, phelloderm; pg, phellogen; s, starch; s.c, secretion cell; s.t, sieve tube; st.c, stone cell; s.p, sieve plate.

faintly pink with Sudan III solution (Fig. 1, E,F; 2, A,B,C). Individual cells can be isolated from alkali macerates (Fig. 4, s.c).

Starch granules, similar in size and composition to those in the phelloderm, occur throughout the phloem; they are usually most abundant in the outer region but in some roots may be almost entirely absent (Fig. 1, E,F; 2, A,B,C,F; 5, J). Calcium oxalate occurs abundantly throughout the phloem and is most evident in longitudinal sections in which long vertical rows of crystals may be observed as elongated monoclinic prisms, often twinned on one or more of the hemi-pyramid faces. Large well-formed prisms may be embedded in masses of sandy crystals. When examined in polarised light the twin crystals show a bicolouration effect. For the distinct prisms, length = 6 to **16** to **28** to 36 μ, breadth = 3 to **4** to **8** to 12 μ (Fig. 2, G).

The completely lignified, radial xylem consists of vessels, tracheids, fibres, xylem parenchyma and medullary rays (Fig. 3, A,B,C). The vessels occur solitary or in pairs and in transverse section are radially elongated or rounded, R = 32 to **48** to **64** to 104 μ, T = 28 to **40** to **56** to 80 μ. Isolated vessel segments show transverse or oblique perforation plates also obvious in radial longitudinal sections and, characteristic elongated apices. The walls possess numerous, alternately arranged, bordered pits (Fig. 3, C; 4, V). Yellow and orange tyloses, staining red with phloroglucinol and concentrated hydrochloric acid are frequent; in the larger roots the tyloses may contain masses of starch. Other vessels are occluded with amorphous brown or black material. The apotracheal xylem parenchyma is seen in transverse sections as isolated cells or more usually, short uniseriate, rarely biseriate tangential rows connecting the vessels and medullary rays and, in longitudinal sections as vertical rows of cells (Fig. 3, A,C). Simple, oval or rounded pits are present on the walls except with cells adjacent to vessels when the pits are bordered. R = 12 to **20** to **28** to 40 μ, T = 8 to **16** to **24** to 36 μ and L = 40 to **72** to **94** to 192 μ. Numerous xylem fibres occur associated with the other wood elements and in transverse section appear either rounded or polygonal in outline with thick lignified walls. Fibres isolated by maceration are somewhat irregular in outline with apices which may be acute, obtuse or occasionally bifurcated. Some apices are formed by a cross wall set at an angle of about 60° to the longitudinal plane. The walls bear slit-like or funnel-shaped pits. Tracheids and intermediate fibre-tracheids are also present (Fig. 3, B, C; 4, f, tr). Fibres isolated from wood macerated in potassium chlorate and concentrated hydrochloric acid measured 310 to **620** to **1,050** to 1,387 μ in length and 8 to **16** to **24** to 36 μ in breadth. The heterogeneous medullary ray cells resemble those of the bark being composed of a core of small, radially elongated procumbent cells, three to six cells wide and up to 15 cells in height, with upper and lower extensions of elongated erect cells which extend vertically to connect with other groups of procumbent cells. Groups of the smaller procumbent cells, when viewed in tangential longitudinal sections, where they appear to be almost circular in outline, are partially or completely enclosed by the larger erect cells. For the procumbent cells, R = 20 to **44** to **84** to 128 μ,

T = 8 to **12** to **20** to 28 μ and L = 8 to **12** to **24** to 40 μ; for the erect cells R = 16 to **28** to **44** to 56 μ, T = 20 to **24** to **48** to 64 μ and L = 28 to **40** to **72** to 104 μ (Fig. 3, A, B, C; 5, E, L). Single prisms of calcium oxalate are present mainly in the procumbent cells of the medullary rays. Length of prisms = 12 to **20** to **24** to 40 μ, breadth = 8 to **12** to **20** to 36μ.

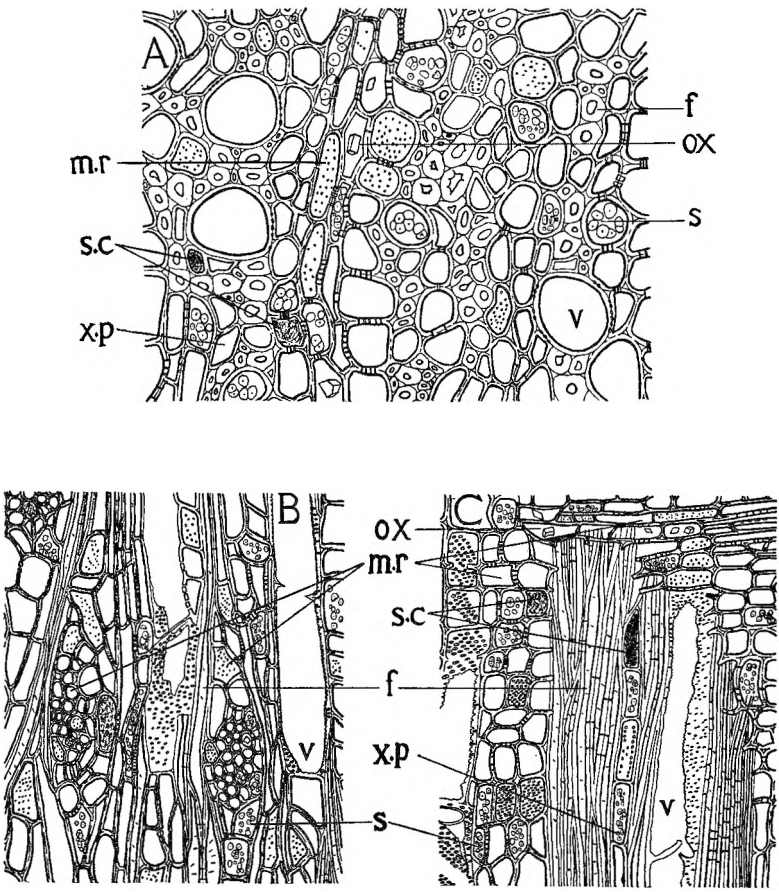

FIG. 3. *Rauwolfia ligustrina* Roem. and Schult. Root wood. Diameter of root 3 cm. A, transverse section × 200. B, tangential longitudinal section × 100. C, radial longitudinal section × 100. f, fibre; m.r, medullary ray; ox, calcium oxalate crystal; s, starch; s.c, secretion cell; v, vessel; x.p, xylem parenchyma.

Xylem parenchyma and medullary ray cells may contain material which with solution of iodine stains orange-brown to purple and other medullary ray cells may possess deep brown amorphous contents. Rounded, oval and plano-convex starch grains, diameter 3 to **8** to **12** to 24 μ, and compound grains of up to five components occur in variable amounts. The hila, as central points or stellate clefts, are more obvious on these grains than on those from the phloem and phelloderm (Fig. 2, E).

POWDERED ROOT

The principal histological features of the powdered root are:

1. Masses of thin-walled, yellow cork cells, polygonal in surface view (Fig. 5, B).

2. Thin-walled cellulosic elements of the phelloderm, phloem and medullary rays containing starch grains, calcium oxalate crystals and resinous material (Fig. 5, C, F, G, J, K).

3. Almost circular or elongated stone cells, either singly or in groups. Length 36 to **60** to **160** to 280 μ, breadth 20 to **32** to **72** to 116 μ (Fig. 5, M).

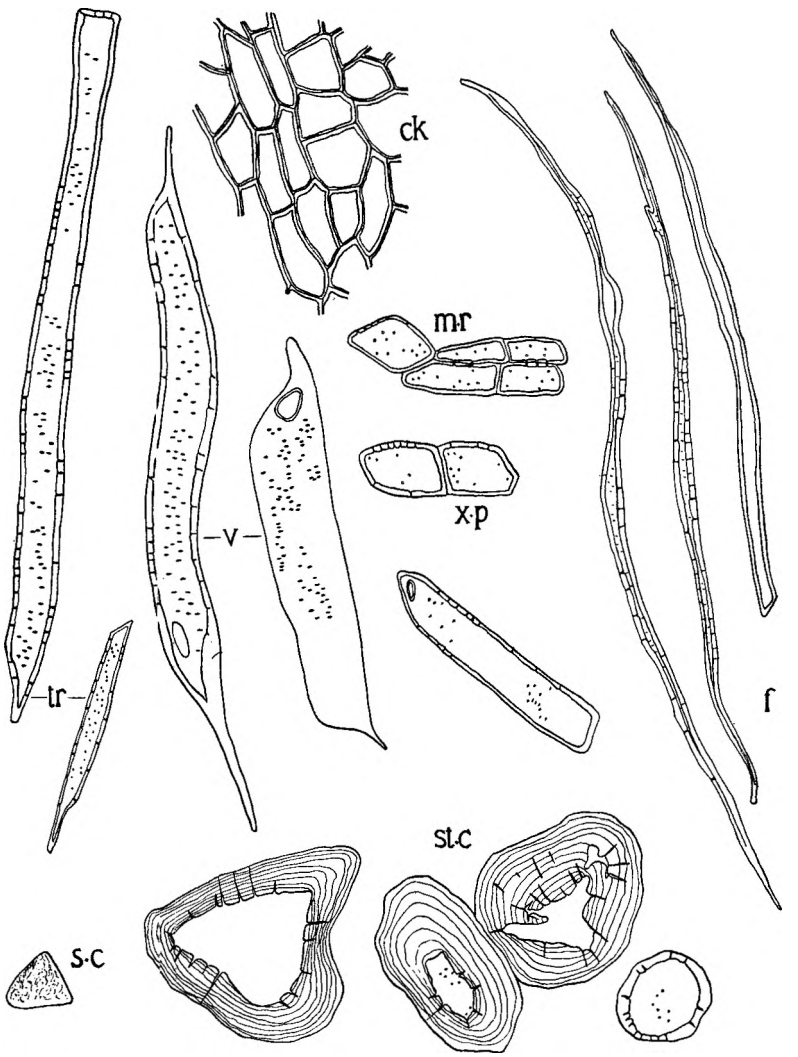

FIG. 4. *Rauwolfia ligustrina* Roem. and Schult. Isolated elements of the root. ck, cork cells; m.r, medullary ray cells; s.c, secretion cell; st.c, stone cell, tr, tracheids; v, vessels; x.p, xylem parenchyma; all × 200. f, xylem fibres × 100.

4. Large quantities of lignified xylem elements composed of fibres, elongated xylem parenchyma cells, two types of medullary ray cell and a relatively few vessels with bordered pits. The parenchyma and medullary ray cells usually contain starch grains (Fig. 5, A, D, E, H, I, L).

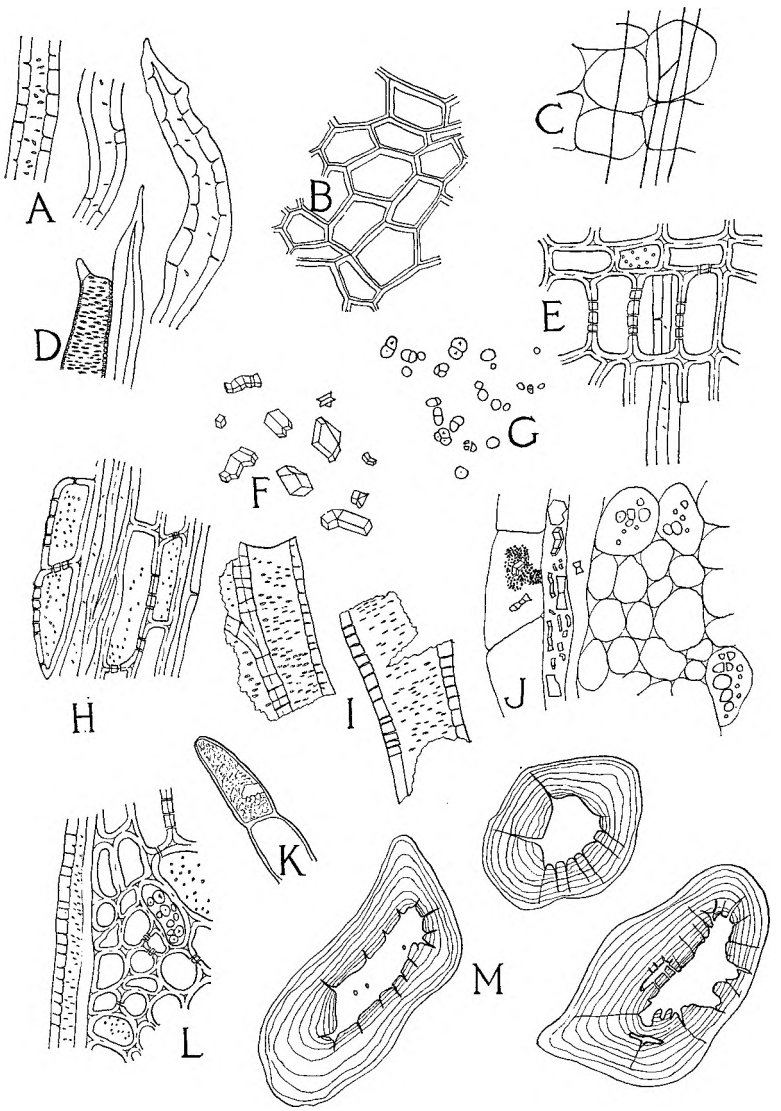

FIG. 5. *Rauwolfia ligustrina* Roem. and Schult. Elements of the powdered root. A, fragments of xylem fibres. B, cork cells. C, portion of medullary ray (as in radial longitudinal section) and sieve tubes. D, vessel and fibre. E, medullary ray cells (as in radial longitudinal section). F, calcium oxalate crystals. G, starch grains. H, xylem parenchyma and fibres. I, vessel fragments. J, medullary ray cells (as in tangential longitudinal section), parenchyma and sieve tube, K, secretion cells. L, medullary ray (as in tangential longitudinal section). M, stone cells. All × 200.

STEM

The stems are cylindrical and, in the samples examined, 0·5 to 3 cm. in diameter at the junction with the rootstock. The buff coloured cork, showing numerous lighter circular scars, is somewhat smoother than that of the root with continuous slight furrows and ridges along its length. A smoothed transverse surface of the stem is similar to that of the root but shows in addition a pith, growth rings which are more distinct. The taste, particularly of the bark, is bitter.

Transverse sections of the stem show variations depending on the presence or absence of sclereids in the phelloderm, outer phloem and pith and upon the number of pericyclic fibres. The brown cork consists of radially arranged, usually unlignified, suberised tabular cells, polygonal in surface view, R = 6 to **8** to **19** to 23 μ, T = 12 to **19** to **38** to 46 μ and L = 15 to **19** to **38** to 55 μ (Fig. 6, A), and in older stems there are intermittent tangentially, elongated groups of lignified cells surrounded by cork cells. The phelloderm is a narrow layer of up to about 12 cells in radial width. Corners of individual cells may be lignified and contiguous cell walls may possess brown unlignified thickening giving the appearance of tangential brown bands in transverse section. R = 11 to **15** to **30** to 38 μ, T = 19 to **34** to **62** to 76 μ and L = 15 to **27** to **40** to 46 μ (Fig. 6, B). In transverse section the pericycle is indicated by scattered, highly refractive fibres, circular, oval or subreniform in outline and giving either no or a slight reaction for lignin. Diameter = 16 to **19** to **27** to 31 μ. In longitudinal sections or in macerated material, the fibres show intermittent swollen portions, 23 to **31** to **58** to 105 μ in width (Fig. 6, B, I). Groups of sclereids, similar to those found in the root may occur in the phelloderm and outer phloem (Fig. 6, G, H) and a crystal sheath occasionally surrounds them.

The inner phloem consisting of thin-walled sieve tubes, companion cells and phloem parenchyma together with the medullary rays resemble the corresponding tissue of the root (Fig. 6, B). The rows of calcium oxalate crystals, often embedded in granular material, are again evident. Starch occurs in variable amounts and is completely absent from some stems. Single grains are circular, ovoid or plano-convex in outline with a central hilum appearing as a point or stellate cavity. Diameter = 3·8 to **6** to **14** to 19 μ. Compound grains of up to five components are frequent. Secretion cells, having somewhat granular contents which stain yellow-brown or purplish with an iodine solution, occur throughout the phloem and phelloderm (Fig. 6, B). The number of cells containing such material was very variable in the different plants examined. Latex ducts occur infrequently as circular or oval cavities and in the specimens examined were much more numerous in the pith.

The stem-wood resembles the root-wood in structure and cell contents (Fig. 7, A, B, C). The main differences lie in the diameter of the vessels, R = 24 to **32** to **48** to 56 μ, T = 20 to **28** to **44** to 60 μ, the somewhat larger fibres, length = 474 to **632** to **1027** to 1454 μ, breadth 11 to **15** to **21** to 30 μ and the generally smaller cells of the wood parenchyma

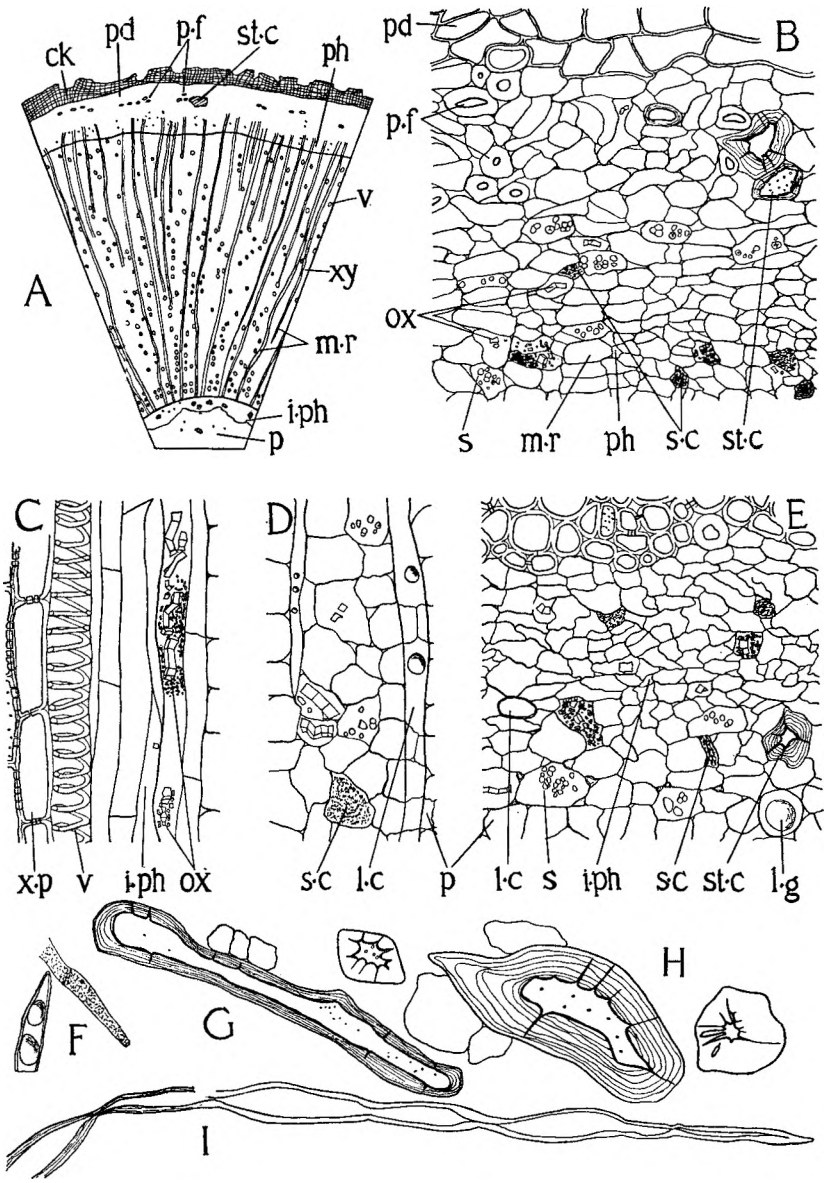

FIG. 6. *Rauwolfia ligustrina* Roem. and Schult. Stem. A, general diagram of transverse section × 15. B, transverse section of phelloderm and phloem. C, radial longitudinal section of xylem and internal phloem. D, ditto pith. E, transverse section of xylem, internal phloem and pith. B–E, all × 200. F–I, isolated elements of bark and pith. F, latex tissue × 200. G, elongated stone cell × 100. H, stone cells × 200. I, pericyclic fibre × 100. ck, cork; i.ph, internal phloem; l.c, latex canal; l.g. latex globule; m.r, medullary ray; ox, calcium oxalate crystal; p, pith; pd, phelloderm; p.f. pericyclic fibre; ph, phloem; s, starch; s.c, secretion cell; st.c, stone cell; v, vessel; x.p, xylem parenchyma; xy, xylem.

and medullary rays. Small groups of spiral vessels constitute the primary xylem (Fig. 6, C).

An unlignified central area consists of large parenchymatous cells of the pith and an internal peri-medullary phloem arranged either as an almost continuous circle or as isolated groups of elements. Calcium oxalate, similar to that of the outer phloem is present in vertical rows (Fig. 6, C, E). Latex tubes permeate between the pith cells, the contents appearing as refractive globules in Berlese mountant and staining yellow-brown with iodine solution. Isolated sclereids or groups of up to three cells radially, five tangentially and eight vertically occur in the pith and are similar to those of the outer phloem (Fig. 6, D, E, F).

Distribution of Reserpine throughout the Main Axis

For the determination of the reserpine distribution, roots and stems for assay were divided into segments corresponding with the numbered

TABLE I

Reserpine content of *R. ligustrina* Roem and Schult

	Plant	Segment No. of bark, Fig. 8	Reserpine per cent		Plant	Segment No. of bark, Fig. 8	Reserpine per cent
A.	Main root	1	0·05	D.	Main root	1	0·32
	" "	2	0·05		" "	2	0·32
	Crown	3	0·03		" "	3	0·44
	"	4	0·01		" "	4	0·46
	Stem	5	0·01		" "	5	0·21
	"	6	0·01		" "	6	0·22
	"	7	0·02		" "	7	—
	"	8	0·02		" "	8	0·19
	"	9	0·01		" "	9	—
B.	Main root	1	0·02		" "	10	0·16
	" "	2	—		" "	11	0·18
	Crown	3	0·01		" "	12	0·18
	"	4	0·01		" "	13	0·13
	Stem	5 to 12	All <0·01		" "	14	0·11
C.	Main root	1	0·06		" "	15	0·09
	" "	2	0·05		Crown	16	0·02
	" "	3	0·05		"	17	0·01
	Crown	4	0·02		"	18	0·01
	"	5	0·01		Lateral root	19	0·29
	"	6	<0·01		" "	20	0·21
	Lateral root	7	0·04		" "	21	0·18
	" "	8	0·09		" "	22	0·22
	" "	9	0·02	E.	Main root	1	0·01
	Stem	10	0·03		" "	2	0·11
	"	11	0·01		" "	3	0·01
					Crown	4	<0·01
					Stem	5	0·01
					"	6 to 12	<0·01
					Lateral root	13	0·02

sections shown in Fig. 8. As preliminary experiments employing paper chromatography had shown the reserpine content of the wood of both roots and stems to be negligible, quantitative determinations were made on the bark only, using a modification of the fluorimetric procedure described for the evaluation of *R. caffra* (Court, Evans and Trease, 1958). With *R. ligustrina* paper chromatography indicated that the reserpine fractions contained other fluorescent substances which interfered with the assay. It was therefore necessary to submit each fraction to paper chromatography and to elute the reserpine band from the chromatogram

with 5N acetic acid. These operations were carried out in the dark and the conditions were standardised. A standard curve was prepared by subjecting pure reserpine to the same procedure. Any rescinnamine present, which under these conditions of chromatographic separation has an R_F value similar to that of reserpine, would also be determined by the method and is consequently included in the reserpine figures. The results of the assays are recorded in Table I. Two other Brazilian root samples were analysed, the bark of one from a root 1 cm. diameter contained 0·17 per cent reserpine, and the other consisting of powdered whole roots, 0·13 per cent.

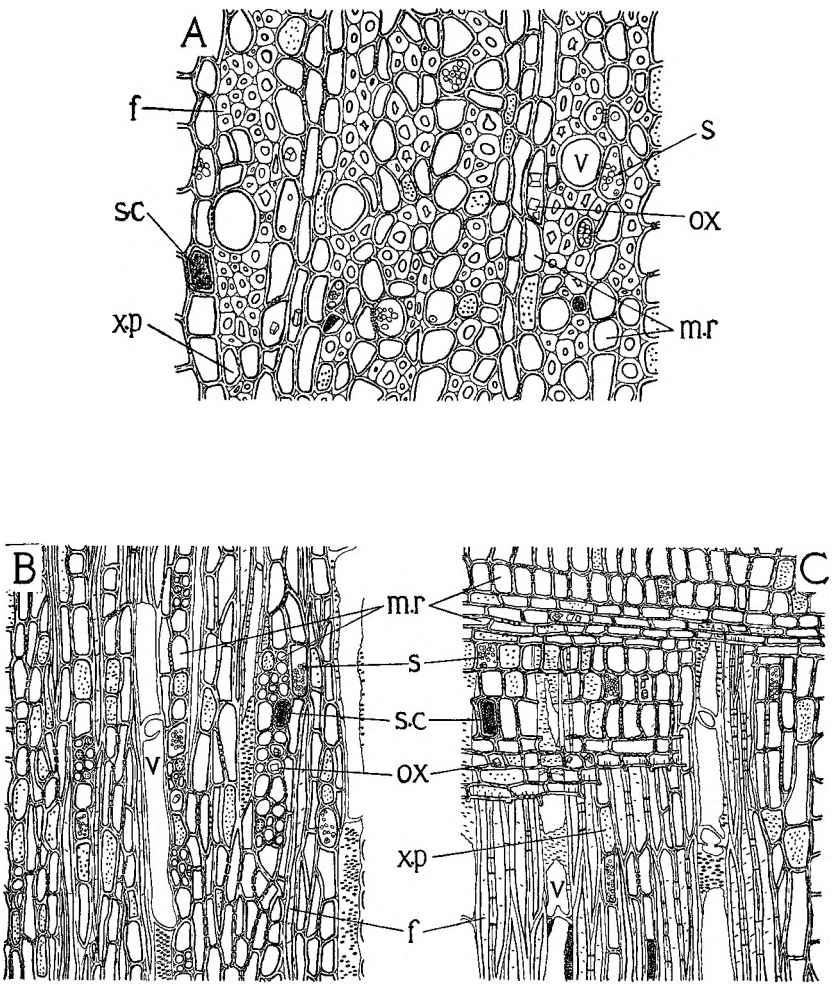

FIG. 7. *Rauwolfia ligustrina* Roem. and Schult. Stem wood. Diameter of stem, 8 mm. A, transverse section × 200. B, tangential longitudinal section × 100. C, radial longitudinal section × 100. f, fibre; m.r, medullary ray; ox, calcium oxalate crystal; s, starch; s.c, secretion cell; v, vessel; x.p, xylem parenchyma.

Discussion

The histological characters of *R. ligustrina* are typical of the family Apocynaceae, characteristic features being latex canals and pericyclic fibres in the stem and vessel segments with peg-like projections and large communication pores in the root and stem woods. Characteristic of the genus *Rauwolfia* are the non-septate fibres, phloem sclereids,

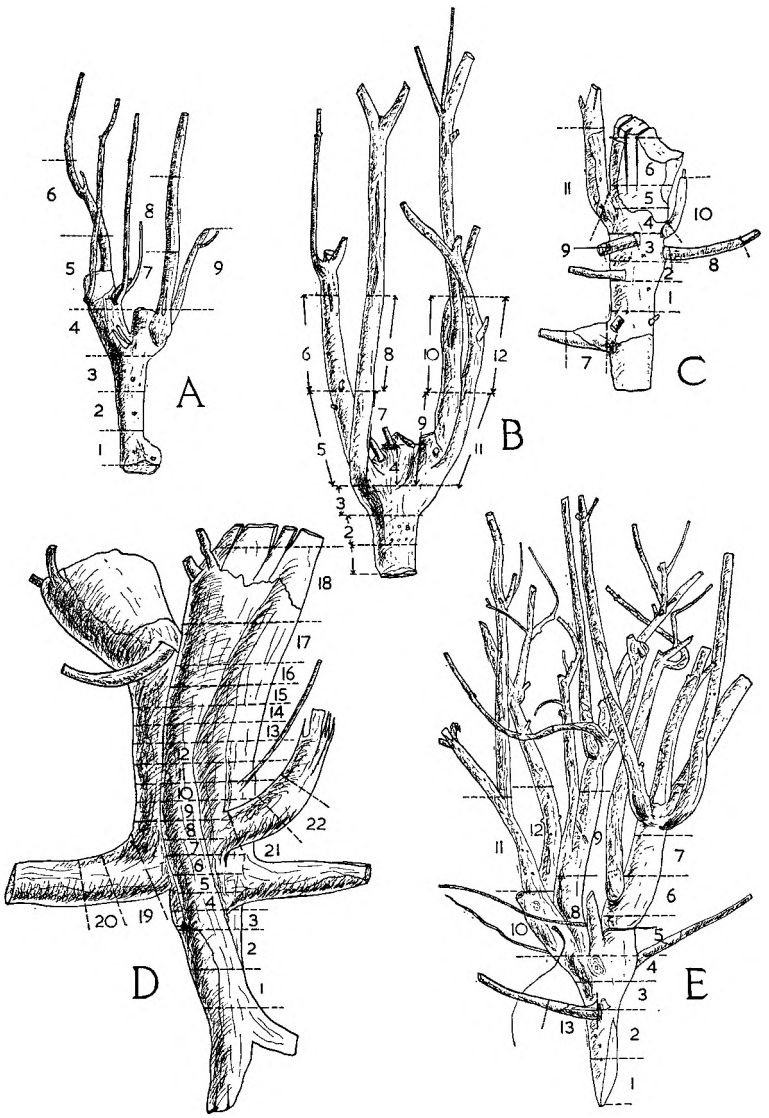

Fig. 8. *Rauwolfia ligustrina* Roem. and Schult. Roots, crowns and attached stems × ¼. Numbers indicate segments analysed for reserpine (see Table I). A, B, E Trinidad samples. C, D Brazilian samples.

laticiferous tubes and heterogeneous rays. The anatomy of the root shows features that are intermediate between those described by Woodson (1957) as being characteristic of arboreal and those of subherbaceous tendency; these include distribution of phloem and xylem, nature of phloem components and vessel diameter.

A considerable variation in cell contents, particularly with regard to starch and resinous secretory material was noted between various specimens, similar variations having been recorded for *R. caffra* (Court and others, 1958) and *R. vomitoria* (Evans, 1956). Woodson has suggested that the starchy tyloses of the vessels of *R. ligustrina* might be used as a diagnostic feature of the root. We have, however, by the examination of more samples than were available to Woodson (private communication), found that such tyloses were only present in the larger roots and were not of such uniform occurrence as to be of great diagnostic value.

R. ligustrina can be readily differentiated from *R. serpentina* as the latter has small vessels of about 36 to 54 μ diameter, no sclereids and an extensive stratified cork consisting of alternating zones of lignified and non-lignified cells (Wallis and Rohatgi, 1949). *R. vomitoria* root possesses larger and more sclereids than *R. ligustrina* root, length 28 to 288 μ and breadth 14 to 56 μ, and vessels of greater diameter, 36 to 180 μ (Evans, 1956). The difference between the roots of *R. ligustrina* and those of the closely related *R. tetraphylla* is less marked and comparison is complicated by the fact that *R. canescens*, *R. heterophylla* and *R. hirsuta* although considered synonymous on gross morphological characters (Woodson 1957; Rao, 1956), do differ in certain histological details (Youngken, 1954, 1955; Dillemann and Paris, 1958; Paris, private communication), particularly vessel size. Like *R. ligustrina*, all have a single unstratified cork layer, although large roots of *R. ligustrina* may exhibit a rhytidoma. Sclereids are present in these species and elongated forms, intermediate between sclereids and fibres, are recorded for *R. heterophylla* and *R. canescens*; these are found only rarely in *R. ligustrina* as are the irregularly lobed sclereids having pointed projections and interrupted lumina which Youngken records (1954, 1955) for *R. canescens*. Compared with the results of Dillemann and Paris (1958), the vessel diameters of *R. ligustrina* (32–**48**–**64**–104 μ) are larger than those of *R. canescens* (56–73 μ maximum sizes) and slightly smaller than those of samples labelled *R. tetraphylla*, *R. heterophylla* and *R. hirsuta* (maximum sizes 100 to 120 μ). Intermediate forms of *R. tetraphylla* would therefore be impossible to distinguish from *R. ligustrina* on this basis. Other S. American species, including *R. biauriculata* Muell.-Arg., *R. cubana* A.DC., *R. littoralis* Rusby, *R. nitida* Jacq. and *R. viridis* R. and S., having a similar habit and geographical distribution to *R. ligustrina* do not yet appear to have been examined in detail.

R. ligustrina stems admixed with root can be detected by the smaller vessels of the xylem, a pith possessing sclereids and latex tubes and, a ring of scattered pericyclic fibres either unlignified or slightly lignified, the individual fibres usually showing well marked swellings along their length.

The assays for individual plants indicate that the reserpine content of the root bark increases with distance from the crown and, in one instance (Plant D), reaches a maximum at about 15 cm. below the bifurcation of the crown and then decreases. From the crown to the stem-bases the reserpine content falls to a negligibly low proportion. Should *R. ligustrina* be used as a commercial source of reserpine, it would be desirable that roots only be collected. It is evident, too, that some plants are richer in alkaloids than others, thus Plants A, B and E would be of little economic use but in contrast the powdered root from Brazil supplied by Professor H. T. Cardoso has a very high reserpine content considering that the bark, which contains the majority of the alkaloids, would form probably only 5–10 per cent of the powder. Thus factors such as habit, time of collection and variety may have a marked effect on the pharmacological activity of the plant.

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A NOTE ON IRRADIATED HEPARIN: SOME BIOLOGICAL AND CHEMICAL PROPERTIES

By S. S. Adams, B. V. Heathcote and P. E. Macey

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The effect of gamma radiation and high energy electrons on the anticoagulant and antilipaemic properties of heparin has been investigated. There was a fall in both activities, but the anticoagulant was reduced more readily than the antilipaemic. After electron bombardment the anticoagulant activities of two different batches of heparin were reduced almost identically, an unexpected finding since such a high degree of reproducibility is not possible with chemical degradation.

In the course of investigations into the effects of irradiation on the stability of a number of pharmaceutical products, it was noted by our colleague, Mr. K. L. Smith, that there was a decrease in the anticoagulant activity of heparin after electron bombardment. More recently Howe, Balazs and Laurent (1958) and Horne (1958) have briefly mentioned a similar finding, and the former workers have also shown that there is a decrease in anionic groups and an increase in reducing substances.

It therefore seemed of interest to examine more fully the effects of electron bombardment and of exposure to gamma rays on the biological properties of heparin, and in particular to observe any possible changes in the antilipaemic activity during these reactions. Zollner, Rothemund and Seitz (1954), by a chemical degradation of heparin, claimed to have reduced its anticoagulant activity to 12·5 per cent without affecting the antilipaemic activity. Houck, Morris and Lazaro (1957) were able by alkaline degradation to decrease the anticoagulant activity without altering the antilipaemic activity, but it is impossible to make any quantitative estimates from their results. A modified form of heparin with reduced anticoagulant activity but unchanged antilipaemic effects would be of interest as a potential therapeutic agent.

METHODS

Irradiation

Sodium heparin of B.P. quality, and a 25 per cent solution of sodium heparin in distilled water, were irradiated by gamma rays from a 100 curie ^{60}Co source. High energy electrons were produced by a 4MeV linear accelerator at the Atomic Energy Research Establishment, Wantage.

The gamma radiation dose rates were about 1,000 rads/min. and were measured with ferrous sulphate solution. The electron dose rates were about 3 megarads/min. and had been measured calorimetrically. Air cooling prevented the temperature from rising more than 10°.

Anticoagulant activity. This was determined *in vitro* by the sulphated whole blood method of Adams and Smith (1950).

Antilipaemic activity. We wish to emphasise that in this communication the term antilipaemic refers to the ability of heparin to liberate clearing

factor *in vivo*. A method based on the liberation of clearing factor in the rat was used. Rats were injected subcutaneously with heparin, and 1 hr. later were bled under ether anaesthesia from the carotid artery into ice-cooled oxalated tubes. Plasma was obtained by centrifugation at 1,500 r.p.m. for 30 min. at a temperature of 0 to 5°. One ml. aliquots of plasma were diluted with 1 ml. of distilled water and the temperature raised to 30°. 0·1 ml. of a 1 per cent dilution of "Ediol"* emulsion in water was then added, and the optical density immediately measured on a Hilger absorptiometer using a neutral filter and a 2 cm. cell. After incubation at 30° for 30 min. the optical density was measured again. The decrease in optical density after this incubation was taken as a measure of clearing factor activity in the plasma. By comparing the effects produced by three doses of the untreated heparin, 1·25, 2·5 and 5 mg./kg.

TABLE I

THE ANTICOAGULANT ACTIVITIES EXPRESSED AS A PERCENTAGE OF THE ORIGINAL UNTREATED MATERIAL OF A NUMBER OF HEPARIN PREPARATIONS AFTER ELECTRON BOMBARDMENT FROM A LINEAR ACCELERATOR

Irradiation dose in megarads	Heparin solution (25 per cent)		Heparin powder (5–6 per cent moisture)		Dried heparin powder (1 per cent moisture)	
	Batch 680 N	Batch 176 P	Batch 680 N	Batch 176 P	Batch 680 N	Batch 176 P
0	100	100	100	100	100	100
40	23	23	—	—	—	—
60	10	11	—	—	—	—
80	6	7	19	17	26	30
120	—	—	9	8	18	16
160	2	1	5	5	8	9

subcutaneously, with those produced by three doses of irradiated materials, an assessment of the antilipaemic activity of the latter was made; six experiments were usually carried out on each sample.

There was evidence to suggest that irradiated samples showed some instability, since a fall in anticoagulant activity was noted on prolonged storage. For this reason all samples were examined as soon as possible after irradiation, and were always stored at 0 to 4°.

Reducing power. The reducing power of all samples was estimated by the method of Hagedorn and Jensen (1923).

Metachromatic activity. This was determined by the method described by MacIntosh (1941), except that all heparin solutions were made in distilled water instead of 0·2 per cent saline.

RESULTS

In Table I are shown the results of exposing two different batches of heparin and their solutions to electron bombardment by the linear accelerator. Similar results were also obtained using ^{60}Co as the source of irradiation. The loss of activity was greatest in the heparin solutions and lowest in dried heparin which contained only 1 per cent moisture. Having established that these reactions could be readily reproduced we

* Ediol, oral fat emulsion manufactured by Schenlabs, New York.

then proceeded to examine the effects of irradiation on both antilipaemic and anticoagulant activity. Most of these experiments were conducted with a source of ^{60}Co, and the effects of this gamma radiation on the biological and chemical properties of heparin are given in Table II. Similar results could also be produced by electron bombardment.

DISCUSSION

It can be seen from Table II that the anticoagulant activity of the irradiated samples fell more rapidly than did the antilipaemic: this has been a consistent finding in all our experiments. These results indicate that the chemical pattern responsible for the antilipaemic activity of heparin is not exactly the same as that producing the anticoagulant effects. Unfortunately the fall in anticoagulant activity relative to antilipaemic

TABLE II

SOME BIOLOGICAL AND CHEMICAL PROPERTIES OF A 25 PER CENT SOLUTION OF SODIUM HEPARIN, SUBJECTED TO VARYING DOSES OF IRRADIATION FROM A SOURCE OF ^{60}CO

Irradiation dose in megarads	Anticoagulant activity*	Antilipaemic activity*	pH	Reducing power†	Metachromatic activity‡
0	100	100	6·0	1·0	114 μg.
12·7	65	93 (74–114)	5·5	6·3	120 μg.
28·0	35	62 (50–78)	4·7	9·6	121 μg.
41·0	25	40 (32–50)	4·6	12·0	152 μg.
59·9	10	19 (15–24)	3·8	16·9	188 μg.

* Expressed as percentage of the activity of the untreated material. Figures in parenthesis are approximate limits of error, P = 0·95.

† Expressed in relation to that of original material = 1.

‡ The amount of heparin in μg. required to reduce by 50 per cent the colour of 10 ml. of a 0·0025 per cent solution of toluidine blue.

activity was not as great as we had hoped, and this type of product has, therefore, no value as a therapeutic agent.

A number of other observations seem worthy of note. A fall in the biological activity was accompanied by a fall in pH and an increase in reducing power. A decrease in the metachromatic reaction also occurred but was less marked than we expected and indicates that the measurement of metachromasia bears no relationship to biological activity (Table II). Thus heparin retaining only 10 per cent of its anticoagulant activity nevertheless produced a fairly strong metachromatic reaction. These results agree with those of Wolfrom, Weisblat, Karabinos, McNealy and McLean (1943) who reported similar findings when using acid hydrolysed heparin.

Table I indicates that the biological properties of two different batches of heparin were reduced almost identically by electron bombardment. It is surprising that this type of reaction can produce such a precise and reproducible biological change in a molecule as complex as heparin, in view of the difficulty of achieving this degree of reproducibility by chemical means.

Acknowledgement. We are indebted to Mr. G. S. Murray, and staff of the A.E.R.E., Wantage, for advice and for arranging the electron bombardment, and to the Bioassay Division, Standards Department of Boots Pure Drug Co. Ltd., for the anticoagulant assays.

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A NEW ANALEPTIC: 5,5-DIETHYL-1,3-OXAZIN-2,4-DIONE (DIOXONE)

I. Toxicity and Convulsant Activity

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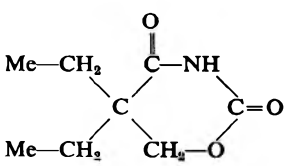
Received December 5, 1960

Dioxone (5,5-diethyl-1,3-oxazin-2,4-dione) is a new substance possessing convulsant properties qualitatively similar to leptazol and bemegride. Its convulsant properties have been studied quantitatively in mice and rats. By comparison with the other two analeptics, dioxone shows the highest ratio of lethal to convulsive doses. Dioxone is active orally in mice, rats and dogs. Experiments on spinal cats and rabbits have shown that it does not act on the spinal cord. In the decerebrated animals its effects appear limited and inconsistent. It has a relatively low toxicity, is well tolerated by rats and dogs when administered for up to 6 months either subcutaneously or orally.

The discovery of the convulsant properties of some compounds belonging to a series of 5,5-disubstituted 1,3-oxazin-2,4-diones has been reported by Maffii and Silvestrini (1961) and of these 5,5-diethyl-oxazin-2,4-dione, Dioxone, proved to be most active.

Contrary to the actions of 5-phenyl-5-alkyl-1,3-oxazin-2,4-diones which prevent experimentally induced seizures in mice, dialiphatic substituted derivatives including dioxone produce clonic and clonic-tonic convulsions.

The antagonism exhibited by some substances of the former group both towards dioxone and leptazol suggested a similarity between the convulsant activity of these two substances.



Dioxone

We now report the results of the pharmacological investigation of the convulsant activity and toxicity of dioxone in different species.

Materials and Methods

Dioxone is a white crystalline substance that melts at 97°, and is readily soluble in ethanol, ether, chloroform and propylene glycol. A 1 per cent aqueous solution may be obtained at room temperature giving a pH of

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4·3, and, with warming to 50°, 2 per cent aqueous solutions may be prepared.

CF-1 Mice, CF-Wistar rats, mongrel dogs, domestic cats and rabbits were used in this work.

In acute toxicity studies mice and rats within a limited weight range were chosen, and the volume of injected solution was kept constant. Intravenous administration was at a rate of 0·1 ml./sec. The volumes administered were in mice per 20 g. weight, 0·5 ml. i.v., 0·2 ml. i.p. and orally; in rats per 200 g. weight: 0·8 ml. s.c., 0·4 ml. i.p. and orally. Four or 5 doses were used and 10 animals for each dose. Deaths occurring within 72 hr. were considered to be due to the compound.

In subacute and chronic toxicity studies in rats, complete blood counts were made before, and every 20 days during the treatment. With dogs receiving dioxone in chronic toxicity tests urine analysis was made every week, glucose and nitrogen blood serum levels every two weeks, as well as Takata-Ara reaction, paper-electrophoretic analysis of plasma proteins, complete blood cell count and prothrombin time tests.

In studies on convulsant activity, the 50 per cent effective dose was determined in rats and mice by administering 3–5 doses of the compounds to groups of 10 animals each and then observing them continuously for clonic and tonic seizures. *For the studies on decerebrated animals*, section of the brain stem was made under ether anaesthesia at intercollicular level. In spinal preparations the section was preceded by injection of a few drops of 1 per cent procaine solution into the medulla to reduce traumatic shock.

The method of Litchfield and Wilcoxon (1949) was followed for estimating the LD50 and ED50.

Results

Effect of Dioxone on the Behaviour and Convulsant Action

Mice. A 1 mg./kg. dose of dioxone intraperitoneally produces only a slight increase in the animals' reactivity, especially revealed by behaviour with the investigator, particularly when attempting to catch the animal. The hyper-reactivity becomes more marked after doses of 3 mg./kg., but the spontaneous activity does not appear to increase. After 10 mg./kg., the spontaneous activity was often reduced, always accompanied by hyper-reactivity. Tremors, clonic convulsion and motor incoordination phenomena occur after the administration of 30 mg./kg. Higher doses provoke generalised tonic-clonic attacks with intervals of prostration and death of some animals.

Preliminary systematic studies of the effects on mice, made according to the method used in this laboratory (Maffii 1959), showed that the effects of dioxone could not be distinguished from those of leptazol at twice the dose.

The spontaneous motility of mice receiving 5 and 25 mg./kg. of dioxone and 10 and 50 mg./kg. doses of leptazol intraperitoneally was registered by a cage with a moving floor. No increase of the spontaneous activity in mice from the effect of subconvulsive doses of the two compounds was

observed during the 6 hr. after administration, the activity being reduced in some instances.

The tracings after the two treatments were not distinguishable.

The convulsant action in mice has been studied quantitatively, determining the ED50 effecting clonic and clonic-tonic attacks obtained through different routes of administration.

Table I compares the data obtained with leptazol, bemegride and dioxone and shows that dioxone is much more active than leptazol and slightly less active than bemegride. The ratios between this ED50 of dioxone and that of leptazol for different routes of administration, were found to vary between 1 : 1·7 and 1 : 2·25. The corresponding ratios

TABLE I

CONVULSANT ACTION IN MICE

Compound	Route of administration	No. of dose	Animals/ dose	ED50 (Fiducial limits: P = 0·05) Clonic seizures	ED50 (Fiducial limits: P = 0·05) Tonic seizures
Dioxone	i.v.	3	8	11·8 (9·7–14·3)	24·6 (19·7–30·7)
	i.p.	3	8	24·0 (21·7–26·6)	50·0 (45·5–55·0)
	oral	3	10	41·5 (29·0–59·3)	108 (84·5–138·2)
	s.c.	6	10	31·0 (26·9–35·6)	58·1 (52·8–63·9)
Leptazol	i.v.	3	8	23·0 (19·8–26·7)	42·0 (37·2–47·5)
	i.p.	5	10	54·0 (47·4–61·6)	92·0 (81·5–103·9)
	oral	3	10	91·0 (65·5–126·6)	—
	s.c.	8	10	62·0 (59·0–65·1)	101·0 (90·1–113·1)
Bemegride	i.v.	6	13	10·6 (10·1–11)	14·9 (13·3–16·6)
	s.c.	6	10	23·0 (21·1–25)	39·5 (36·4–42·8)

between the ED50 of bemegride and dioxone are 1 : 1·12 (i.v.) and 1 : 1·34 (s.c.). Increase in dosage causes a proportional increase in the duration and frequency of the convulsive attacks, while the latent period between administration and onset of the convulsion tends to be shortened.

Rats. Dioxone at 1 and 3 mg./kg. i.p. produces only slight reduction of the spontaneous activity. Tremors and some isolated clonus of the muscles of the limbs are observed with 10 mg./kg. Generalized clonic convulsions, repeating until death intervenes, occur with doses above 15–20 mg./kg. Clonic convulsions occurred in all animals at 30 mg./kg.; in 33 per cent they turned into tonic-clonic attacks. On oral administration, the first symptoms (tremors and muscular twitches) of the action of dioxone appeared 10 min. after a dose of 50 mg./kg. 60 mg./kg. produced clonic convulsions in 4 animals out of 9 and, in one, clonic-tonic attacks were followed by death. The duration of the single attacks increases regularly with repetition of the paroxysms.

The ED50 values for clonic seizures, as produced by different routes of administration, are gathered in Table II.

Rabbits and cats. In rabbits, intravenous administration of 5 mg./kg. of dioxone produces clonus of the masticatory muscles and some respiratory excitation immediately after injection. A generalized clonic attack was exceptionally rare. Higher doses (8–10 mg./kg.) led to fully clonic convulsions and, less frequently, to tonic-clonic attacks. All the animals survived after a period of prostration after the convulsive attack. In cats, intraperitoneal administration of 15 and 20 mg./kg. caused clonic and tonic-clonic convulsions preceded by a period of excitation and accompanied by salivation and sometimes vomiting. The animals survived the convulsive phenomena.

Dogs. Intraperitoneal administration. 5 mg./kg. of dioxone administered to dogs produced only slight muscular twitches and a small increase in respiratory frequency in one dog out of three; these effects disappeared after 50 min. In two experiments, 10 mg./kg. caused an increase in respiratory frequency and a few weak clonic contractions even 10 min. after the injection. An attack of vomiting occurred after 1 hr. in one animal. The animals appeared normal after 120 min. Respiratory excitation, salivation and vomiting appeared at a dosage of 20 mg./kg.

TABLE II

CONVULSANT ACTION OF DIOXONE AND BEMEGRIDE IN RATS

Compound	Route of administration	No. of doses	Animals/dose	ED50 mg./kg. (approximate) Clonic seizures
Dioxone	s.c.	4	10	16
	i.p.	3	15	15
	oral	3	10	55
Bemegride ..	s.c.	5	10	17

One dog out of two receiving this dose displayed generalised clonic convulsions 15 min. after administration, but both the animals appeared to be normal within 1 hr. of injection.

A 30 mg./kg. dose administered to several animals produced repeated clonic and tonic-clonic attacks, starting from 4 to 30 min. after injection.

Nevertheless, there was great variation in the susceptibility to convulsions. That the convulsive effects are linked to individual characteristics has been confirmed in cross-over tests in which the same animal received leptazol or dioxone. After several days the alternative drug was then given. The dogs displayed identical symptoms at the effective doses. Leptazol comparatively tested in some of the animals, displayed a series of symptoms identical with those observed after dioxone, but only at higher doses. Slight muscular twitches, increase of the respiratory frequency, salivation and attacks of vomiting were observed at doses of 10 and 20 mg./kg. Clonic convulsions were observed at 30–40 mg./kg. with a wide variation in incidence and degree. Repeated tonic-clonic attacks, accompanied by salivation and motor incoordination, and followed by prostration, were seen with 60 mg./kg. As with dioxone, the animals appeared to be normal within about 2 hr. after administration.

Oral administration. By this route dioxone also produces the characteristic convulsive symptoms. Two animals displayed clonic convulsions

with respiratory excitation and vomiting at 30 and 50 mg./kg. It has been observed that the latent period before the appearance of an attack varied from 35 to 60 min.

Persistence of the action and effects of successive administration. To determine the persistence of the effect, subconvulsive doses of dioxone (10 mg./kg.) and of leptazol (20 mg./kg.) were administered subcutaneously at 20, 40, 60, 90 and 120 min. Experiments on the same animal, with dioxone and leptazol, were made at 7 days' interval. Three of the 6 dogs used for this "cross-over" test were treated first with dioxone and secondly with leptazol. The results confirmed that the equivalent effective doses of dioxone and leptazol are in a ratio of 1 : 2 for dogs, as well as for mice and rats. The persistence of the effects, is about the same for both compounds and depends much upon the animals. For this reason it is difficult to establish the interval of time necessary between separate doses to avoid summation of effects. Greater responses were observed after the second administration with intervals of 20, 40, 60 and 90 min., and in some days even when the interval was prolonged to 2 hr.

Effects of Dioxone in Decerebrate Animals

The experiments were carried out on rabbits, cats and dogs. Dioxone and the other compounds, were administered intravenously at doses between 2·5 and 20 mg./kg. In rabbits, dioxone produces accentuation of the rigidity which may be followed by contractions of the muscle of limbs, isolated or grouped in deambulatory movements, and accompanied by respiratory stimulation. Only in 2 animals out of 10 were obvious convulsive attacks seen. The general picture of the changes produced by dioxone was substantially the same in cats except that movements of deambulation were usually absent. Also, in one rabbit out of 9, true convulsive phenomena were seen. This animal had received only 2·5 mg./kg. The reasons for these three erratic results are difficult to explain as the experimental conditions were the same and on autopsy the sections appeared to be complete and at the intercollicular level. Also in 2 out of 5 cats given bemegride (1–5 mg./kg. i.v.) we observed clonic seizures followed by death. As with dioxone, the animals which had convulsions were not those receiving the larger dose.

In the dogs neither dioxone nor bemegride (in doses from 2·5–7mg. /kg.) produced convulsions. Occasionally some tremors and increase in rigidity were seen. In 5 of 6 amimals both dioxone and bemegride produced marked stimulation of respiration.

Effects on Spinal Animals

In spinal rabbits and cats dioxone injected intravenously up to 20 mg./kg., produces intense clonus of the muscles with cranial innervation, but no effect on the limbs and trunk.

Antagonism by Anticonvulsant Agents toward the Convulsant and Lethal Effects of Dioxone

The action of some drugs noted for their anticonvulsant effect and their antagonism towards leptazol has been studied on the convulsive and

lethal results produced by dioxone. We considered trimethadione, meprobamate, phenobarbitone (leptazol antagonists) and diphenylhydantoin, which, has an anti-epileptic action, but which is inactive against leptazol-induced convulsions. Table III gives the 50 per cent protective doses (PD50) for the 4 drugs used against the lethal phenomena produced by 70 mg./kg. doses of dioxone intraperitoneally and 140 mg./kg. doses of leptazol. It would therefore appear that substances capable of antagonising the effects of leptazol also exert this action with dioxone. Diphenylhydantoin was found to be equally inactive in both instances.

Acute Toxicity

Table IV compares the LD50 values of dioxone, leptazol, and bemegride administered by different routes in various species. All produced death after tonic convulsions and generally within a short time of administration (from 20 min. i.v. to 3 hr. orally). Apart from the similarity in

TABLE III

ANTAGONISM BY DIFFERENT ANTICONVULSANT AGENTS TOWARDS LETHAL EFFECTS OF DIOXONE AND LEPTAZOL

Anti-convulsant	No. of doses	No. of mice/ dose	ED50 (protective) (70 mg./kg. dioxone)	No. of doses	No. of mice/ dose	ED50 (protective) (140 mg./kg. leptazol)
Trimethadione	5	6	269 mg./kg.	3	7	320 mg./kg.
Meprobamate	5	6	38 "	3	18	39 "
Phenobarbitone	4	6	~ 10 "	2	12	~ 14 "
Diphenylhydantoin	1	5	>200 "	1	5	>200 "

symptoms and death rate a quantitative difference among the three analeptics was evident, bemegride being the most toxic and leptazol the least. It may be observed, that in the rat the oral LD50 is relatively lower than in the mouse for all the compounds tested.

Subacute and Chronic Toxicity

Dogs. A preliminary test was made on 2 dogs, each of which received 5 mg./kg. of dioxone (1 per cent solution), daily intraperitoneally for a month without signs of toxicity. Haemochromocytometric tests, and prothrombin times before and after treatment, did not reveal any significant changes. A weekly examination of the urine did not disclose any pathological symptoms. The glycaemia and the Takata-Ara reaction, determined 15 and 30 days after treatment, were normal.

Two dogs received for 6 months oral doses of 30 mg./kg. of dioxone daily in 3 doses at 4 hr. intervals. Both animals survived. Alteration of character, sociability, appetite, or motor functions was not observed. In one dog, isolated clonus of the muscles of the head and the neck occurred three times during the 6 months. Being fully grown animals, their weights remained constant. Only occasional attacks of vomiting occurred during treatment 3 and 4 times respectively in the 6 months. No changes of a pathological nature were observed in the tests on urine and blood, made as described under "methods".

At autopsy, no gross pathological signs were observed in lungs, heart, spleen and kidneys. Liver was found slightly augmented in volume. Some slight subacute inflammation was found in the stomach and small intestine of both animals. Microscopically slight albuminous degeneration of the liver and kidneys of one dog were the only pathological findings.

Two dogs received 10 mg./kg. of dioxone twice daily subcutaneously for 6 months. The changes produced differed substantially in the two animals. In one dog repeated emesis was observed (53 days in 182),

TABLE IV

ACUTE TOXICITY IN MICE AND RATS

Compound	Animal	Route of administration	No. of doses	Animals/dose	LD50 mg./kg. (Fiducial limits)
Dioxone	mouse	i.v.	4	10	31·5 (26·7–39·9)
		i.p.	4	10	52·0 (45·7–59·3)
		oral	7	13	130 (106·6–158·6)
		s.c.	3	10	60·5 (54–67·7)
	rat	i.p.	4	10	31·6 (26·11–38·24)
		oral	5	10	70·5 (50·7–98)
		s.c.	4	8	39·4 (35·8–43·3)
Leptazol	mouse	i.v.	5	11	51·0 (45·65–56·96)
		i.p.	5	10	96 (88·8–104·6)
		oral	4	10	162 (135–194·4)
		s.c.	5	10	101 (93–109·5)
	rat	i.p.	4	10	70 (63·2–77·5)
		oral	4	10	140 (115·7–169·4)
		s.c.			100 (4)
Bemegride	mouse	i.v.	6	10	18·5 (15·9–21·4)
		s.c.	3	10	40·5 (36·1–45·3)
	rat	s.c.	4	10	30·5 (32·1–28·9)

together with anorexia and a moderate loss of hair, that appeared on the 35th day of treatment. The other dog did not show any peculiar symptoms. Vomiting occurred on 2 days in 183. Neither loss of hair, nor diminution in appetite was noted. Both animals kept a constant weight. At macro- and microscopic examination at the end of the 6 months no serious pathological signs were detected. Only signs of albuminous degeneration of some zones of hepatic parenchyma were observed in the first dog. The site of injection showed only minor signs of local irritation. The blood counts and analysis of urine and blood did not show any deterioration during the treatment, except for a sporadic appearance of a reducing substance in the urine of the first dog during the 2nd and 4th months.

Rats. Two groups of male rats received oral doses of 15 and 30 mg./kg. 6 days a week for 6 months. The compound was dissolved in distilled water, at 0·5 per cent for the lower dose and 1 per cent for the higher dose. The volume administered was 0·3 ml./100 g. weight. Few symptoms were observed during the period of treatment. On the 36th day one animal showed a small zone of alopecia that disappeared spontaneously within a week. One rat of the group treated with 30 mg./kg. showed three episodes of clonic seizures on the 71st, 124th and 141st day of treatment. No significant changes were observed in the test animals during and at the end of treatment, compared with the controls receiving only distilled water though the increase in weight was higher in the animals receiving dioxone. Complete blood counts of every animal did not show any significant changes from pretreatment levels.

The macroscopic examination and the weights of kidney, liver, spleen and adrenals did not differ from untreated animals. Histological examination showed albuminous degeneration of liver in 2 animals treated with 15 mg./kg., and in 4 animals treated with 30 mg./kg. Kidneys, spleen and heart did not show any sign of damage.

The same experiment was made over 6 months on groups of 10 animals each animal receiving dioxone intraperitoneally at daily doses of 10 and 25 mg./kg. Those receiving 25 mg./kg. were given the analeptic in two fractions at eight hourly intervals.

Dioxone was given in 0·416 per cent aqueous solution (pH = 4·8) and the injected volume was 0·3 ml./100 g. weight.

Rats receiving 10 mg./kg. did not show any unusual pathological symptoms except for a clonic attack in one animal during the last week of treatment. In the group receiving 25 mg./kg., tremors and muscular twitches were observed especially after the second daily half-dose. After the 23rd day of treatment clonic seizures were occasionally observed until the end of the treatment.

No animals died among those receiving up to 10 mg./kg. Seven out of 10 rats given 25 mg./kg. died at the 28th, 37th, 58th, 75th, 86th, 95th and 113th day respectively. All deaths occurred after the second daily half dose, during clonic seizures. Blood counts, made during and at the end of the experiment did not show any deviation from normal. At the end of the experiment the weights of animals receiving 10 mg./kg. were not significantly different from the controls given distilled water, and the three survivors of the group treated with 25 mg./kg. showed a normal increase of weight. Microscopic examination of the principal organs at the end of the treatment showed no sign of irritation in the peritoneal cavity. Kidneys and liver were augmented in volume in the animal receiving the higher dose.

Discussion

Dioxone is a new compound which exhibits interesting neurophysiological properties. It possesses a definite and noticeable convulsant action which is similar in many respects to that of leptazol and bemegride.

In mice and rats, dioxone produces clonic convulsions in sub-lethal doses but tonic seizures are induced by doses approaching the lethal ones as occurs with leptazol and bemegride. By comparing the ratios between the LD50 and ED50 of the three convulsant agents as shown in Table V it appears that the figures for LD50:ED50 ratios for tonic seizures are uniform both amongst the three analeptics and between different routes of administration, indicating that in animals given dioxone or the other two convulsant agents the tonic extensor phase of convulsions is related to severe toxic phenomena and mortality either produced by the convulsive status itself or—less likely—produced directly by the agents. Moreover as far as the LD50:ED50 ratios for clonic seizures are concerned a

TABLE V

RATIOS BETWEEN ACUTE TOXICITY AND CONVULSANT ACTION OF DIFFERENT AGENTS IN MICE

Compound	Route of administration	LD50 ED50 (clonic seiz.)	LD50 ED50 (tonic seiz.)
Dioxone	i.v.	2·66	1·28
	s.c.	1·93	1·03
Leptazol	i.v.	2·21	1·21
	s.c.	1·62	1·00
Bemegride	i.v.	1·76	1·24
	s.c.	1·76	1·02

quantitative difference has been found between dioxone and the other two drugs, which suggest that dioxone has a more specific activity than either bemegride or leptazol.

The ratio found with dioxone, by i.v. administration, is 2·66, a figure higher than any other known synthetic drug with similar properties. The convulsant activity of dioxone is also easily demonstrated in rabbits, cats and dogs.

In an attempt to investigate the origin of the convulsive phenomena and thereby approach the problem of site of action of dioxone, it can be concluded from the results obtained in spinal animals, that the new analeptic does not act on the spinal cord, since high doses do not produce convulsions or any other muscular activity in muscles with spinal-innervation.

In this respect dioxone—like bemegride and leptazol—differs from strychnine and also from nikethamide which possess a marked action on the spinal cord at convulsant dosage (Han and Schuk, 1956).

In decerebrate animals the effect of dioxone appeared less clear-cut. Although experiments were made on a relatively large number of animals the results were not so consistent as in spinal animals. In the dogs and in rabbits and cats, only minor motor phenomena were seen as well as an increase in the rigidity. In a few rabbits and cats we were able to produce true, generalised convulsions both clonic and tonic-clonic. We cannot explain at present the reason for these results on the basis of data available.

The convulsive effect of dioxone is counteracted by phenobarbitone, meprobamate and trimethadione, and this evidence further confirms its similarity with leptazol. As the seizures induced by dioxone may also be antagonised by the phenyl-4-alkyloxazindiones active against leptazol-induced convulsions (Maffii and Silvestrini 1961) and since the active doses of the anticonvulsant agents are about the same when dioxone and leptazol are used in isodynamic dosage, it seems reasonable to suppose that the antagonism between dioxone and anticonvulsants is functional rather than competitive.

Experimental evidence shows dioxone has a prompt and relatively long-lasting action, and is well absorbed when administered orally. Its acute toxicity is low especially if one considers the high level of activity of the compound, and chronic toxicity studies in rats and dogs did not reveal severe pathological features. This fact is particularly significant in considering that in the 6 month treatments, maximal subconvulsive doses were administered both daily and twice daily.

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LETTERS TO THE EDITOR

Histamine Releasers and Histamine Sensitivity

SIR,—Harvey (1961) reported a decreased sensitivity to histamine 6 hr. after 48/80 administration. This present report is to confirm and extend her results using different animals and methods.

Normal female guinea-pigs and those desensitized to histamine by daily injection of progressively higher doses of histamine until the original LD 95 (4·0 mg./kg. subcutaneously) was tolerated were subjected to histamine aerosol before and after the administration of n-octylamine (0·3 mg./kg. subcutaneously) and 48/80 (4·0 mg./kg. subcutaneously).

The Aerosol Reaction Time (A.R.T.) was determined by averaging the durations of exposure to histamine aerosol before (*a*) cough, and (*b*) dyspnoea, according

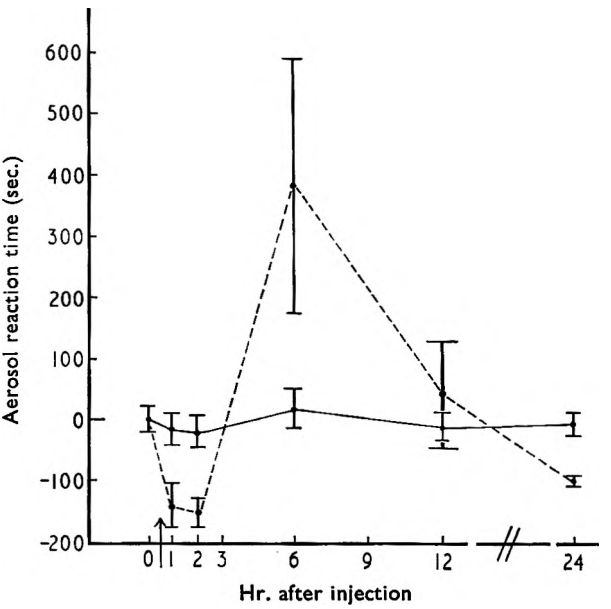

Fig. 1. The effect of n-octylamine on normal guinea-pigs. n-Octylamine given at arrow.——control – – – experimental.

to Kallos and Pagel (1937). Each point on the curves represents the mean of at least 5 determinations on 5 animals. The scatter for each point is quite wide, nevertheless all curves drawn from determinations on single animals have the same general shape, indicating significant differences from point to point. The actual A.R.T.s were adjusted arithmetically so that the "0" hour pre-injection A.R.T. coincides with zero on the Y-axis.

It can be seen that the curves (Figs. 1 and 2) are biphasic. A period of increased sensitivity to histamine aerosol occurs 1 to 3 hr. after n-octylamine injection, while 6 hr. after injection the animals are less sensitive, than in the pre-injection period. Similar, though not as well defined, biphasic curves are produced by 48/80 injection.

The inverse relationship between tissue content of histamine and that tissue's tolerance to histamine as suggested by Harvey (1961), several authors quoted by her, and Ambrus, Ambrus and Harrison (1951) would seem to be borne out. Initially, when the releasers lower the tissue content of histamine, the sensitivity of the guinea-pig to histamine (aerosol) increases. Perhaps the mechanism is

similar to that suggested by Straub (1907) whereby the amount of a particular drug crossing the cell membrane is inversely related to the intracellular concentration of that drug. This possibility was suggested by Ambrus and others (1951).

The secondary decrease in sensitivity to histamine at 6 hr. and more after injection to the releaser is more difficult to explain although it agrees with Miss Harvey's (1961) findings and the findings of several authors quoted by her. The secondary decrease in sensitivity might depend on the increased ability of

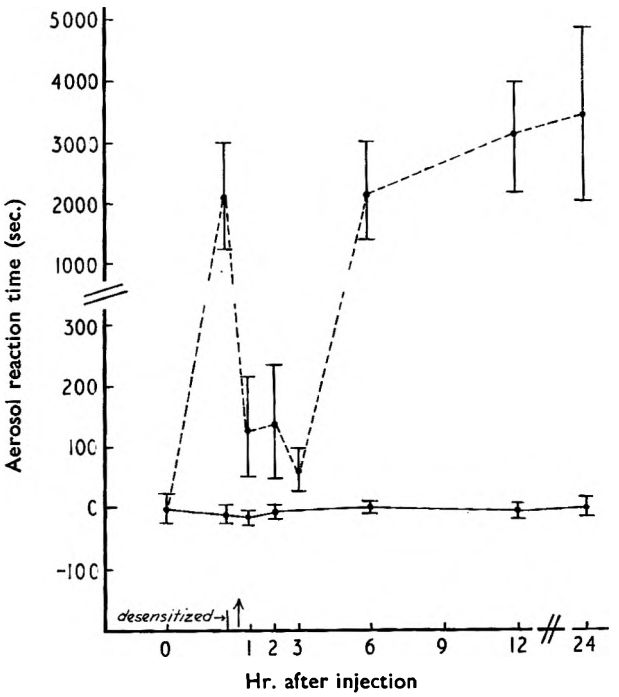

Fig. 2. The effect of n-octylamine on histamine-tolerant guinea-pigs. n-Octylamine given at arrow.——control – – – experimental.

histamine-depleted tissues to bind histamine (Schayer, Davis and Smiley, 1955). The period of maximum sensitivity reflects the time of lowest tissue histamine concentration. After this maximally-sensitive period the tissues gradually replenish their histamine store both by synthesis and binding of exogenous histamine. This replenishment is reflected in the return to normal A.R.T. The secondary decrease in sensitivity (higher A.R.T.) indicates that the tissues have bound more histamine than in the normal state. This explanation is purely conjectural and is subject to confirmatory tissue histamine determinations.

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Carcinoid Tumours and Pineapples

SIR,—In a letter to the Editor in this Journal, West (1960) claims that he has found only traces of indole derivatives in pineapples, and that Foy and Parratt (1960) found likewise. In fact, Foy and Parratt claim that they found none. I find quite high levels in Australian pineapples (25 μg./ml. in canned juice) and have the confirming finding of raised indole excretion after the ingestion of pineapple juice (Bruce, 1960). My result is confirmed by Sjoerdsma (personal communication) working with canned pineapple juice available in Maryland, and in which he claims to have detected 35 μg./ml.

The possible reasons for this discrepancy seem to be twofold:

(1) the geographical or ripeness differences in the fruit, and
(2) differences in extraction procedures or in chromatographic, bioassay or colorimetric methods.

I have written to Dr. West with a view to resolving the differences, and suggesting the exchange of samples and assay methods. I shall communicate my result to this Journal as soon as possible.

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February 2, 1961.

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