REVIEW ARTICLE

RECENT ADVANCES IN FREEZE-DRYING*

By R. I. N. Greaves, M.D.
Professor of Pathology, University of Cambridge

In 1946, the Medical Research Council published their Special Report Series, No. 258, on "The Preservation of Proteins by Drying" (Greaves, 1946). This Report summarised the work of the M.R.C. Drying Unit which had been responsible for the preparation of dried plasma for transfusion purposes during the War.

The First International Symposium on Freezing and Drying (1951) was held in London in that year to be followed by a Second Symposium in 1958 (Parkes and Smith, 1960) and a Symposium organised by the New York Academy of Sciences, in 1959 (Review, 1960). During these post war years the technique of freeze-drying has been applied more and more widely and, with rapid technical improvements, has now reached the stage when it is becoming a commercial proposition in the preserved food industry.

In the pharmaceutical industry, freeze-drying has already become an important stage in the manufacture of certain products such as streptomycin and for the distribution of unstable products, such as hormone preparations, in a stable form. It has also enabled stable vaccines to be prepared, in particular B.C.G. (Obayashi, 1960; Muggleton, 1960) and vaccinia (Collier, 1955).

In the course of this review it is impossible to consider all the advances which have been made since the end of the war, and I shall limit myself to a discussion of those advances which I consider are of particular importance to the pharmacologist and the pharmaceutical industry. Advances in vacuum and refrigeration techniques, which have enabled drying chambers of almost any size to be made and have led to reliability and ease of operation, will not be discussed here. Instead, I wish to discuss Professor Louis Rey's work on Thermal Analysis (Rey, 1959, 1960a,b) which enables us to predetermine the optimal drying conditions for a particular substance. In addition, I wish to discuss the drying of living organisms and finally the application of heat in freeze-drying systems.

Thermal Analysis

If a simple solution of a salt such as NaCl is cooled, when it reaches 0° pure ice starts to separate as crystals. This removal of pure water from the system leads to concentration of the NaCl until a certain concentration known as the eutectic concentration is reached. The temperature then drops to −21·6°, at which temperature the eutectic concentrate freezes. This temperature of −21·6° is known as the eutectic temperature of NaCl.

* Based on two Special University of London lectures given at the School of Pharmacy, Brunswick Square, London, on March 6 and 8, 1962.

Obviously, if the material we wish to dry is in a solution of NaCl. and we dry at a temperature above $-21{\cdot}6°$, then there will be some drying from the liquid phase.

Again, many substances, such as glycerol-water solutions, do not crystalise entirely when they are frozen. The remaining fluid concentrates progressively and its viscosity becomes greater and greater until it hardens and becomes, at least partially, a glass. This glass, although it is metastable, may persist indefinitely. On warming the reverse process occurs, the glass progressively losing its high viscosity and returning to the liquid state. Many compounds which we may wish to dry may show these properties; in particular, sugars and alcohols and some vitamins. Such substances are very difficult to dry as the continuous softening of the glass leads to "puffing" of the product with some degree of melting and denaturation. But, thermal analysis will enable us to discover the devitrification temperature, and preliminary thermal treatment of the substance may enable a satisfactory freeze-drying of the substance to be accomplished.

The Optimal Drying Procedure

In the past we have faced the freeze-drying of a new product by the empirical method of trial and error. In the light of Rey's work, it is now possible to forecast the precise optimal drying procedure. The method is basically extremely simple; all that is necessary is to plot the temperature of the material when it is cooled and warmed at a constant rate and to compare its temperature with that of distilled water treated in an exactly

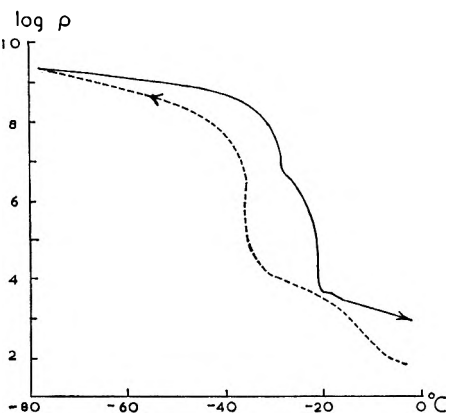

FIG. 1. Variation of the electric resistance with temperature of a solution of 10 parts per 1,000 of NaCl in distilled water. *Broken line* measurements taken during cooling. *Solid line* measurements taken during warming. *By courtesy of Professor L. R. Rey.*

similar way. At the same time the electric resistance of the product should also be measured. Single estimations may be plotted by hand, but when routine measurements are to be made, it is a great convenience to record them automatically.

When a solution of an electrolyte is completely frozen, its electrical resistance becomes infinitely great. In order to measure this resistance, we need a resistance bridge measuring up to at least 100 megohms, and in order to avoid polarisation, this bridge must be fed with alternating current, preferably at about 1,000 cycles/sec., preferably square wave. Fig. 1 shows the resistivity measurements on cooling and warming a solution of 10 parts per 1,000 of NaCl in distilled water. The discrepancy between the two curves is due to supercooling on freezing so that a totally solid phase is not achieved until the temperature reaches $-40°$; the correct eutectic thawing temperature of $-21{\cdot}6°$ can be confirmed only on the warming curve.

This experiment shows the importance, when freeze-drying material containing NaCl, of first prefreezing to a very low temperature before drying at a temperature just below the eutectic, otherwise, at the same drying temperature, there might be some supercooled liquid present.

Automatic Control of Drying Temperature

This experiment also suggests a method of automatic control of the drying temperature. Just before the eutectic temperature is reached, there is a sudden fall in resistance. This fall could be made to cut off the heating circuit. This has a distinct advantage over control simply by the

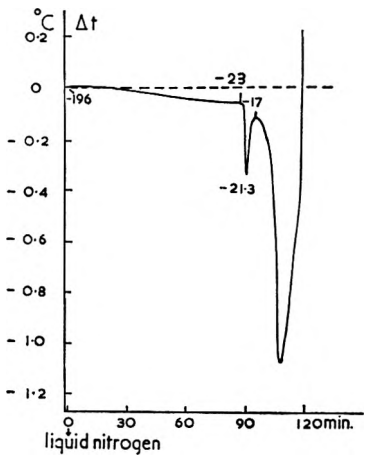

FIG. 2. Differential thermal analysis diagram of a solution of NaCl in distilled water. *By courtesy of Professor L. R. Rey.*

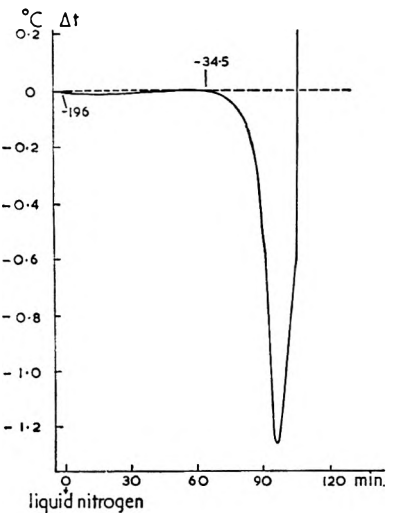

FIG. 3. Differential thermal analysis diagram of normal horse serum. *By courtesy of Professor L. R. Rey.*

temperature of the product. First, it enables drying to be carried out at the maximal permissible temperature and second, when the temperature starts to rise towards the end of the drying cycle, instead of the heat being automatically reduced as it would be if one were controlling by the temperature of the product, the heat will remain maximal. Thus, the duration

of the late stages of drying is greatly reduced, though a second relay controlled by the temperature of the product must operate to cut off the heat as soon as the dried product has reached its maximum safe temperature. This very elegant method of automatic heat control has been described by Rey (1961).

I have quoted only the example of a simple salt solution. With biological material, such as animal sera, the picture is much more complicated. The presence of proteins masks the salt eutectics and many of the salts are in very small concentrations. As a result, the electric resistance falls gradually with no very sudden steps.

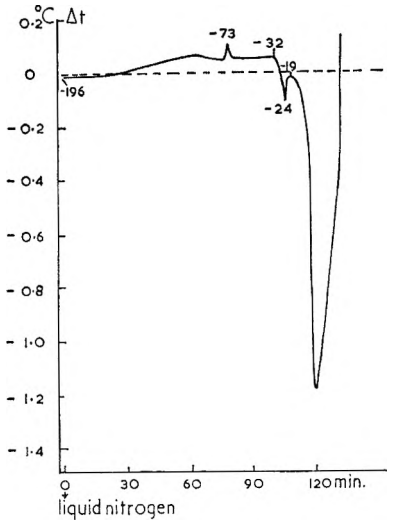

FIG. 4. Differential thermal analysis diagram of Earle's salt solution. *By courtesy of Professor L. R. Rey.*

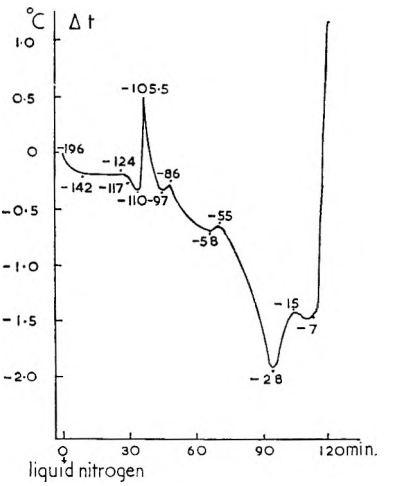

FIG. 5. Differential thermal analysis diagram of a 50 per cent solution of glycerol in Earle's salt solution. *By courtesy of Professor L. R. Rey.*

Another approach is by direct temperature measurement during constant warming. The principle behind these measurements is the continuous comparison on a warming curve between the solution being investigated and distilled water ice. Phenomena such as devitrification of a glass involving crystalisation will be shown as an exothermic reaction, the curve rising, whilst thawing of a eutectic mixture will be shown as an endothermic reaction, the curve falling. These changes are correlated with an absolute measurement of temperature.

Fig. 2 shows such a curve for NaCl with its eutectic at $-21{\cdot}3°$. Fig. 3 shows a similar curve for horse serum with eutectic melting starting at $-34{\cdot}5°$, and the curve for chick embryo extract shows eutectic melting starting at $-17{\cdot}5°$.

Fig. 4 shows the more complicated thermal analysis of Earle's solution. Note particularly the exothermic reaction at $-73°$ due to crystalisation followed by eutectic melting starting at $-32°$ and being maximal at $-24°$.

Fig. 5 shows an even more complex picture of Earle's solution containing 50 per cent of glycerol. The first endothermic reaction is due to a vitrous transformation at $-117°$; at $-105°$ there is devitrification to cubic ice. At $-86°$ there is an exothermic crystallisation from cubic to hexagonal ice. At $-58°$ there is thawing of the glycerol: water eutectic and final thawing at $-28°$.

Since nearly all biological solutions contain salts and undergo considerable supercooling during freezing, and since many form glasses on freezing, difficulties are likely to be encountered unless a complete thermal analysis has previously been made. Some general rules can, however, be devised. First, it is wise to prefreeze to a very low temperature, at least to $-80°$ in "dry-ice", preferably to $-196°$ in liquid nitrogen, to be certain that no supercooled liquid remains. Second, if glass is formed, it is necessary to ensure devitrification before drying starts; this is done by raising the temperature to a point above the lowest temperature at which exothermic reactions can be observed, and holding at this temperature sufficiently long for crystallisation to occur. Third, after the previous treatment, drying must be carried out below the lowest eutectic temperature. If the electric resistance of the material is known, then this resistance may be used to control the heat input during drying.

The Drying of Living Organisms

In the M.R.C. Report No. 258 (Greaves, 1946) there was no reference to the preservation of living organisms by freeze-drying. Several papers had appeared by that time, notably by Elser, Thomas and Steffan (1935), suggesting that the method was of value for this purpose. Since the war, however, a considerable amount of work on the preservation of viruses and bacteria by freeze-drying has been published. The position up to 1954 has been admirably reviewed by Fry (1954).

The most important factor in deciding the percentage survival both immediately after drying and for long term storage is undoubtedly the medium in which the organisms are suspended for drying. Very many different media have been reported to have been used successfully, such as serum, broth, skimmed milk and gelatin, but seldom has success been verified by viable counts on a variety of organisms. In 1949, Fry and Greaves suggested that a medium consisting of 75 per cent serum and 25 per cent broth, containing 7·5 g. of glucose per 100 ml. gave satisfactory viable counts with a number of organisms, including such delicate organisms as *Neisseria gonorrhoeae* and *Vibrio cholerae*, both immediately and also after long term storage at room temperature. At that time they suggested that the serum acted as a protective colloid and as a support medium to give a final dried cake, and that the glucose exerted its effect by acting as a buffer of the residual moisture content preventing the organisms from becoming too dry. They had no explanation why broth was so necessary. They also showed that the glucose effect was shared by many other sugars such as lactose and sucrose. Since its description, this medium has been used widely and successfully for the drying of many different species of organism.

But this medium is not suitable for all purposes. For instance, neither serum nor broth would be very suitable for human injection of a living vaccine. Moreover, although this medium gives excellent long term survival at room temperature, and we have results now of up to 15 years duration, at high ambient temperatures survival is poor. Consequently, over the years, the Fry–Greaves medium has been considerably modified.

It was Obayashi (1960) who first showed that the use of sodium glutamate greatly increased the stability of B.C.G. vaccine at high temperatures. Muggleton (1960), also working with B.C.G., found that the effect of sodium glutamate was neutralised by glucose and not by sucrose. He therefore suggested that a B.C.G. vaccine for human use should be dried in a mixture of 5 per cent glucose-free dextran, 1 per cent sodium glutamate and 5 per cent sucrose. He showed that the dextran was a satisfactory non-antigenic substitute for the serum and the sodium glutamate a satisfactory substitute for the broth in Fry and Greaves' medium, and that 5 per cent sucrose buffered the residual moisture content at 1 per cent.

Meanwhile, Scott (1960), in Australia, was approaching the problem from a different angle. He dried his cultures in a papain digest broth and then stored at varying water activities over salts at varying hydration values, and at various temperatures, in vacuum, air and nitrogen. He found that at low water activities and low temperatures the good initial survival with glucose was well maintained, but that such dried cultures were unstable at high temperatures. Sucrose gave much better protection at high temperatures; ribose was poor at all temperatures and water activities. Scott concluded from his experiments that the instability of dried cultures at high temperatures was due to the presence of carbonyl groups, that the amino-acids of the broth in Fry and Greaves medium neutralised carbonyl groups, but that glucose added carbonyl groups whereas sucrose did not.

In testing a drying medium, a good initial survival and long term storage at room temperature is not enough. A slightly less good initial survival if it gave better long term survival at higher temperatures would be preferable. For this reason I introduced the empirical method of heating in which the dried material was subjected to a temperature of 100° and the time necessary to reduce the viable count to half was estimated.

Under these conditions the Fry and Greaves medium showed up very poorly at high temperatures. 5 per cent peptone was poor for immediate survival, but excellent at high temperatures, which supported Scott's hypothesis. Sodium glutamate alone worked best at 5 per cent; at lower percentages, as used by Obayashi, the initial survival was poor, suggesting that the cultures were too dry; at higher percentages the glutamate appeared to have the dual effect of neutralising carbonyl groups and also acting as a buffer of the final moisture content. The glutamate was greatly improved by adding a colloid such as 5 per cent glucose-free dextran.

These studies, Greaves (1960), led to the conclusion that a drying medium for bacteria should contain (1) a protective colloid, i.e. 5 per cent

dextran containing no glucose. (2) A buffer to control the residual moisture content around 1 per cent, i.e. 5–10 per cent sucrose or 5–10 per cent sodium glutamate. (3) A neutraliser of carbonyl groups, i.e. broth or 1 per cent sodium glutamate.

Recently Obayashi, Ota and Shiro (1961) have suggested the use of polyvinylpyrrolidone as the protective colloid instead of dextran. From a limited experience, I have had good results using the K 30 fraction of polyvinylpyrrolidone with a mean molecular weight of 45,000 and also with Bayer's New Periston with a mean molecular weight of 11,500. When made up, the polyvinylpyrrolidone is acid and, in my experiments, was neutralised before use.

Obayashi and his colleagues in the same paper have used my high temperature survival experiments and consider that for a particular organism it is possible to get a fair idea of the likely storage time at any temperature by extrapolation from the results obtained at high temperatures.

An important paper is that of Collier (1955), who recommends the use of 5 per cent peptone alone for preparing dried vaccinia virus. As already stated, this is not very good for bacteria, for the organisms get too dry. But I have confirmed Collier's findings with other viruses, in particular influenza virus, and the virus of *Herpes simplex*.

Could there be a fundamental rule of nature here that the more simple the form of life the more water it is possible to remove without causing death? Some of my recent work would confirm that this is so.

Drying Higher Forms of Life

Two possible break-throughs in the search for a method of drying higher forms of life have occurred in recent years. The first was a paper by Annear (1956a), in which he claimed an 80 per cent recovery on drying *N. gonorrhoeae* as opposed to the more usual 10–15 per cent of freeze-drying techniques. He obtained a similar recovery with *V. cholerae* and also claimed the successful drying of leptospira (1956b) and the protozoon *Strigomonas oncopelti* (1956c). In his method he first freeze-dried plugs of 10 per cent peptone, 7 per cent glucose and 0·5 per cent soluble starch. Onto the freeze-dried plug he placed a small drop of his culture and immediately placed the ampoule on a vacuum manifold and evacuated it. The drop of culture liquified part of the plug, and when a vacuum was applied this liquid foamed in the ampoule and the foam rapidly dried. Unfortunately, Annear made no measurements of temperature so it was impossible to decide whether this was freeze-drying or rapid drying of a liquid film.

The second break through was a paper by Meryman (1959), in which he claimed the successful freeze-drying of bovine sperm. The basis of his method was the very rapid freeze drying of the sperm which was suspended as a thin film on nylon mesh. Freezing was by evaporation, and he stated that the time taken to bring the specimen temperature from ambient to -35° was critical, and for sperm was between 2 and 3 sec.

Three questions seemed to require investigation.

The first was this. If a protozoon would not survive freezing, was it possible to dry it at a temperature above freezing? Was this why Annear's technique was successful with *S. oncopelti*? And, was Annear's technique really not freeze-drying?

The second question followed from the first. Was residual moisture content critical with higher forms of life? By analogy with the viruses ought we to leave much more than the 1 per cent residual moisture necessary for bacteria when drying higher forms of life?

The third question was: could other critical freezing curves, similar to Meryman's curve for spermatozoa be found for higher forms of life?

In an attempt to answer these questions, I reproduced Meryman's apparatus at Cambridge and, with the help of Dr. Polge, tried to repeat his results with sperm, but with no success. My experience is not unique for, though several other workers have also tried, no one except Tokio Nei (Nei and Nagase, 1961), on one occasion, has been successful. Indeed Meryman himself cannot now get any success. The reason for this is a mystery, as yet unsolved.

I was, however, able to repeat his success in drying red blood corpuscles which remained intact if they were resuspended after drying in a 40 per cent solution of polyvinylpyrrolidone.

I have also done many experiments to try to dry *Euglena gracilis* and *Strigomonas oncopelti*, and see whether a critical freezing curve could be obtained for them. These experiments were very frustrating. Occasionally some success resulted, but could never be repeated with certainty.

However, using this apparatus, I was able to make the temperature measurements that Annear had failed to do, and was able to show that his drying was probably at a temperature below 0° in a supercooled state. It was very easy to supercool Annear's foams to −9° and, under these circumstances, the method gave good survival of *S. oncopelti*, but not *E. gracilis*. If freezing occurred, *S. oncopelti* was killed.

In an attempt to discover when *E. gracilis* was dying during the drying process, counts were done at 2 min. intervals. These showed a continuous slow loss of viability until 80 per cent of the water was removed, at which point total loss of viability occurred. Similarly, counts of *E. gracilis* were made after the various stages of the Meryman freeze-drying curve, which showed clearly that *E. gracilis* could not be successfully frozen in the absence of some protective agent.

Looking at the series, virus, bacteria, protozoa, it seems that as the organism becomes more complex so more water must be left if viability is to remain. But if a high water content is necessary for a good survival, is this likely to be compatable with long term preservation?

I was feeling rather pessimistic as a result of these experiences when, at this time I was given a sample of dried Nigerian mud. This mud contained the desiccated forms of small nematode worms. When water was added to this dried mud, the worms swelled and swam away.

Nature has succeeded where man has failed. But this experience must be accepted as a challenge for the future.

Heat Transfer in Freeze-Drying Systems

The most common criticism of the freeze-drying process is made on the grounds of slowness of drying. The necessary apparatus for freeze-drying is costly, and if the throughput is small, drying costs are high. This does not greatly matter if the cost of the product is high, as it usually is with medical products, but it becomes decisive if cheap products, such as foods, are to be dried.

The rate of drying depends directly on the rate of application of that amount of heat which is required to supply the latent heat of sublimation of the ice. Much of the recent research on the freeze-drying process has, therefore, been directed into investigating methods of heat transfer and identifying the factors which limit the rate of heat transfer.

In my 1946 review (Greaves, 1946) I pointed out that in a freeze-drying system one had a water vapour pressure difference between the water vapour pressure of the drying material and the condenser, a resistance to the flow of vapour and a rate of flow. This may be expressed thus:

$$\frac{\text{Vapour pressure difference}}{\text{Resistance to flow}} = \text{Rate of flow.}$$

As the vapour pressure of ice is a function of temperature, the formula may be rewritten:

$$\frac{\text{VP. } T_1 \text{ (drying material)} - \text{VP. } T_2 \text{ (condenser)}}{\text{Resistance to flow}} = \text{Rate of flow}$$

$$= \text{Constant K} \times \text{watts.}$$

If you wish to dry at a low temperature, then VP $(T_1 - T_2)$ must be kept small so that the drying temperature approximates to the condenser temperature, and if drying is to be fast, the heat input must be high. This, in turn, means that we must keep all obstruction to the flow of vapour as low as possible.

The Importance of Design

In a freeze-drying system, factors which lead to high degrees of obstruction are poor design, such as restricted vapour paths, or poor construction causing leakage and so producing a high partial pressure of non-condensable gases. But in any well designed plant, these factors are of small magnitude compared with the obstruction to the flow of water vapour caused by the dried material as the drying boundary receeds from the surface. Thus, the very nature of the dried material places a limiting factor on the speed of drying and, since this limitation is a function of the density of the dried product, it does not necessarily follow that an advantage in speed of drying will result from concentrating the liquid before freeze-drying. An extreme example of obstructive resistance to vapour flow is caused by the intact membranes of cells and, as everybody knows who has used the freeze-drying technique for preparing histological preparations of tissues, drying becomes very slow indeed.

Methods of Applying Heat

Heat may be applied by conduction, radiation or convection or by dielectric heating or by a combination of any of these methods.

An analysis of the problem as to the most suitable method of applying heat is best made by considering the simple case of subliming distilled water ice.

Assuming a condenser temperature of −40°, the temperature at the surface of the drying ice will be around −35° in a well designed apparatus. Supposing we have a block of ice, 2 in. thick, frozen on a metallic heating plate. As melting will not occur till the temperature of the heating plate has risen to 0°, it is safe to raise the temperature to −5°. This will give a gradient of 30° through the ice to the drying surface. As drying proceeds, the thickness of the ice will decrease; if the heat input is kept constant, the heater temperature will fall. Alternatively, the heater temperature could be kept constant at −5°, in which case the heat input would constantly increase, and with it the drying rate.

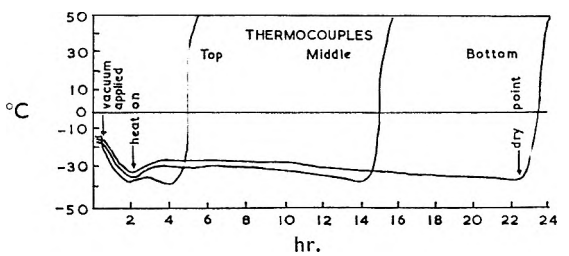

FIG. 6. Drying curves for 2 litres of distilled water ice prefrozen in a rectangular tin giving a depth of 3 in. Heating was by radiant heat from above. Heat input 100 W. Vacuum 0·05 mm./Hg. Condenser temperature −50°.

If heating was by radiation, the conditions would be very different. There would be no gradient through the ice, and provided the obstructive resistance of the system was low, vast amounts of heat could be applied without significantly raising the drying temperature (Fig. 6). Alternatively, as it would be quite safe to dry at −5°, it would be possible to raise the condenser temperature to say −10° with a big increase in the economic efficiency of the process.

An additional advantage of radiant heating would be that since one side of the block no longer needed to be in contact with the heater plate, drying could take place from both sides of the block, thereby effectively halving its depth.

With distilled water ice, there is a very obvious advantage for radiant as opposed to conductive heating. However, the advantages when drying a biological solution are not as great due to the limitation imposed by the danger of overheating and even burning the dried material on the surface of the block.

Suppose we wish to dry a protein solution which has shown eutectic melting at −21·6° on previous thermal analysis. With conductive heating

it will not be safe to raise the heater temperature above $-25°$, so that the maximum permissible gradient across the frozen block will be 10°. This means that either we must dry slowly with a thick block or faster with a thin block, and the calculation of the optimal thickness for a maximum efficiency of operation becomes difficult.

As drying proceeds, the increasing layer of dried material causes increasing resistance to the flow of vapour, and if the heat input remains constant, the drying temperature will rise and the temperature at the point of contact with the heater may rise above the eutectic temperature causing local melting. This will completely upset the uniformity of heating and wet patches will probably be found in the dry product.

With radiant heating there will be no gradient across the frozen block so that it will be impossible to cause melting of the eutectic mixture. The limitation of heat input will be set by the highest safe temperature to which the dried material may be raised. This temperature is usually about $+80°$, and if the radiant heater plates are automatically controlled at this temperature, and if radiant heat is applied to both sides of the block, it is found that a $1\frac{1}{2}$ in. thick block can be dried in 38 hr., whereas a $\frac{3}{4}$ in. block dried in 11 hr. These figures show the advantage of keeping thickness to a minimum.

The advantages of radiant heat on both sides of the block are very obvious, but this may not always be convenient. Conductive heat from the bottom of the block, coupled with radiant heat from above may be a satisfactory compromise.

When exact experiments are made on the absorption of radiant heat, it is found that more heat is absorbed at a given heater temperature than theoretically it should be. The probable explanation for this discrepancy is that the water vapour being evolved from the drying product becomes superheated, and some of this heat is returned to the drying product by convection. Rey and Rieutord (personal communication) have examined this phenomenon and find that by controlled injection of non-condensable gas into the vacuum they can increase this convection heating and can transfer as much heat from heater plates at $+80°$ as would be possible without convection with plates at $+200°$. This gives a considerable increase in the rate of drying without running into danger of burning the dried product. The only disadvantage is that the increase in non-condensable gases increases the resistance to flow of the water vapour. This, as I have already shown, will cause a rise of the drying temperature. Thus, there is an upper limit to the amount of injection which can be used for a particular product.

In the Accelerated Freeze-Drying (A.F.D.) method of food preservation (Hanson, 1961) the food is placed between heater plates and is separated from them on both sides by expanded aluminium sheets. This gives a combination of radiant and conductive heating but because it causes considerable obstruction to vapour flow the drying temperature is high, but the drying rate is fast. Presumably much of the vapour is superheated and returns part of this heat in the form of additional convection heating.

Continuous Removal of the Dried Product

Another approach to avoiding the danger of burning the dried product with radiant heating occurred to me, namely, to remove the dried product continuously by scraping so that the radiant heat contacted only a frozen surface. Theoretically, it should be possible to dry at an extremely fast rate, and to test this theory I built the apparatus shown in Figs. 7 and 8.

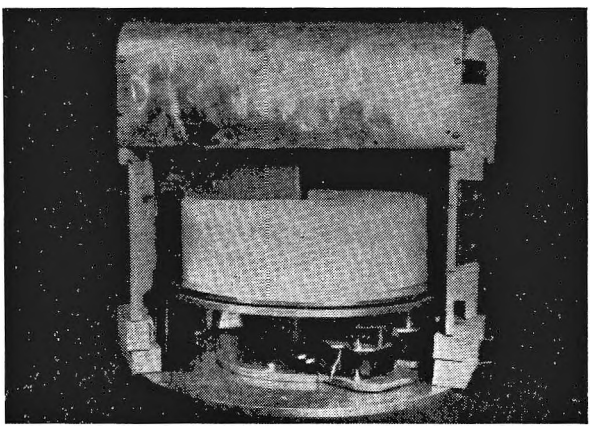

FIG. 7. Apparatus for the continuous scraping and removal of the dried surface.

This apparatus proved that the theory was correct. The limitations which I met with were firstly when I burnt out the heating elements and, secondly, at very high rates of drying, the dried particles were carried away in the vapour stream to the condenser. Nevertheless, drying rates 200 times as fast as conventional drying rates were achieved.

Dielectric Heating

Finally, dielectric heating. The dielectric constants of frozen and dried material differ considerably, and it should be possible to put heat into the the frozen material without any danger of heating the dried material. There are certain technical difficulties in applying this type of heat, mostly concerned with ionisation and flash-over in the vacuum chamber. These difficulties have been largely overcome now that it is possible to develop considerable power at a frequency around 1,000 mcs./sec. But, unfortunately, the ice block is heated fairly uniformly and gradients develop much as with conductive heating. Moreover, there is a tendency for heating not to be uniform, leading to melting at a point. At once considerable positive feedback occurs and the whole block of ice explodes. Possibly as still higher frequencies become available a skin effect may result so that all the energy is absorbed at the drying surface when enormous drying speeds should be achieved without the danger of burning the dried product.

However, these high drying rates could only be achieved with very thin layers as the obstructive resistance of the dried material would cause melting of the frozen product.

Microwave heating might, however, prove very useful in the period of desorption, when the removal of the last traces of water from the dried material proves a slow process using conventional methods of heating.

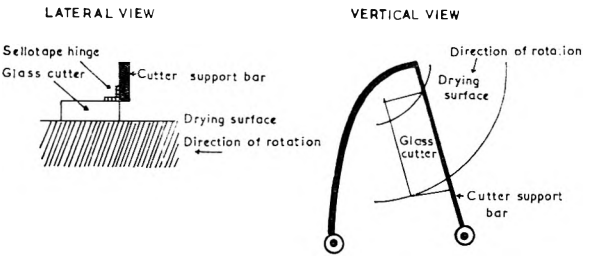

FIG. 8. Schematic diagram of surface scraping mechanism.

HIGH VACUUM SPRAY FREEZE-DRYING

The most successful method for drying foods has been spray drying of liquids in a current of hot air at atmospheric pressure. Owing to the large surface for evaporation from the small particles, drying is very rapid at a temperature well below that of the hot air stream. Such spray drying plants being continuous in their operation and constant in their energy requirements, have proved to be economical to operate. But there is usually a loss of flavour and solubility of the products dried by this process. A lowering of the air temperature leads to an improvement in quality of the dried product but at the expense of an increase in drying time and an increase in the size of the apparatus.

Improved products can be obtained by drying at lower temperatures by reducing the pressure. Such plants usually distribute the liquid as a thin layer on a belt in a vacuum chamber. If the pressure is low enough, the liquid may froth or foam but this, by increasing the drying surface, may be an advantage. Usually the lower the drying temperature the better the dried product, but a lower limit in temperature is set by the temperature at which the product freezes, for if this occurs the feed mechanism also freezes.

A continuous batch process for the freeze-drying of solid foods has been devised. In this process a batch of prefrozen material is introduced through a lock into a vacuum tunnel through which it progresses, the dried material being removed from time to time by way of a vacuum lock at the far end of the tunnel. Liquid foods prefrozen in blocks could also be dried in such a tunnel.

Nevertheless, a process for freeze-drying liquids, in which the liquid could be continuously introduced, the dried powder being removed by way of a vacuum lock from time to time as required for packaging, would offer great attractions to the food industry and also in the pharmaceutical

industry when freeze-drying is necessary as a step in manufacture, as in the production of streptomycin.

The problems of spraying liquids into a freeze-drying chamber are associated with the extremely rapid evaporative freeze which occurs and which leads to the freezing and complete occlusion of the jet.

My early attempts to overcome this problem (Greaves, 1946), led me to conclude that a very high velocity of the liquid as it left the jet orifice was essential. Some success was achieved by making a jet from a minute hole in a piece of brass foil. Such jets could be made so fine that they discharged only about 100 ml./hr., but were very apt to be blocked by particulate matter, and the frozen particles were very variable in size.

After the war, the American Chain Belt Co. patented a process for continuous freeze drying in which the liquid was continuously introduced t hrough a jet and fell as liquid onto a moving belt on which it froze.

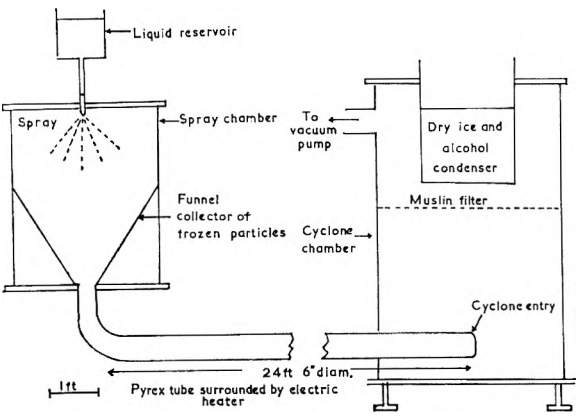

FIG. 9. Schematic diagram of experimental apparatus for vacuum spray drying.

I was able to experiment with one of the jets, as used by the Chain Belt Co. It is the type which discharges the liquid as a cone, the peripheral edge of the cone breaking up into droplets. The smallest size passed 1·6 gal./hr. The main feature of this type of jet is that the liquid leaves the jet orifice at high velocity. Using this jet to spray into a freeze-drying chamber with a low vacuum, it was possible to produce frozen particles. If the vacuum improved, freezing took place at the edge of the cone, the frozen material looking rather like corn-flakes. Further improvement of the vacuum led to freezing nearer and orifice of the cone and eventually to freezing at the orifice and total blockage of the jet.

Nevertheless, by careful control of the vacuum, it was possible to keep this jet running continuously, and an apparatus was built to study the possibility of continuous freeze-drying using this jet for introducing the liquid (see Fig. 9). The idea was that the difference in pressure between the spraying chamber and the cyclone collecting chamber would drive the particles through the long narrow Pyrex tube, during which period they would be subjected to intense radiant heat. The dry particles should then

be separated from the vapour stream in the cyclone chamber being deposited as a dry powder on its base. But the enormous velocity of the particles through the heater tube had not been anticipated. Nothing could ever be seen, but at the start the tube emitted a musical note which rapidly rose in pitch till it became ultrasonic. It is not surprising that very little heat was absorbed in this very short space of time. Attempts to apply further heat were made by heating the walls of the cyclone, but were not very effective, nor was the cyclone very effective in separating the particles from the vapour stream.

With the jet passing a minimum of 1·6 gal./hr., any experiment was a major engineering project and was costly and time consuming, and it was decided that further experiments must cease until such time as a jet which would pass small quantities could be devised. It is a pity that micro-wave heaters were not available when these experiments were being made, for this apparatus would have been admirably suited for testing its efficiency; but even if it were effective, the problem of removing the particles from the vapour stream would still remain.

It is against this background that I have been working to produce a jet which would allow liquid to be continuously introduced into a freeze-drying chamber. Such a jet should be able to pass very small quantities of about 100 ml./hr. so that small scale apparatus could be used in development, but it should also be able to pass large quantities if required. It should also be possible to vary the particle size over a large range at will.

Principles on Which the Experiments were Based

If the jet was to work with small volumes, high velocity at the orifice could not be obtained with small holes in thin membranes due to the danger of blockage. Speed away from the orifice must therefore be obtained by a moving member.

The rate of flow should be variable and adjustable externally.

The bore of the jet should be reasonably wide and the end should be flat and adjustable so that it is very close to the moving member. This should produce a zone of high vapour pressure so that freezing between the jet and the moving member should be avoided.

Experimental

All the experiments were conducted in a small glass cylinder $6\frac{1}{4}$ in. internal diameter, $10\frac{1}{2}$ in. high, mounted over a hole in the top plate of a small freeze-drying plant, having a condenser mechanically refrigerated to $-40°$ by a quarter h.p. Freon 12-Compressor.

Experiments with High Speed Moving Member

Fig. 10 is a photograph of the apparatus. The moving member is a brass disc of 4 in. in diameter mounted on the spindle of a small motor rotating at a speed of 1,450 rev./min.

The jets which have been used all have an internal bore of $\frac{1}{25}$ in., their ends varying between $\frac{1}{10}$ in. and $\frac{1}{4}$ in., and are of brass, steel or rubber.

The flow rate is controlled externally by rubber pressure tubing squeezed in a differential thread clamp.

In operation, when the vacuum has fallen to 0·1 mm. Hg, the motor for the brass disc is started and the clamp on the liquid line slowly opened. Small particles of ice will be discharged from the jet orifice, and it is important at this stage not to increase the flow too much until the disc has been cooled, when an ice track will form on the disc. The flow may now be increased till the maximum required rate is achieved.

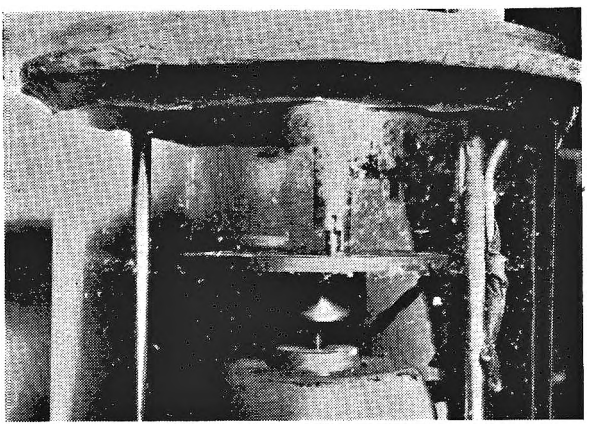

FIG. 10. Electronic flash photograph showing ice track forming on high speed horizontal moving member.

From direct observation, it would appear that most of the liquid leaves the jet as a fine liquid spray which rapidly freezes and drops as frozen particles. This is confirmed by the fact that a proportion of the larger particles reach the glass cylinder which is very close to the jet, and freeze on it. Some of the liquid also freezes on the ice track and is scraped off as it comes round to the back of the jet. This is confirmed by raising the jet when the ice track merely becomes thicker; this is convenient for it ensures that the gap at the orifice of the jet is always kept very small.

As the flow rate is increased, the particle size becomes progressively larger and more and more will freeze on the wall of the glass cylinder. It would be an obvious necessity to work with a much larger cylinder if large volumes were to be processed. The maximum flow of milk, using this small cylinder, was $\frac{1}{2}$ litre/15 min. Beyond this, a large proportion froze on the cylinder wall.

The minimal flow was about 100 ml./hr. Below this rate the flow became intermittent, probably due to intermittent freezing of the jet orifice from direct contact with the ice track as it was no longer being warmed by a sufficient passage of liquid.

The mean particle size is the product of the rate of flow and the speed of the moving member. The slower the speed or the greater the flow the larger the particle.

Drying experiments were carried out with milk. The frozen particles were collected in a metal tin in which was a rotating comb to keep the powder stirred. The tin was heated from below.

If drying was allowed to take place very slowly with no stirring, a very beautiful dried product resulted. But when stirred, large amounts of heat could be absorbed and drying was very rapid, but all the finer particles were carried away in the vapour stream and were lost.

No doubt means could be devised to overcome this difficulty, and it had been intended to experiment along these lines, but further experiments with the jet at slow speeds have shown a much simpler solution of this problem.

Experiments with Slow Speed Moving Member

A second apparatus was constructed with the brass disc on a shaft passing to the exterior so that it could be driven with a reduction gear motor.

At 56 revs./min. the jet operated very much as at high speed but the particle size was very much greater. Less material was thrown off the disc centrifugally; much more freezing on the ice track on the disc which consequently got much colder. It was, therefore, necessary to have a higher rate of flow to prevent the jet freezing than was necessary at high speed.

At 1 rev./min. all the liquid froze on the disc once it became cold and could be removed by a scraper.

At this speed difficulties start to occur. If the disc is too hot the ice does not stick to it and so does not get carried away. It is, therefore, necessary to start the flow very slowly. When the disc is about 5°, the ice gets carried away from the jet satisfactorily, but has separated by the time it arrives at the scraper which merely has to push the ice over the edge of the disc. If the disc gets too cold considerable power is necessary to separate the ice from it. There is also a danger of the jet freezing if the disc gets too cold and, although this may be cured by using a rubber tipped jet, the rate of flow is critical. Suitable thermostatic control of the disc temperature should cure this, but has not been tried.

This continuous extrusion of ice into a freeze-drying chamber does give a product very suitable for drying which has sufficient weight to prevent it being carried away in the vapour stream. But for continuous extrusion the radial drag of a rotating horizontal disc appears incorrect. It was, therefore, decided to construct a drum rotating in a vertical plane. The speed of rotation was 1 rev./min. and the diameter of the drum 4 in.

Fig. 11 shows that if the drum is at the correct temperature no scraping device is necessary to separate the ice from the drum which extrudes as a continuous helix, giving the perfect product for drying.

This continuous extrusion is fascinating to watch. The jet is flat and not shaped to the radius of the drum. It is mounted slightly forward so that the back edge is in contact with the drum. The ice seems to form continuously on the front edge of the jet, and bubbles of gas and liquid can be seen inside the frozen tape immediately on extrusion.

In order to observe this phenomenon more clearly, a $1\frac{1}{2}$ in. diameter drum was constructed so that, with a $\frac{1}{4}$ in. jet, there was considerable discrepancy between the front edge of the jet and the drum. Under these conditions liquid could be seen flowing forward along the surface of the jet then freezing up the front edge and continuously pushing forward only making contact with the surface of the drum about $\frac{1}{10}$ in. in front of the jet. On stopping the drum, ice continued to be extruded for a while. It is possible that working on these lines it might be possible to make a jet which would continuously extrude ice without a moving member.

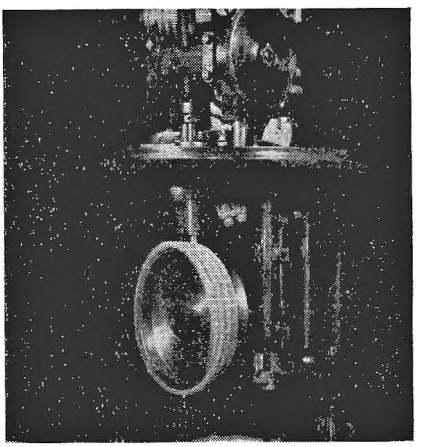

FIG. 11. Ice forming as a continuous helix on slow speed drum.

Using this smaller drum, much greater latitude in operation was obtained by increasing the linear speed from 12 in./min. to 48 in./min. (Fig. 12).

A scraper bar was fitted so that the ice was separated from the drum after three-quarters of a revolution. A radiant heater was focused on the remaining quarter of the drum and adjusted so that the temperature of the drum remained constant at 0° and a small heater placed round the jet to maintain its temperature at +15°.

Under these conditions extrusion could be maintained for a very long period with complete constancy.

CONCLUSION

This review is based on two lectures given at the School of Pharmacy, London University. This explains why my own work figures so largely. I have, however, tried only to describe recent advances in fundamental work and have made no attempt to describe the enormous amount of developmental and applied work which has been done since the war.

In the laboratory and in the pharmaceutical industry, the freeze-drying procedure is widely used for the preservation of very valuable materials.

The cost and time of drying is relatively unimportant provided a perfect product results. Too often freeze-drying is carried out as a purely empirical procedure, and if the result is not satisfactory, the process of freeze-drying is condemned. I would like to see the term freeze-drying reserved for a procedure in which drying takes place below the lowest eutectic temperature of a material which has been treated during pre-freezing so as to ensure that no metastable glassy material is present.

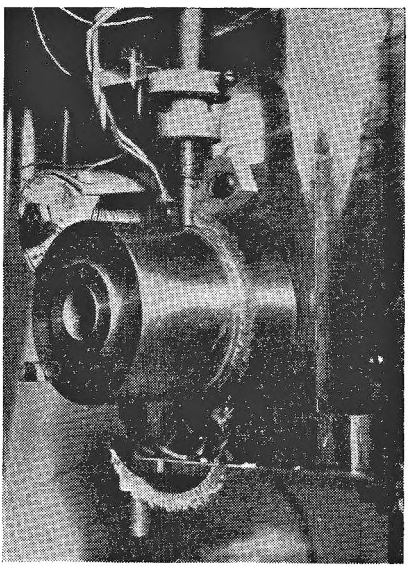

FIG. 12. Final form of apparatus. Linear speed of drum 48 in./min. The ice is removed from the drum after three-quarter revolution. The radiant heaters for the drum and jet can be seen. Electronic flash photograph. Note the section of ice falling from the drum.

A very different problem is posed in the drying of foods. Here we are attempting to preserve, by drying, a relatively cheap commodity, and the cost of achieving the previous criteria would usually be prohibitive. Foods must be dried as rapidly as possible and at the highest temperature consistent with obtaining an acceptable product. Most of the dried foods could not claim to be freeze-dried if my definition is accepted and, for this reason, the freeze-drying of foods has been omitted from this review.

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RESEARCH PAPERS

THE PHOSPHORYLATION OF ANTI-ADRENERGIC QUATERNARY AMMONIUM SALTS RELATED TO CHOLINE

By F. C. Copp, T. S. G. Jones and A. McCoubrey*

From The Wellcome Research Laboratories, Beckenham, Kent

Received April 30, 1962

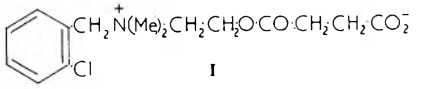
Quaternary ammonium salts related to choline with anti-adrenergic properties have been phosphorylated. The resulting betaines were used to investigate a possible means for improving the oral absorption of quaternary ammonium salts. One of these was converted to the α-glycerophosphate for similar study.

The utility of bretylium for the treatment of hypertension has been criticised because of poor and possibly erratic absorption of oral doses (Dollery, Emslie-Smith and McMichael, 1960). The degree of absorption of oral doses of guanethidine, a drug often compared to bretylium, may be little better than that of bretylium (Dollery, Emslie-Smith and Milne, 1960) but so far there has been no suggestion of an erratic absorption of this drug. Some evidence for uneven absorption of oral bretylium can be found in the results of Duncombe and McCoubrey (1960) and this could arise from the peculiar absorption characteristics of quaternary ammonium salts in general (Levine, Blair and Clark, 1955). They appear to be rapidly absorbed by the intestine for a short time but the rate soon declines to a very low value. The greater part of a dose is not absorbed.

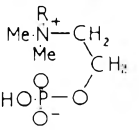
In attempts to overcome this difficulty, analogues of bretylium bearing a choline unit of structure were esterified to give betaines which were anticipated to have pK values more suited to intestinal absorption (Hogben, Tocco, Brodie and Schachter, 1959) yet suffer hydrolysis by tissue enzymes after absorption to regenerate the parent quaternary ammonium salt.

Initial experiments were made with a succinyl derivative (I) (Coker and Copp, 1960) but the pharmacological results were disappointing. Phosphorylcholine is hydrolysed at a moderate rate by non-specific phosphomonoesterases (Roche and Bouchilloux, 1947) and better results were obtained with a weakly acid phosphoric ester betaine (II; R = *o*-chlorobenzyl) (Boura and McCoubrey, 1962). Conversely, the strongly acid diphosphoryl compound (III), given orally, was less readily absorbed than the compound (II; R = *o*-chlorobenzyl) though it was hydrolysed *in vitro* by both acid and alkaline phosphatase from rat liver and intestine respectively. It had moderate activity by the subcutaneous route.

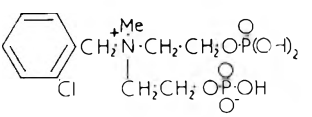
* Present address: Research and Development Laboratories, Reckitt and Sons, Ltd., Hull.



I



II



III

Choline can be phosphorylated by various methods (Baer, 1952), the simplest by heating with polyphosphoric acid (Beznák and Chain, 1937). The compounds chosen for phosphorylation gave moderate yields of betaines by this method. During purification, the removal of the excess of phosphoric acid was troublesome. Part of the barium phosphate produced by neutralisation with baryta assumed a colloidal state, though on a few occasions it came down wholly as a heavy precipitate. The conditions for complete precipitation were not discovered. Preliminary boiling to hydrolyse meta- and pyro-phosphoric acids had no influence. In initial experiments the products were difficult to crystallise. Paper chromatography showed that they contained traces of phosphoric acid and when this was removed by passing methanolic solutions down a column of Amberlite IR 45 (OH^- form) they crystallised readily. In this process methanol was used to suppress ionisation of the betaine while allowing the first ionisation of phosphoric acid so that the betaine could pass through the column whilst phosphoric acid was retained.

For the preparation of a ^{14}C-labelled betaine the excess of phosphoric acid was neutralised by ammonia and the bulk of the ammonium phosphate was precipitated by ethanol. Ammonia was then removed by passage down a column of Amberlite IR 120 (H^+) and residual phosphoric acid precipitated by shaking with silver oxide. The soluble silver salt of the betaine was then decomposed by hydrogen sulphide.

Glycerophosphates are not hydrolysed by phosphomonoesterases (Schmidt, Greenbaum, Fallot, Walker and Thannhauser, 1955) though they are readily attacked by phosphodiesterases. It was considered that presentation of an anti-adrenergic agent as a glycerophosphoryl ester betaine, a neutral molecule, might alter the pattern of distribution of the drug, and, in particular, might allow the agent to penetrate into the brain, which contains a phosphodiesterase (Webster, Marples and Thompson, 1957). BW 171C60 (II; R = *o*-chlorobenzyl) was therefore converted into the α-glycerophosphate. The method was not sterospecific and involved preparation of the allyl ester of BW 171C60 and subsequent hydroxylation by alkaline permanganate. Hydroxylation by the method of Woodward, Gunstone and Morris (1957) was unsuccessful and led to loss of the phosphoric ester group, possibly by intramolecular cleavage of a carbonium ion arising by abstraction of bromide ion. The glycerophosphate (BW 564C61) was purified as its cadmium chloride complex by the method used for glycerophosphorylcholine (Tattrie and McArthur, 1955).

EXPERIMENTAL

The dihydroxyphosphinyloxyethylammonium betaines of Table I were prepared in 60–70 per cent yield by the method of Beznák and Chain (1937). The bulk of the barium phosphate was removed by centrifuging and the cloudy supernatant was clarified by filtration through a Sterimat pad. The filtrate was adjusted to pH 4 by 2N sulphuric acid, barium sulphate filtered off and the filtrate evaporated to dryness. The residue was dissolved in 3 parts warm anhydrous methanol and the solution passed through a column (30 × 1 cm.) of Amberlite IR 45 (OH^-) that had been previously washed with water and methanol. The effluent was collected from the first appearance of the betaine and it was washed through by 6 parts of methanol. A small quantity of the betaine remained on the column and could be recovered by further washing. The column could be used repeatedly without regeneration. Evaporation of the effluents gave white residues that crystallised readily from the appropriate solvent (see Table I).

Aqueous solutions of the betaines (1 per cent) had pH approximately 4.

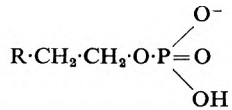
N-o-*Chlorobenzyl*-NN-*di*[2-(*dihydroxyphosphinyloxyethyl*]-N-*methyl*-*ammonium betaine* (BW 293C60) was prepared in 40 per cent yield by phosphorylation of *N-o*-chlorobenzyl-*NN*-di(2-hydroxyethyl)-*N*-methylammonium iodide by the method mentioned above. It crystallised readily from ethanol without need for removal of adsorbed phosphoric acid. The white prisms had m.p. 220–223°. (Found: P, 14·8. $C_{12}H_{20}ClNO_8P_2$ requires P, 15·2 per cent.) An aqueous solution (0·2 per cent) had pH 2·4.

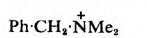
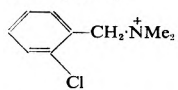
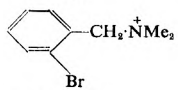
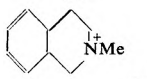
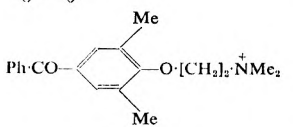
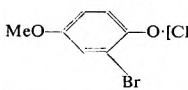
N-o-*Chlorobenzyl*-N-2-*hydroxyethyl*-N-*methyl*-N-[^{14}C]*methylammonium iodide* (BW 329C57). *N-o*-Chlorobenzyl-*N*-2-hydroxyethyl-*N*-methylamine (Coker and Copp, 1960) (211 mg.) was dissolved in ethyl methyl ketone (1 ml.). [^{14}C]Methyl iodide (142 mg.; 1 mc) was condensed on to the frozen solution at −80°. The mixture was allowed to regain room temperature in a stoppered tube, when a crystalline mass formed within 3 hr. After keeping overnight, ethyl acetate (0·5 ml.) was added and after 1 hr. the precipitated solids were centrifuged down, washed with ethyl methyl ketone and finally dried *in vacuo*. The product had m.p. 85–88° (335 mg.; 95 per cent).

N-o-*Chlorobenzyl*-N-2-(*dihydroxyphosphinyloxy*)*ethyl*-N-*methyl*-N-[^{14}C]*methylammonium betaine* (BW 171C60). The above ^{14}C-labelled material (335 mg.; 1 mmole) was heated on a steam-bath with phosphoric acid (90 per cent; 2 g.) under vacuum with a coarse air leak directed to the top of the liquid until the theoretical loss in weight (0·33 g.) had occurred (1 hr.). Phosphorus pentoxide (1 g.) was added and the heating continued for 8 hr. The product was dissolved in water (3 ml.) and warmed gently for 30 min. Aqueous ammonia (30 per cent) was added slowly with cooling until the solution was alkaline. Ammonium phosphates were precipitated by ethanol (75 ml.). The mixture was cooled to 0° and centrifuged and the residue washed with ethanol. The combined supernatants were evaporated to small bulk and passed down a column (10 × 1 cm.) of Amberlite IR 120 (H^+). The effluent was shaken with

TABLE I

2-(DIHYDROXYPHOSPHINYLOXY)ETHYLAMMONIUM BETAINES



R	M.p.	Solvent	Formula	Found			Required		
				C	H	H_2O	C	H	H_2O
	215–216°	ethanol	$C_{11}H_{18}NO_4P \cdot H_2O$	47·1	7·2	6·7	47·6	7·2	6·5
 (*a*)	222–223°	ethanol-ethyl acetate	$C_{11}H_{17}ClNO_4P \cdot H_2O$	41·9	6·0	6·7	42·4	6·1	5·8
 (*b*)	218–219°	ethanol-acetone	$C_{11}H_{17}BrNO_4P \cdot H_2O$	37·1	5·2	5·5	37·1	5·3	5·1
	237–238°	methanol-ether	$C_{12}H_{18}NO_4P \cdot H_2O$	49·1	6·6	5·0	49·8	6·9	6·2
	206–207°	ethanol-ethyl acetate	$C_{21}H_{28}NO_6P \cdot H_2O$	57·7	7·1	4·7	57·4	6·9	4·1
 (*c*)	49–51°	—	$C_{13}H_{21}BrNO_6P \cdot 3H_2O$	34·4	5·7	12·0	34·5	6·0	12·0

(*a*) Cl: Found, 11·0; Required: 11·0 per cent
(*b*) N: Found, 4·0; Required: 3·9 per cent
(*c*) Dried Material—Br: Found, 20·0; Required: 20·1 per cent

fresh silver oxide (from silver nitrate, 0·5 g.) and solids were centrifuged down. The soluble silver salt of the betaine was decomposed by hydrogen sulphide and the mixture filtered and evaporated to dryness. The residue was diluted with unlabelled BW 171C60 (160 mg.) and crystallised from ethanol. Radio-assay of the product (280 mg.) indicated 0·475 mc/mmole (48 per cent). Autoradiographs of chromatograms showed freedom from starting material and one spot at the position occupied by BW 171C60.

N-o-Chlorobenzyl-N-2-[(±)-α-*glyceryloxy(hydroxy)phosphinyloxy*]-*ethyl*-NN-*dimethylammonium betaine* (BW 564C61). BW 171C60 (3 g.) in water (15 ml.) was shaken with fresh silver oxide (from 1·8 g. silver nitrate) for 30 min. and the solids were filtered off. The solution was evaporated to dryness and suspended in ethanol (45 ml.) and allyl bromide (0·9 ml.) added. The mixture was heated at 50° for 1·5 hr., filtered, and the filtrate was evaporated to dryness. The residue was dissolved in water (5 ml.) and passed down a column (10 × 1 cm.) of Amberlite IRA 400 (OH^-). The effluent was collected so long as small portions decolourised bromine water. It was evaporated to dryness and the allyl ester crystallised from acetone-ethanol in white needles, m.p. 96–97° (2·2 g.; 73 per cent). (Found: C, 47·5; H, 6·6; N, 3·8; Loss at 100°, 4·6. $C_{14}H_{22}ClNO_4P\cdot H_2O$ requires C, 47·2; H, 6·5; N, 4·0; Loss at 100°, 5·1 per cent).

TABLE II

AVERAGE R_F VALUES FOR THE ABOVE PRODUCTS VISUALISED BY DRAGENDORFF'S REAGENT

Solvent system	BW 329C57	BW 171C60	BW 171C60 allyl ester	BW 564C61
Propanol-ammonia (25 per cent) (6:4) ..	0·84	0·51	—	0·63
s-Butanol-acetic-water (12:5:3)	0·75	0·47	0·73	0·53
Pyridine-n-butanol-ammonia (1:1:1)	0·75	not visualised	0·66	not visualised

The above ester (2·9 g.) was dissolved in water (150 ml.) and 2N sodium carbonate added to give pH 8–9. The solution was cooled to 0° and potassium permanganate (1·8 g.) in water (50 ml.) added with stirring during 15 min. The mixture was adjusted to pH 7 and manganese oxides filtered off. Traces of permanganate in the filtrate were destroyed by bisulphite and the solution was evaporated to dryness. The residue was extracted with hot ethanol and the extract evaporated to small bulk before adding saturated ethanolic cadmium chloride solution. The crystalline precipitate was collected, washed with ethanol, and dried *in vacuo* over phosphorus pentoxide. It melted over the range 112–176°. (Found: C, 24·0; H, 3·9; N, 2·0; P, 4·1; total Cl, 21·2; ionisable Cl, 16·5; loss at 100°, 5·0. $C_{14}H_{23}ClNO_6P\cdot 1\frac{3}{4}\ CdCl_2\cdot 2H_2O$ requires C, 24·2; H, 3·7; N, 1·9; P, 4·2; total Cl, 21·8; ionisable Cl, 17·0, loss at 100°, 4·9 per cent).

The product gave a positive chromotropic acid test and was oxidised by periodate. For pharmacological study the product was dissolved in water and shaken with excess silver carbonate at room temperature for

1 hr. Solids were removed and soluble silver precipitated by hydrogen sulphide. The solution was shaken with activated charcoal and filtered, evaporated to dryness and redissolved in butanol. A small amount of solid was removed and the butanol was removed *in vacuo*. The product was a colourless syrup. Yield, 2·25 g. (74 per cent). Chromatograms revealed a trace of BW 329C57 due to hydrolysis.

Chromatographic information is given in Table II.

Acknowledgements. The authors gratefully acknowledge the determination of the specific activities of labelled products by Dr. W. G. Duncombe, and analyses by Mr. P. R. W. Baker. Skilled technical assistance was provided by Mr. A. Lane.

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THE ABSORPTION OF BRETYLIUM AND RELATED QUATERNARY AMMONIUM SALTS FROM THE ALIMENTARY TRACT

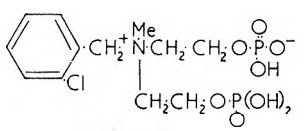
BY A. L. A. BOURA* AND A. MCCOUBREY*

From The Wellcome Research Laboratories, Beckenham, Kent

Received April 30, 1962

N-Methyl-^{14}C-labelled bretylium was injected into the lumen of segments of the gastrointestinal tract of cats prepared so that blood draining from these segments could be collected for analysis. The drug was not absorbed from the stomach. The concentration of drug in the venous blood from the duodenum rose steadily during 1·5–2 hr. but then declined to a value slightly exceeding that in the systemic blood. The greater part of the dose was not absorbed. Bretylium slowly traversed rat duodenum (mucosa to serosa) *in vitro* and the rate was not affected by factors that can influence carbohydrate metabolism. Transfer proceeded equally well from serosa to mucosa. To investigate means of obtaining better absorption of quaternary ammonium anti-adrenergic drugs a close analogue of bretylium bearing a choline residue was phosphorylated to give a phosphoric ester betaine (III). This was about twice as active by the oral route in cats as the parent drug, in which form it was excreted in the urine.

ONLY a small proportion of oral doses of quaternary ammonium salts is absorbed by the intestine (Levine, Blair and Clark, 1955). The effective oral to subcutaneous dose ratio of bretylium (II; R = H, X = Br) was about 5:1 in cats (Boura and Green, 1959) but a suspected erratic absorption of oral doses in man has been a source of criticism in the therapeutic application to treatment of hypertensive disease (Dollery, Emslie-Smith and McMichael, 1960). The absorption of bretylium was therefore studied *in vitro*, using rat duodenum, and *in vivo*, using cats.



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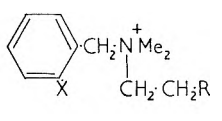

II

An analogue of bretylium, *o*-chlorobenzyl-*NN*-dimethyl-*N*-2-hydroxyethylammonium iodide (BW 329C57; II; R = OH, X = Cl) had comparable adrenergic neurone blocking activity to bretylium in the Finkleman preparation and in intact cats (Boura and Green, unpublished). Since the rate of intestinal absorption of weak acids and bases has been related to their pK values (Hogben, Tocco, Brodie and Schachter, 1959), it was considered that the phosphoric ester betaine of BW 329C57 (II; R = O·PO(OH)O$^-$; X = Cl), a weak acid, might have more favourable ionisation characteristics for absorption from the intestine. It was further

* Present address: Research and Development Laboratories, Reckitt and Sons, Ltd., Hull.

surmised that body phosphatases could hydrolyse the ester after absorption to liberate the active quaternary ammonium salt. The concept was extended to a brief study of the more strongly acid betaine, BW 293C60 (I), and to the neutral α-glycerophosphate ester betaine BW 564C61 (II; R = $O{\cdot}PO(O^-){\cdot}O{\cdot}CH_2{\cdot}CHOH{\cdot}CH_2OH$, X = Cl).

Methods

New drugs are described in the preceding paper (Copp, Jones and McCoubrey, 1962). All chromatograms were run in s-butanol-acetic acid-water (12:5:3) unless stated otherwise, and drugs were visualised either by spraying with Dragendorff's reagent diluted to pale orange colour or by autoradiography.

Assay of BW 329C57 (II). Since this compound did not form complexes with sulphonic acid dyes soluble in halogenated solvents the method used for bretylium (Duncombe and McCoubrey, 1960) could not be applied. It was assayed nephelometrically by titrating Dragendorff's reagent with the drug eluted from paper chromatograms. Using a reagent (1 ml.) containing one molecular proportion of bismuth trichloride to four of potassium iodide a minimum of 25 μg. BW 329C57 could be assayed.

Samples of urine from cats that had received doses of BW 329C47 or BW 171C60 were depleted of salts and protein by addition of 2 volumes of alcohol and cooling to 0°. The solutions were filtered and aliquots chromatographed on Whatman No. 1 paper. Marker spots were visualised by spraying with a dilute Dragendorff's reagent (sensitivity 1 μg.) and the indicated area of paper was eluted by not more than 0·2 ml. water. The volume of eluate was measured in a graduated pipette (0·2 ml.) adapted as a burette, the tip of which had been ground to minimum area and waxed. The eluate was added to the reagent (1 ml.) contained in a scratch free tube in approximately 0·01 ml. amounts until the Tyndall effect due to a suitable beam of light persisted. Frequent controls were necessary to compensate for temperature changes. The reagent was prepared fresh for each titration by mixing bismuth trichloride (4 per cent in 2N hydrochloric acid; 0·5 ml.) and potassium iodide (8·4 per cent in 0·2N sodium hydroxide. Use of N hydrochloric acid increased the sensitivity slightly but the reagent became too unstable. Results were calculated from the expression

$$\mu\text{g. BW 329C57 in eluate} = VS\left(\frac{1+y}{y}\right)$$

where V = eluate volume, $S = \frac{100v}{1+v}$, v = standard titration volume using 100 μg./ml. BW 329C57, y = titration volume.

Recovery of 100 μg. amounts of drug averaged 86 ± 7 per cent in 8 trials.

Assay of BW 171C60. A satisfactory chemical assay was not devised. Perchloric acid oxidation (Hanes and Isherwood, 1949) of eluates from paper chromatograms and assay of inorganic phosphate gave erratic

results. A minimum of 20 μg. drug could be visualised on paper chromatograms by Dragendorff's reagent. It was finally assayed by use of ^{14}C-labelled material.

Assay of radioactive drugs. Blood samples were dried to constant weight (4 days) over phosphorus pentoxide under reduced pressure. The residues were powdered and plated on polythene planchettes for counting at infinite thickness under a mica end window counter. Urines, intestinal contents or salines were depleted of salts and protein if necessary by the addition of two volumes of ethanol and cooling to 0°. Aliquots (usually 40 μl.) of the filtrates, concentrated if necessary, were plated on lens paper for counting. Similar aliquots with added radioactive drug sufficient roughly to double the counting rate (usually about 200 counts/min.) were used for calculation of results.

Enzymes

An alkaline phosphatase was prepared from rat intestinal mucosa (Long, 1953). An acid phosphatase was prepared from rat liver (Goodlad and Mills, 1957). In both instances the rate of hydrolysis of phosphoric ester betaines (60 μmoles) was followed by assaying aliquots of the incubated mixtures for inorganic phosphate at suitable time intervals. The activity of the enzyme preparations was assessed by measuring the rate of hydrolysis of β-glycerophosphate.

A phosphodiesterase was prepared from rat liver (Dawson, 1956). The rate of hydrolysis of BW 564C61 was assessed qualitatively by examination of visualised spots of BW 329C57 (Dragendorff's reagent) and α- and β-glycerophosphates (acid molybdate reagent) on paper chromatograms.

Pharmacological Evaluations

Experiments in vitro. The preparation described by Finkleman (1930) was used to study the effects of drugs on the function of inhibitory postganglionic sympathetic nerves supplying rabbit intestine. The mesenteric nerve was stimulated from platinum electrodes with supramaximal shocks at 50 pulses/sec. for 15 sec. every 3 min. The effect of drugs on motor actions of postganglionic sympathetic nerves was studied, using the hypogastric nerve-ductus deferens preparation of the guinea-pig (Huković, 1961). Supramaximal stimulation was applied to the nerve, using saline electrodes at 50 pulses/sec. for 1 sec. every min.

Experiments in vivo. Changes in sympathetic tone in groups of 5 cats were observed by measuring with calipers the portion of the nictitating membranes exposed across the palpebral fissure. Before dosage with drugs the cats were restricted to milk and water for 24 hr. and for oral dosage were lightly anaesthetised with ether to facilitate passage of a stomach tube.

Absorption from the Alimentary Tract in vivo

Cats were fasted for 18 hr. and anaesthesia was induced by ether and maintained by chloralose (60 mg./kg. intravenously).

Absorption from the stomach. The abdomen was opened in the midline and the cardiac and pyloric sphincters ligated. The intestines and spleen were removed after tying all vascular connections including those between the stomach and omentum. A loose ligature was placed round the portal vein just caudad to its bifurcation before entering the liver. The peritoneal cavity was filled with warm liquid paraffin. Heparin (10 mg./kg.) was given intravenously and the stump of the superior mesenteric vein was cannulated retrogradely with polythene tubing. By pulling on the ligature the venous outflow from the stomach could be diverted into the cannula and the blood collected in tared tubes. The right carotid artery was cannulated for collection of systemic blood. The drug was injected into the lumen of the stomach and blood samples were collected during 30 sec. to 1 min., depending on flow rate, every 15 min., 1–2 ml. of blood was collected at each sampling.

Absorption from the intestine. The technique described for the stomach was applied to the study of intestinal absorption. A 10 cm. portion of the duodenum or colon was isolated between ligatures. The remaining portions of the intestine, the stomach and spleen were removed, preserving the vascular connections of the isolated segment. Blood draining from the intestine to the liver was collected as described above.

In other experiments cats were given oral doses of drugs and urine was collected, by suprapubic puncture if necessary, every 24 hr.

Absorption from the Intestine in vitro

A portion of rat duodenum was everted, weighed and suspended in bicarbonate saline (50 ml.) at 37°. The tissue was cannulated at each end so that the contents from the serosal side could be washed out with warm saline (1 ml.) for analysis. In some experiments the tissue was not everted. Labelled bretylium was added to the bath to a final concentration, usually 10^{-3}M. The serosal fluid was assayed for radioactivity by evaporation to dryness, dissolution in 0·2 ml. water and plating in 40 μl. aliquots on lens paper in planchettes. Samples were taken every 30 min. The results were calculated by reference to a standard calibration curve and expressed as mg. bretylium absorbed/g. tissue/hr.

Results

BW 171C60, LD50 approximately 80 mg./kg. intravenously, was less toxic in mice than its hydrolysis product BW 329C57, LD50 40 mg./kg. The betaine did not cause appreciable block of adrenergic neurones within 30 min. in the Finkleman and ductus deferens preparations at 100 μg./ml., whereas BW 329C57 was active at 3 μg./ml. within 10 min. Fig. 1 shows that BW 171C60 relaxed the nictitating membranes of cats after oral doses of 5 mg./kg., whereas BW 329C57 was inactive at 10 mg /kg. At higher dose levels, 10 mg./kg. BW 171C60 had a similar degree of activity to 20 mg./kg. BW 329C57.

BW 293C60 was inactive in 3 cats given oral doses of 20 mg./kg., but relaxed the nictitating membrane of 1 cat at 10 mg./kg., s.c.

BW 564C61 had a similar degree of activity to BW 171C60 after oral dosage in cats. There were no signs of any central effect to suggest that the drug had entered the brain.

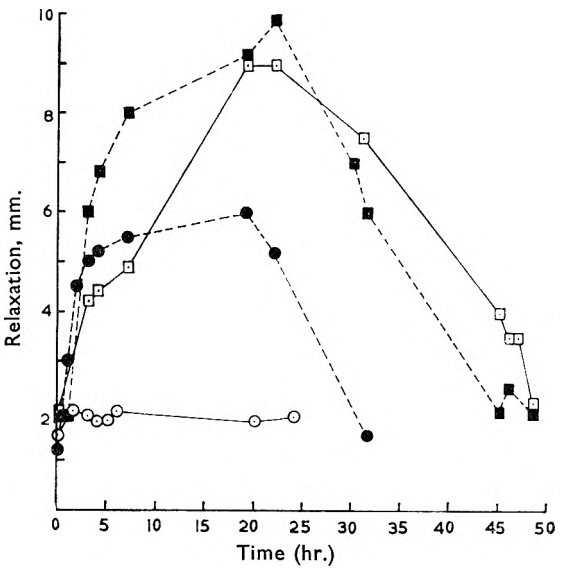

FIG. 1. Relaxation of the nictitating membranes of cats given oral doses of *N-o*-chlorobenzyl-*NN*-dimethyl-*N*-2-hydroxyethylammonium iodide (BW 329C57) and its phosphoric ester betaine (BW 171C60).

⊙——⊙ BW 329C57, 10 mg./kg. ●——● BW 171C60, 5 mg./kg.
⊡——⊡ BW 329C57, 20 mg./kg. ■——■ BW 171C60, 10 mg./kg.

Absorption of Bretylium by the Gastrointestinal Tract of Cats

Stomach. There was no detectable radioactivity in the blood draining from the stomach of a cat during one hour after injection into the lumen of 50 mg. ^{14}C-labelled drug (approximately 42 μc).

Duodenum. Fig. 2 shows that the rate of absorption of ^{14}C-labelled bretylium (50 mg.; approximately 21 μc) into blood draining from the duodenum was initially slow but that it began to rise fairly rapidly to reach a peak at 90–120 min. and then declined equally rapidly so that the concentration in the blood leaving the duodenum was scarcely higher than that in systemic blood. In this experiment 20·6 mg. of drug was absorbed during 210 min. In a second experiment, 6·0 mg. were absorbed during 165 min. leaving 32·0 mg. in the intestinal contents. A fairly high proportion (9·4 mg.) was found in the intestinal tissue. The amounts of drug absorbed were calculated by estimating the area enclosed by the curve relating μg./ml. in blood (corrected for systemic blood content) to time and multiplying by the average rate of blood flow from the duodenum.

Colon. A very slow rate of absorption was found during 3 hr. when the drug was injected into the lumen of the colon. The level in the blood draining from the tissue rose slowly during the experiment, finally

reaching 5·4 μg./ml. The level in systemic blood at this time was 0·6 μg./ml.

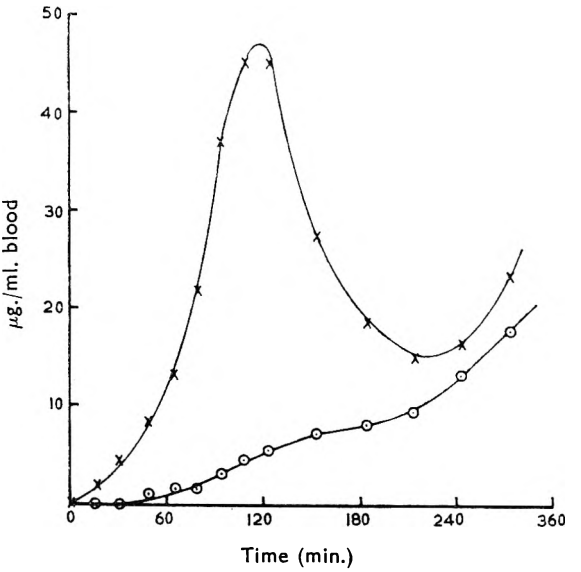

FIG. 2. The absorption of bretylium from the duodenum of cats. ^{14}C-*N*-Methylbretylium (50 mg.) was injected into the lumen of an isolated segment of cat duodenum. Samples of blood draining from the tissue and carotid blood were assayed for radioactivity. ×——× duodenal blood. ○——○ carotid blood.

Absorption of BW 171C60 by the Gastrointestinal Tract of Cats

Stomach. There was barely detectable radioactivity in the blood leaving the stomach during 200 min. after injection of 40 mg. of ^{14}C-labelled drug into the lumen (approximately 60 μc). No radioactivity appeared in systemic blood. Radioassay of the stomach contents indicated a recovery of 38 mg. of the dose and autoradiographs of chromatograms showed one spot at R_F 0·54, at the same position as a marker spot of BW 171C60.

Duodenum. No BW 329C57 could be detected by nephelometric assay in the blood draining from the duodenum after injection of 15 mg. BW 171C60 into the lumen (<2·5 μg./ml.). Chromatography of the intestinal contents after 180 min. showed that both BW 329C57 and BW 171C60 were present. Nephelometric assay indicated that 30 per cent of the dose had been hydrolysed.

There was very little absorption during 90 min. of 40 mg. of ^{14}C-labelled drug (approximately 60 μc). The maximum concentration of drug in the blood draining from the tissue was 4·4 μg./ml. at a time when the level in systemic blood was 1·2 μg./ml. Assay of the lumen contents showed that 35·8 mg. of the dose remained unabsorbed and this was accounted for on autoradiographs by BW 329C57 (R_F 0·71). There was no trace of BW 171C60. Extraction of the pooled dried blood samples coming

from the intestine with ethanol and autoradiography of the extract revealed a weak spot at R_F 0·55 due to BW 171C60. Incubation of heparinised cat blood (1 ml.) with BW 171C60 (1 mmole) at 37° for 2 hr. caused an increase of inorganic phosphate equivalent to 5·5 per cent of the added drug.

After oral dosage in conscious cats. A cat, deprived of food for 24 hr. received 52 mg. of drug orally. Urine was collected by suprapubic puncture at 0, 8, 24 and 32 hr. after the dose. The urine was collected in a 6 per cent trichloroacetic acid, centrifuged and assayed for BW 329C57 nephelometrically. Recoveries were respectively 0, 12·6, 10·8 and 3·6 mg., equivalent to a total of 24·6 mg. of BW 171C60. Chromatograms revealed no trace of BW 171C60.

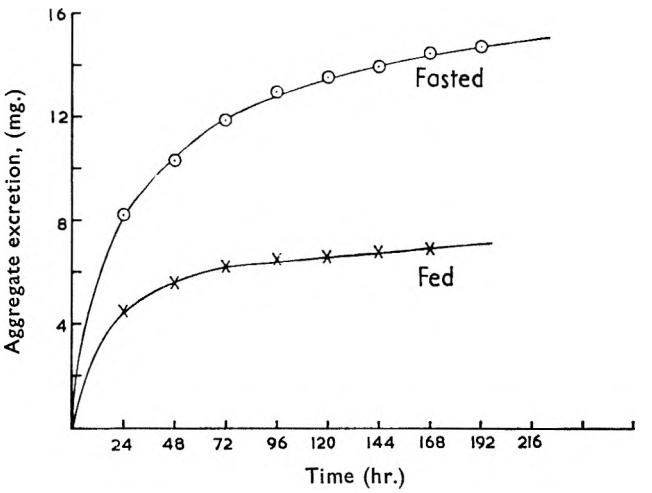

FIG. 3. Excretion of radioactivity in the urine by cats after oral doses (30 mg.) of *N*-*o*-chlorobenzyl-*N*-methyl-*N*-^{14}C-methyl-*N*-2-phosphatoethyl ammonium betaine (BW 171C60)

In a similar experiment, using a cat fed its normal diet before receiving 46 mg. of drug orally, the respective recoveries were 0, 2·8, 3·3 and 0 mg. BW 329C57, equivalent to 5·5 mg. BW 171C60 absorbed.

These experiments were repeated, using the ^{14}C-labelled material. 30 mg. was given to a cat, fed normally before the dose, and to a cat whose food had been withdrawn for 24 hr. Urine was collected every 24 hr.

Fig. 3 shows that excretion of radioactivity persisted for 7 days and that a greater proportion of the drug was absorbed by the fasted cat. These figures may not represent the total absorption since a small proportion of all similar drugs examined so far has been excreted into the bile and is presumably voided in the faeces. The nictitating membranes of all four cats were relaxed.

Absorption of BW 564C61

A cat received 14 mg. of the drug orally and urine was collected for 24 hr. About 50 per cent of maximal relaxation of the nictitating

membranes occurred. Chromatography of aliquots of the urine in propanol-15 per cent ammonia (6:4) and spraying with Dragendorff's reagent showed 2 spots. That at R_F 0·77 was identified as BW 329C57 by comparison with a marker spot. A spot at R_F 0·65 may have been unchanged drug but the appropriate marker spot was double (R_F 0·57 and 0·67). By rough assessment of the size of spots given by varying amounts of urine it was estimated that about 2·5 mg. of BW 329C57 had been excreted, probably with about the same amount of unchanged drug.

When the above dose (5 mg./kg.) was doubled there was a greater relaxation of the nictitating membranes and chromatograms of urine again revealed two spots, R_F 0·74 and R_F 0·64. The recovery was much smaller in this instance but the urine sample was also very small (47 ml.) and had to be collected by suprapubic puncture.

Absorption of Bretylium by Rat Duodenum in vitro

Fig. 4 shows that the rate of absorption of bretylium by everted rat duodenum (mucosa to serosa) rose to a peak at about 2·5 hr. and then

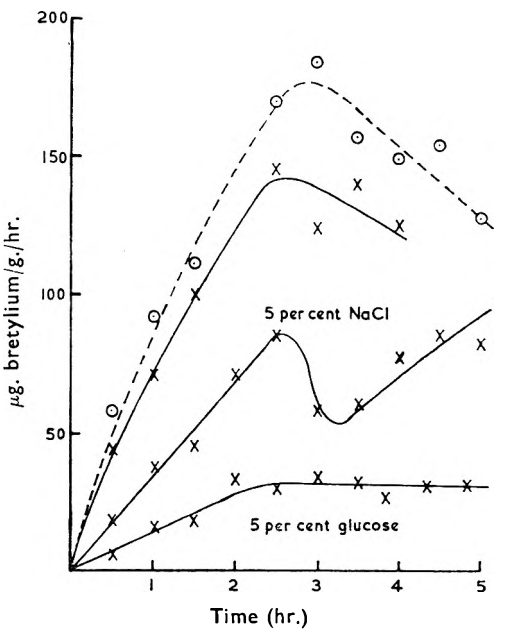

FIG. 4. The diffusion of ^{14}C-*N*-methylbretylium across rat duodenum *in vitro*. ×——× Everted sacs of duodenum were suspended in bicarbonate buffer containing labelled bretylium (10^{-3}M). The serosal fluid was assayed for radioactivity. ○ – – – ○ Duodenal sac not everted.

began to decline, similar to the results found *in vivo* in cats. At the peak rate the concentration of bretylium on the serosal side was approximately half that of the bathing solution. The total amount of drug removed from the bath during the experiments had negligible influence on the

initial concentration present. Fig. 4 also shows that there was no noticeable difference in the rate of passage of drug from serosa to mucosa.

Some attempts were made to influence the rate of transfer by modifying the bathing solution at 90 min. Increasing the glucose concentration five times, replacing glucose by glutamate, omission of glucose, or addition of iodoacetate (10^{-4}M) had no appreciable influence. Placing 5×10^{-4}M bretylium on the serosal side caused a temporary slackening of the appearance of labelled bretylium on the serosal side during 60 min., followed by restoration of the original rate. Adrenaline (10^{-5}M) caused a temporary slackening during 60 min. Lowering the pH of the bath to a value of 5 had little effect. The only agents to cause notable change were 5 per cent glycine and 5 per cent sodium chloride which appeared to prevent further rise in absorption rate. This maintained the value attained before the addition.

The rate of absorption of bretylium from 5×10^{-4}M solution was about twenty times faster than from 5×10^{-5}M solution.

Hydrolysis of BW 171C60, BW 293C60 and BW 564C61 in vitro

No inorganic phosphate was liberated by heating BW 171C60 (3·3 mg.) for 30 min. on a steam bath in either 5N hydrochloric acid or 5N sodium hydroxide. Chromatograms of aliquots revealed only unchanged material after spraying with Dragendorff's reagent.

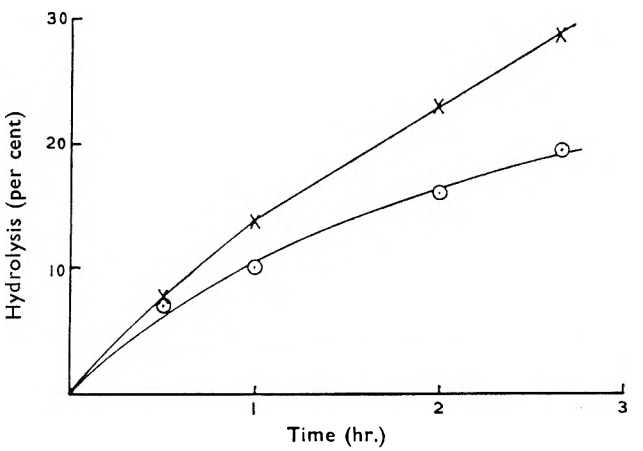

FIG. 5. The rate of hydrolysis of phosphate ester betaines by the alkaline phosphatase of rat intestinal mucosa. Aliquots of the incubated mixture of enzyme plus drug were analysed at intervals for inorganic phosphate. Each point is the mean of 2–3 experiments. ×——× BW 293C60. ○——○ BW 171C60.

BW 564C61 was completely hydrolysed during 15 min. heating on a steam bath in either N hydrochloric acid or sodium hydroxide. Paper chromatograms and suitable sprays revealed α- and β-glycerophosphate, inorganic phosphate and BW 329C57. It was stable to 0·1N acid or alkali at 37° during 2 hr.

Fig. 5 shows the rate of hydrolysis, calculated by analysis for inorganic phosphate, when BW 171C60 and BW 293C60 were incubated with the

alkaline phosphatase of rat intestine, equivalent to 2 per cent of the total extracted activity of the mucosa of one rat. β-Glycerophosphate (50 μmoles) was almost completely hydrolysed during 2 hr. under the same conditions. BW 171C60 (6 μmoles) was 10 per cent hydrolysed during 2 hr. at 37° by the acid phosphatase of rat liver whereas β-glycerophosphate (10 μmoles) was 10 per cent hydrolysed during 1 hr. under the same conditions.

BW 564C61 was unaffected by either alkaline or acid phosphatase from rat intestine and liver respectively but it was appreciably hydrolysed by the phosphodiesterase of rat liver. No attempt at accurate measurement was made but paper chromatograms revealed BW 329C57 and α- and β-glycerophosphate.

Discussion

The rate of absorption of bretylium from the intestine of the cat *in vivo* showed the same peculiarity observed for other quaternary ammonium salts (Levine, Blair and Clark, 1955), that is, a steady rise followed by a steady fall, though most of the dose remained in the lumen. There were indications for similar behaviour *in vitro* using rat duodenum. At peak absorption rate *in vivo* the dose of 50 mg. could have been absorbed within 3 hr. It seems clear that the intestine is capable of absorbing bretylium at an appreciable rate but that the process is subject to self limitation. No notable irregularity in absorption rate was observed in any of these experiments but the occasional erratic control of blood pressure described in clinical use of bretylium (Dollery, Emslie-Smith and McMichael, 1960) could conceivably be due to rapid clearance of blocked absorption channels. Duncombe and McCoubrey (1960) obtained some evidence for erratic excretion of bretylium. From the experiments with rat duodenum *in vitro* the mechanism of absorption seems to be one of simple diffusion. Transfer of drug occurred equally well from serosa to mucosa as vice versa. Absorption was not affected by addition of iodoacetate and transfer of ^{14}C-labelled drug from mucosa to serosa was only temporarily delayed by increasing the serosal concentration of unlabelled drug. It seems reasonable to conclude that the absorption channels become obliterated by the drug during transfer but the nature of the channels remains undefined.

The results from conscious cats given BW 171C60 fulfilled the expectations mentioned in the introduction. The drug was more readily absorbed than was BW 329C57, judging by the effect on the nictitating membranes. Though not proven, there is little reason to doubt that the pharmacological effects of BW 171C60 at the doses used are those of its hydrolysis product, BW 329C57, in which form it was excreted in urine. BW 171C60 was inactive *in vitro*. Nevertheless, assuming that urinary excretion accounts for the greater part of the dose given, the proportion of BW 171C60 absorbed is still relatively low. Several points of interest deserve mention. The drug was not hydrolysed during passage across the intestinal wall and was not actively hydrolysed by blood. Since it was excreted in urine as BW 329C57, the hydrolysis must have occurred in other tissues,

presumably mainly in the liver. Though the drug is a weak acid it was not absorbed appreciably by the stomach. Brodie and Hogben (1957) consider that all weak acids are, at least theoretically, capable of traversing the stomach wall. The drug was not hydrolysed during its stay in the stomach though it was rapidly hydrolysed in the intestine. The failure to observe appreciable absorption by the isolated intestinal loop may be accounted for by the liberation of phosphatase into the lumen during surgical preparation though the point was not investigated experimentally. This may also account for the smaller degree of absorption during digestion, though in unpublished work we have noted a smaller degree of absorption of a similar drug given after feeding and in this instance phosphatase can play no direct part.

The results with BW 564C61 were disappointing in so far as this drug was synthesised with a view to studying the influence on the brain of an anti-adrenergic drug conveyed by a natural as opposed to a parenteral route. Being a neutral molecule it was considered that it might traverse the blood brain barrier to suffer hydrolysis within cerebral tissue by phosphodiesterase. Since it was not feasible to make ^{14}C-labelled material and chemical analysis of brain tissue for small amounts of BW 329C57 is virtually impossible, the brains of animals receiving the drug were not analysed. It is interesting however that the drug appeared to be much more stable to the tissues than BW 171C60. This could be due to its stability to phosphomonoesterases, a property shared by α-glycerophosphorylcholine. Conversely the product was a racemic mixture and one isomer may be less susceptible to enzymic hydrolysis. Further work with this substance was abandoned because of its intractable nature.

Acknowledgements. The authors gratefully acknowledge the counting of our ^{14}C-labelled specimens by Dr. W. G. Duncombe, and the technical assistance of Messrs. J. Harry, A. Lakeman and R. Palmer.

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COMPARATIVE STUDY OF THE CALCIPHYLACTIC CHALLENGING POTENCY OF VARIOUS IRON COMPOUNDS

By Ralph Strebel,* Jaromir Vašků and Hans Selye

From the Institut de Médecine et de Chirurgie expérimentales Université de Montréal, Montreal, Canada

Received May 7, 1962

Systematic experiments in rats sensitised for calciphylaxis by pre-treatment with dihydrotachysterol were made using fourteen different iron compounds as challengers. It was noted that the selective localisation of soft-tissue calcinosis in various organs depends largely upon the carrier to which the iron atom is attached; hence, under otherwise identical conditions, essentially distinct calciphylactic syndromes can be produced in rats given the same amounts of iron attached to different carriers.

Calciphylaxis is a condition of hypersensitivity in which—especially during a "critical period" after sensitisation by a systemic calcifying factor, for example vitamin-D compounds, parathyroid hormone—topical treatment with certain challengers such as egg white, egg yolk or metallic salts, causes an acute local calcinosis (calcification of soft tissues) followed by inflammation and sclerosis. In rats suitably sensitised, for example with dihydrotachysterol, such calciphylactic reactions can also be elicited selectively at predetermined sites (in the skin, joints, pancreas, bile ducts, uterus, spleen, lung, trachea, thyroid, parathyroid, carotid body, Brunner's glands, cells of the reticulo-endothial system) by the intravenous administration of challenging agents that exhibit a particular affinity for certain organs. The techniques for the production of the various calciphylactic syndromes as well as their structural characteristics have been described in detail elsewhere (Selye, 1962).

Among the challengers that can elicit both topical and systemic calciphylactic reactions, iron salts are of special interest for various reasons:

1. Iron-containing complexes are widely distributed in the body and since they often occur in the pathological calcium deposits of man, iron may play a rôle as an endogenous elicitor of calcinosis.
2. When administered intravenously to appropriately sensitised animals, various iron compounds elicit calcification in different parts of the body, depending presumably upon their special affinity for one or the other tissue.
3. Iron preparations are commonly used in clinical medicine, especially in the treatment of anaemias.
4. Under certain circumstances, calciphylaxis can prevent the cardiovascular and renal calcinosis, normally produced by vitamin-D compounds and parathyroid hormone. This is due to a kind of "reverse calciphylaxis" obtainable only through very diffuse iron impreganation

* Fellow of the U.S. National Institutes of Health, Fellowship—No. MF-8987-C3.

of the tissues throughout the body. Here, apparently, innumerable minute foci of iron in various forms cause a widespread and diffuse distribution of the mobilised calcium and thus impede the formation of large circumscribed lime deposits in vital organs (Selye and Strebel, 1962).

In view of these considerations, we felt that it would be rewarding to compare the calciphylactic challenging potency of various iron compounds given under strictly comparable conditions in doses containing equiatomic amounts of metallic iron.

Material and methods

145 female Sprague-Dawley rats of the Holtzman farm, with a mean initial body weight of 99 g. (range 95–105 g.) were subdivided into 15 groups as shown in Table I.

Dihydrotachysterol (Calcamin, Dr. A. Wander, S.A., Basel, Switzerland) was administered at the dose of 1 mg. in 0·5 ml. of corn oil by stomach tube to all animals on the first day of the experiment.

On the second day 14 iron compounds were injected intravenously (jugular) in amounts containing 1 mg. of metallic iron in 1 ml. of water. Since the chemical composition of many among these preparations has not been completely clarified, available information with the name of the suppliers is listed.

Ferrous carbonate saccharated (City Chemical Co., New York): 20 per cent ferrous carbonate, 70 per cent sugar, 10 per cent lactose (N.F. = not less than 15 per cent $FeCO_3$ = 7·23 per cent Fe).

Iron nucleate (National Biochemical Co., Cleveland, Ohio): a complex of iron and nucleic acid said to correspond to 1 ml. of nucleic acid and 2 atoms Fe. The preparation contains 4·6 per cent of metallic iron.

Iron dialysed (British Drug Houses, Poole, England): contains about 3·5 per cent Fe ≡ 5 per cent Fe_2O_3, or 6·7 per cent $Fe(OH)_3$.

Ferric phosphate soluble (Fisher Scientific Co., Fair Lawn, N.J.): a complex salt of sodium ferricitrophosphate, containing 12–15 per cent Fe, 15 per cent P_2O_5, 45 per cent citric acid.

Ferric potassium tartrate or tartrated iron (City Chemical Co., New York): approximate composition K(FeO) $(C_4H_4O_6)$ + water of crystallisation. It contains about 18 per cent Fe, 65 per cent tartaric acid.

Ferric potassium citrate (City Chemical Co., New York): a complex salt containing about 16 per cent Fe and 65 per cent citric acid.

Ferric potassium oxalate (City Chemical Co., New York): $K_3Fe(C_2O_4)_3.3H_2O$. Anhydrous salt 89·00 per cent, H_2O 11·00 per cent, $K_2C_2O_4$ 50·75 per cent, Fe 11·37 per cent, anhydrous oxalic acid 54·98 per cent.

Iron peptonised (City Chemical Co., New York): contains 16–18 per cent Fe.

Iron oxide saccharated or "Fe–OS" (*Proferrin*, Merck Sharp & Dohme, West Point, Pa.): a mixture of ferric saccharate, approximate composition $C_{12}H_{22}O_{11}(Fe_2O_3)_2$ and some sodium saccharate plus free sugar.

Ferric hydroxide dextran complex or "Fe–Dex" (*Imferon, Imposil*, Benger Laboratories, England): each ml. is equivalent to 50 mg. Fe.

Ferric hydroxide dextrin complex or "Fe–Din" (*Ferrigen*, Astra Soedertälje, Sweden): contains 2 per cent Fe.

Ferric chloride (Fisher Scientific Co., Fair Lawn, N.J.): $FeCl_3.6H_2O$.

Ferrous chloride (Fisher Scientific Co., Fair Lawn, N.J.): $FeCl_2.4H_2O$.

Ferrous sulphate (Fisher Scientific Co., Fair Lawn, N.J.): $FeSO_4.7H_2O$.

The animals were maintained exclusively on Purina Laboratory Chow (Purina Co. of Canada) and tap water. On the sixth day the experiment was terminated by killing all surviving animals with chloroform. At autopsy the organs were macroscopically inspected with a stereoscopic loupe and representative specimens from various organs were fixed in ethanol-formol (4 parts absolute ethanol, 1 part 10 per cent formalin) for the subsequent histochemical demonstration of calcium with the von Kóssa or celestine blue techniques as previously described (Selye, 1962). The former technique depends upon formation of a black precipitate between calcium phosphate and silver nitrate, while the latter results from staining the calcified organic matrix with celestine blue under standard conditions.

Results

As can be seen from Table I, the 14 iron compounds used in this study produced very different calciphylactic syndromes (which consist of diverse patterns of organic calcification) although all of them were administered at a dose containing equiatomic amounts of metallic iron. To comment on each of the tabulated organ lesions here would hardly be warranted, but a few of them deserve special mention.

In the dihydrotachysterol-pretreated controls which received no iron compounds (Group 1), calcium deposition was only of moderate intensity. This "nonspecific calcinosis," produced by systemic calcifiers alone, is virtually limited to the naturally predisposed organs namely the heart, kidney and stomach; being independent of challenge, it should not be confused with true calciphylactic reactions.

The most widespread calciphylactic responses were obtained by solubilised ferric phosphate (Group 5) and ferric potassium tartrate (Group 6), which produced calcium deposition in most of the target organs examined. The action of some other compounds is much more organ-specific. For example, Fe–Din (Group 12), which elicits a very obvious anaphylactoid type of swelling of the snout, induced calcification of the lips in the dihydrotachysterol-sensitised animal. This change was extremely pronounced in our experiment although the other organs showed little calcinosis at the low dose level at which Fe–Din was administered here.

It is also noteworthy that the acute toxicity of the iron compounds tested varied between wide limits, some of them being tolerated without causing any immediate disturbance (Groups 3, 4, 9, 10, 11 and 12), while others could be tolerated only if the injections were performed slowly, taking several minutes for the administration of the required

TABLE I

COMPARATIVE STUDY OF THE CALCIPHYLACTIC CHALLENGING POTENCY OF VARIOUS IRON COMPOUNDS

Group	Number of rats	Treatment*	Skin	Salivary gland	Thyroid	Parathyroid	Heart		Oesophagus	Stomach	Duodenum	Pancreas	Thymus	Kidney	Carotid body	Articulation	Lips	Mortality per cent
							Auricle	Ventricle										
1	10	None	0	0	0	0	0	1·0	0	0·2	0	0	0	1·3	0	0	0	0
2	10	$FeCO_3$ saccharated	0·4	0·1	0·1	0	0·2	1·5	0	0·6	0·2	0	0·2	1·5	0	0	0	10
3	10	Iron nucleate	0·1	0	0	0	0	0·7	0	0·5	0	0	0	0·65	0	0·1	0	0
4	10	Iron dialysed	0	0	0·1	0	0	0·7	0	1·1	0	0	0	0·5	0	0	0	0
5	10	Ferric phosphate soluble	0·7	1·6	1·8	2·1	0	0·3	0·4	2·2	2·35	1·15	0·2	2·2	0·5	0·1	0	10
6	15	Ferric potassium tartrate	0	1·2	1·4	1·5	0	1·0	0	2·0	1·8	1·5	0·4	1·6	0·3	0	0	30
7	10	Ferric potassium citrate	1·2	0·5	0·3	0·6	0	0·6	0	0·8	0·5	0	0·25	1·5	0	0	0	10
8	15	Ferric potassium oxalate	0	0·5	1·7	1·5	0·4	1·4	0·2	1·4	1·2	0·8	0·1	2·0	0·4	0	0	53
9	10	Iron peptonised	0·9	0·6	0·8	0·9	0	0·1	0	1·4	0·4	0	0	0·6	0	0	0	0
10	10	Ferric hydroxide saccharated "Fe–OS"	0·3	0·2	0	0	0·5	0·9	0	0·6	0·2	0	0	0·7	0	0	0	0
11	10	Ferric hydroxide-dextran complex "Fe–Dex"	0·3	0·5	0	0	0·2	0·1	0	0·5	0·1	0	0	0·1	0	0	0·1	0
12	10	Ferric hydroxide-dextrin complex "Fe–Din"	0	0	0	0	0	0·7	0	0·5	0·1	0	0·3	0·8	0	0	1·2	0
13	10	Ferric chloride	0	0	0	0	0	0·3	0	1·3	0·5	0·3	0·3	0·5	0	0	0	0
14	10	Ferrous chloride	0	0	1·8	2·4	1·0	0·6	0	0·8	1·8	0	0	0·8	0·6	0	0	80
15	5	Ferrous sulphate	—	—	—	—	—	—	—	—	—	—	—	—	—	—	0	100

* In addition to the agents listed in this column, all animals received a single sensitising dose of dehydrotachysterol as described in the text.

amount (Groups 5, 6, 7, 8 and 13); still other compounds induced a high mortality rate even when thus injected (Groups 14 and 15). Many of the iron preparations in this series, appear to produce tachyphylactic responses in that a second injection given a few minutes after a first dose is better tolerated.

Discussion

It has long been known that the absorbability, stability and the rate of diffusion of various iron preparations depends largely upon the carrier (anion or ligand) to which the metal is attached. Using the Prussian-blue reaction for the demonstration of iron in tissues, it could also be shown histochemically that iron thus administered in various forms, possesses an affinity for different organs depending upon the carrier; the largest amounts of the metal are usually found in the organs that respond with calcification in the sensitised rat. It is difficult to understand how mere differences in the solubility, stability or diffusibility of iron compounds could account for their vastly different specific organ affinities. *A priori*, it might be expected that a stable and highly diffusible compound could best traverse the capillary walls and spread through tissues throughout the organism without organ selectivity. Our observations show, however, that the distribution of the various iron preparations differs not only quantitatively but also qualitatively.

Since ferrification and subsequent calcification are predominantly limited to connective tissue elements, which are structurally quite similar, it may be reasonably assumed that the biochemical constitution of the stroma is largely dependent upon the adjacent parenchymal elements. For example, the connective tissue compounds of the pancreas, salivary glands and thyroid do not differ essentially in their histologic structure. This similarity is even more striking when we compare the stroma of the thyroid with that of the parathyroids, which are situated within the same regional blood vessel, lymph vessel and nervous supply systems. Yet, the calciphylactic sensitivity of these structures is not the same. These findings suggest that through calciphylaxis we may be able to detect differences in the biochemical constitution of the stroma in organs whose connective-tissue framework shows no morphologic evidence of specificity.

Only in one instance was it possible to identify a structural difference in the connective tissue that could account for its selective calciphylactic sensitivity. The "anaphylactoid shock organs" of the rat and particularly the lips are rich in mast cells and these discharge their granules during the anaphylactoid reaction that occurs for example after treatment with a compound such as Fe–Din. Even Fe–Dex can produce such a discharge although only at higher dose levels than those used in the present experimental series. In these cases, the discharge of the mast cell granules appears to be causally related to the development of the anaphylactoid oedema. In the calciphylactically sensitised rat the induction of an anaphylactoid reaction by such an iron-containing compound results in

the excess localisation of iron in the target organ. Histologic examination of the lips in Fe–Din treated rats shows that in fact Prussian-blue positive iron granules tend to localise in and around the walls of small vessels, wherever mast cell discharge is intense and presumably these iron deposits are responsible for the subsequent attraction of calcium.

Acknowledgements. This work was supported by grants from The National Heart Foundation of Canada and The Department of Health for the Province of Quebec.

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STUDIES ON *DATURA LEICHHARDTII* MUELL. EX BENTH.

PART I. THE ANATOMY OF THE LEAF AND STEM

BY W. C. EVANS AND N. A. STEVENSON

From the University, Nottingham

Received January 8, 1962

Datura leichhardtii is readily distinguished from other species of the genus by its general morphology. Although similar to that of *D. stramonium*, the microscopy of the leaf differs in the length of the clothing trichomes and often in the form of the calcium oxalate crystals; it may be distinguished from other members of the Section Dutra, with the exception of *D. metel*, by the form and abundance of trichomes. *D. leichhardtii* differs from *D. metel* in possessing on the lamina of the leaf, glandular trichomes with a uniseriate stalk and a single-celled head. The values for palisade ratio and stomatal index are within the same range as those of allied species.

THE genus *Datura* has been extensively investigated from the viewpoint of taxonomic classification, genetics, commercial production of crude drugs and the study of alkaloid biogenesis. Most attention has focused on those species which are utilised commercially either as sources of alkaloids or for galenical manufacture and adequate descriptions are available for these plants. One species which has received little attention, either anatomically or chemically is *D. leichhardtii* Muell. ex Benth., a plant named by Mueller (1855) and subsequently described in Bentham's (1868) *Flora Australiensis*. Its natural distribution appears to be limited to Australia where it has been cited as occurring along the banks of the rivers of Western Australia and in Queensland (Bentham, 1868; Ewart and Davies, 1917; Hurst, 1942; Gardner and Bennetts, 1953).

In addition to the possible usefulness of *D. leichhardtii* as a source of tropane alkaloids, it would also appear to be potentially useful in the study of the genetics of alkaloid production in the genus and, for breeding experiments involving interspecific hybrids. In this connection Blakeslee (Avery, Satina and Rietsema, 1959) has shown that this plant, used as the female parent, is capable of forming viable crosses with seven other herbaceous *Datura* spp. and with two others by embryo dissection. In this way it has been used as a bridging species for otherwise, often incompatible, crosses. This species forms an obvious link between Safford's (1921) Section I, Stramonium Gaertner and Section II, Dutra Bernhardi; it resembles the former in its general habit, leaves and flowers but is included in the latter because of its nodding capsule. The chromosome-end arrangements of two types of *D. leichhardtii* have been studied by Blakeslee and compared with other species of the genus (Avery, Satina and Rietsema, 1959).

In this paper we record the anatomical characters of the leaves and stems of this plant.

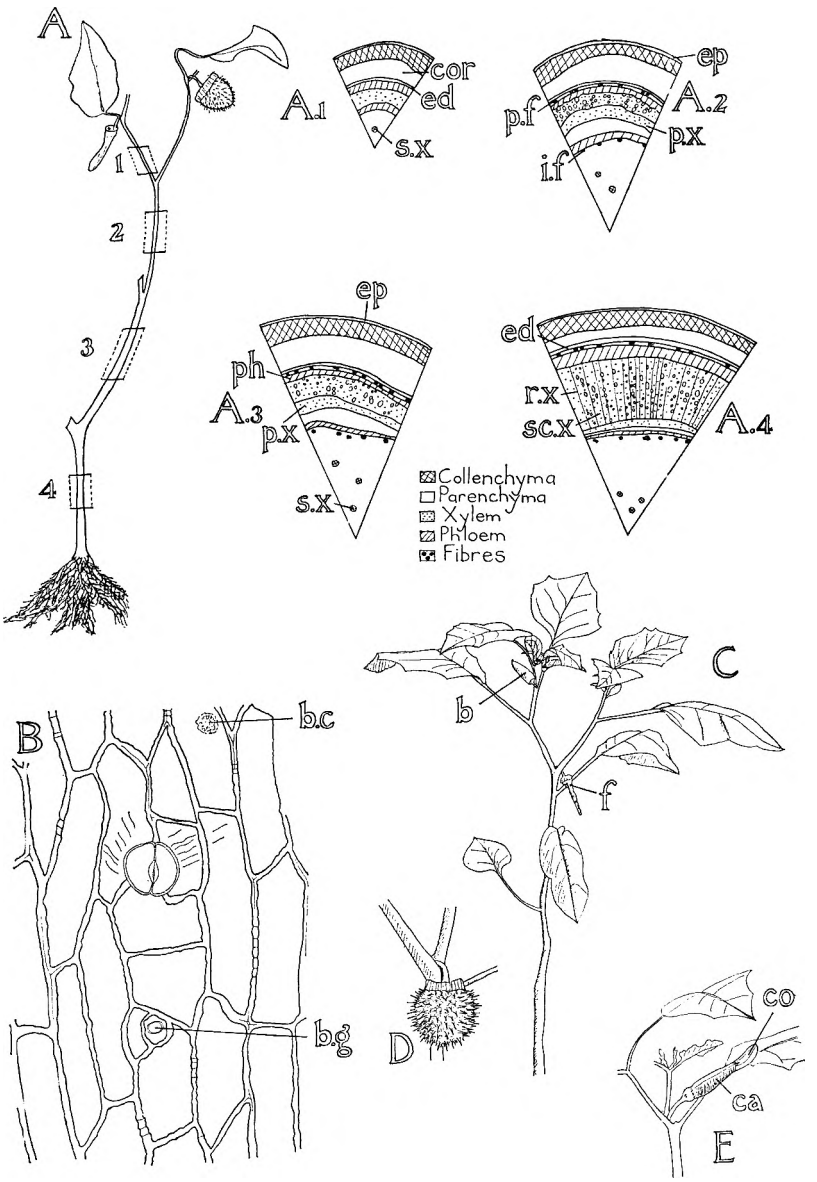

FIG. 1. *Datura leichhardtii* Muell. ex Benth. A1 to A4, general diagrams, × 10 of transverse sections of stem taken at positions 1 to 4 on A. B, epidermis of stem × 180. C, young plant × ¼. D, mature capsule × ¼. E, flower, Nottingham plant × ¼. *b*, bud; *b.c*, base of clothing trichome, *b.g*, base of glandular trichome; *ca*, calyx; *co*, corolla; *cor*, cortex; *ed*, endodermis; *ep*, epidermis; *f*, self-pollinated flower which has become pendant, with shrivelled perianth; *i.f*, internal fibres; *p.f*, pericyclic fibres; *ph*, phloem; *p.x*, primary xylem; *r.x*, medullary ray; *sc.x*, secondary xylem; *s.x*, sandy crystals of calcium oxalate.

Plant Material

The *D. leichhardtii* for this investigation was obtained in 1952 as dried specimens, including seeds, collected from an unspecified source in Australia. From the seeds, mature plants were raised in Nottingham and the propagation continued over a number of years from seed collected annually. The climate proved generally unsuitable for field-work with this species, but in warm dry summers some reasonably mature plants were produced in the open. Most of our material was grown in a temperate greenhouse. We have also examined the type specimen and others at the Kew Herbarium.

Anatomical Structure

Plants grown in Nottingham (Fig. 1, C) possessed the macroscopical characters ascribed by Bentham (1868) to *D. leichhardtii* and were similar to the Kew Herbarium specimens. Except during the hot summer of 1959, we have not succeeded in obtaining flowers with fully expanded corollas. The latter remain folded (Fig. 1, E) and after self-fertilisation the pedicel bends over and the dried remains of the perianth often adhere to the developing fruit. Avery and Satina (1959) also illustrate *D. leichhardtii* with an unexpanded corolla but several of the Kew herbarium specimens, collected in Australia, have fully opened flowers. This would seem to be a character influenced by environment as is also the development and dehiscence of the fruit. After pollination, the lower portion of the calyx develops into a flange which may be appressed to (Fig. 1, D) (also Avery and Satina, 1959) or reflexed away from (Gardner and Bennetts, 1953) the fruit. In dry conditions the capsule dehisces regularly into four valves which hinge back to reveal the seeds tightly packed in two loculi. Sometimes the seeds may be scattered as a result of the rapid splitting of the fruit wall; during or after dehiscence the whole capsule may fall from the plant. Damp conditions tend to promote less regular dehiscence and the pericarp disintegrates into a number of irregular soft portions which fall with the seeds leaving only the flange and septum attached to the axis.

Stems

The herbaceous stem has a bicollateral vascular system and Fig. 1, A1–A4 shows the distribution of tissues at different levels of the plant axis; the principal variation occurs in the amount of secondary xylem and presence of pericyclic and internal fibres at the different levels. Except in the instances stated, the following description applies to stems taken at any level.

The epidermis consists of elongated cells showing in surface view occasional striations of the cuticle. Fine pores traverse the unevenly thickened anticlinal walls and calcium oxalate as single prismatic crystals is occasionally evident. Anisocytic stomata are frequent and with older stems, often have associated with them brown amorphous material. Longitudinal cracks which sometimes occur in the epidermal layer just

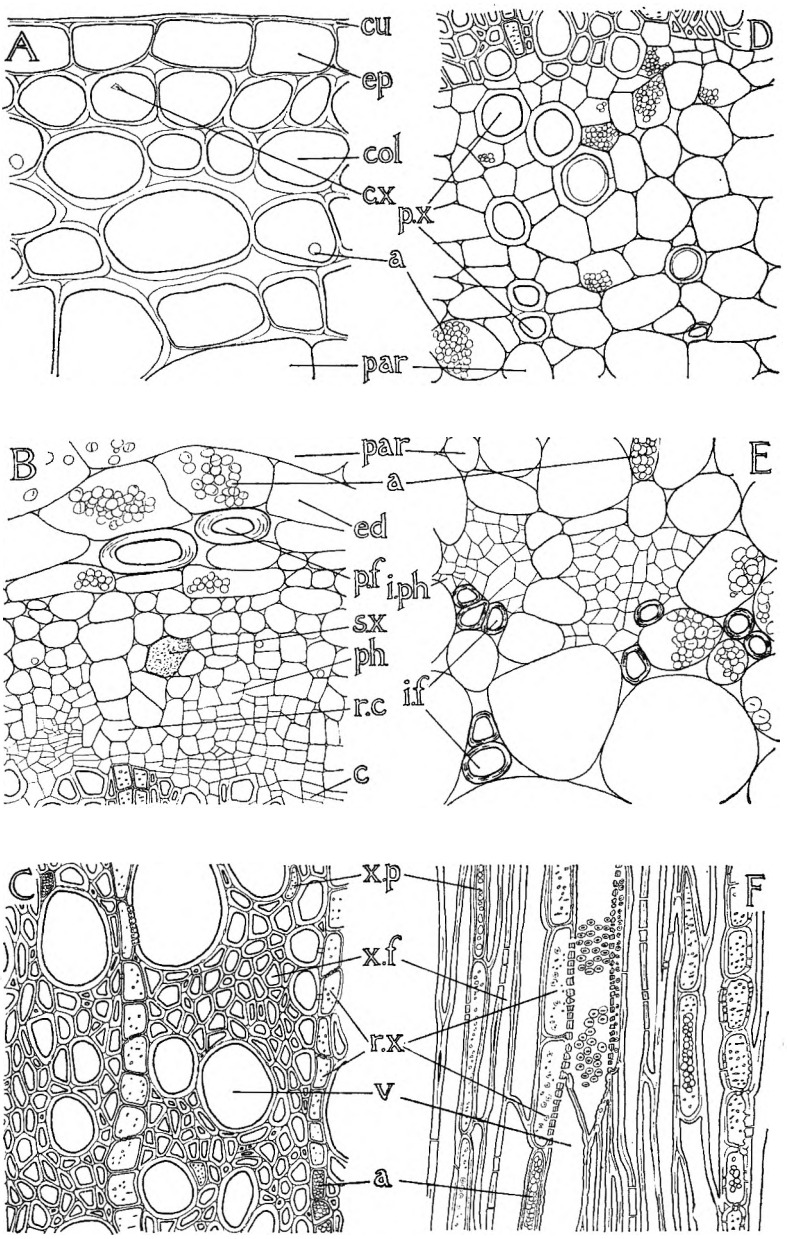

FIG. 2. Stem of *Datura leichhardtii* Muell. ex Benth. A–E transverse sections. A, cortical region; B pericycle and phloem; C, secondary wood; D, primary xylem; E, internal phloem; F, tangential longitudinal section of secondary xylem. All × 180. *a*, starch; *c*, cambium; *col*, collenchyma; *cu*, cuticle; *cx*, calcium oxalate crystal; *ed*, endodermis; *ep*, epidermis; *i.f*, internal fibres; *i.ph*, internal phloem; *par*, parenchyma; *p.f*, pericyclic fibre; *ph*, phloem; *p.x*, primary xylem; *r.c*, medullary ray cell in the phloem; *r.x*, medullary ray in the xylem; *s.x*, sandy crystals of calcium oxalate; *v*, vessel; *x.f*, xylem fibre; *x.p*, xylem parenchyma.

above soil level are filled with a brown disorganised callus tissue. For the epidermal cells, R = 18 to **20** to **30** to 45 μ; T = 24 to **30** to **50** to 60 μ and L = 36 to **60** to **120** to 144 μ (Fig. 1, B; 2, A). The epidermis of older stems bears only the remains of trichome bases (Fig. 1, B), but both clothing and glandular trichomes are present on the young stems. In structure, the trichomes resemble those of the leaves, described below, with the slight exception that those glandular trichomes with a single-celled head and a uniseriate stalk do not usually show warty walls (Fig. 6, A).

The hypodermal collenchyma in the young stems shows thickening in the corners of the cells, whereas in older stems the thickening extends around all the walls. Individual cells are longitudinally and usually tangentially elongated. R = 36 to 84 μ; T = 36 to 168 μ and L = 120 to 360 μ (Fig. 2, A). This layer merges into underlying thin-walled parenchyma of relatively large cells which are oval, round or angularly flattened in transverse section R = 75 to 300 μ; T = 120 to 360 μ and L = 75 to 150 μ. Intercellular spaces are present and single starch grains up to 15 μ in diameter are contained in the cells. The endodermis is clearly indicated as a starch containing sheath, individual starch grains resembling those of the cortex. In transverse section the cortex consists of about eight layers of cells (Fig. 2, A, B).

A discontinuous ring of small groups of, or isolated, fibres, oval or rounded in transverse section with highly refractive walls and staining a pale pink with phloroglucinol solution followed by concentrated hydrochloric acid, indicates the pericycle (Fig. 2, B). These fibres can be isolated from an alkali maceration and show tapered or rounded ends, a length of about 1·8 to at least 3·5 cm., a lumen diameter of 24 to 60 μ, wall thickness of 3 to 8 μ and sometimes marked swellings along their length (Fig. 3, p.f.).

The phloem (Fig. 2, B) consists of sieve tissue traversed by secondary medullary rays, 1 to 3 cells wide with individual cells measuring R = 15 to **21** to **33** to 45 μ and T = 12 to **15** to **30** to 40 μ. The larger sieve tubes are up to about 30 μ in diameter. Idioblasts, R and T = 10 to 96 μ and L up to about 132 μ, containing sandy crystals of calcium oxalate occur scattered throughout the phloem; phloem fibres are absent.

Interior to the cambium the completely lignified secondary xylem forms a cylinder consisting of vessels, fibres, secondary medullary rays and xylem parenchyma (Fig. 2, C). Vessels are scattered evenly throughout the wood either singly, in pairs or in small groups; they are often radially elongated, T = 15 to **25** to **65** to 96 μ and R = 24 to **36** to **100** to 132 μ. Vessel members, excluding the protuberances are 80 to **120** to **300** to 360 μ long and may be isolated for examination, together with other components of the wood by use of Schulze's macerating fluid. The vessel pitting varies from simple slits to alternately arranged bordered pits. Occasional tyloses are evident in the vessels of older parts of the stem.

The wood fibres, length 200 to **350** to **700** to 720 μ, diameter 9 to **15** to **27** to 36 μ and wall thickness 2 to 5 μ have pits ranging from simple slits to others with extended pit apertures. Wood fibres adjacent to a

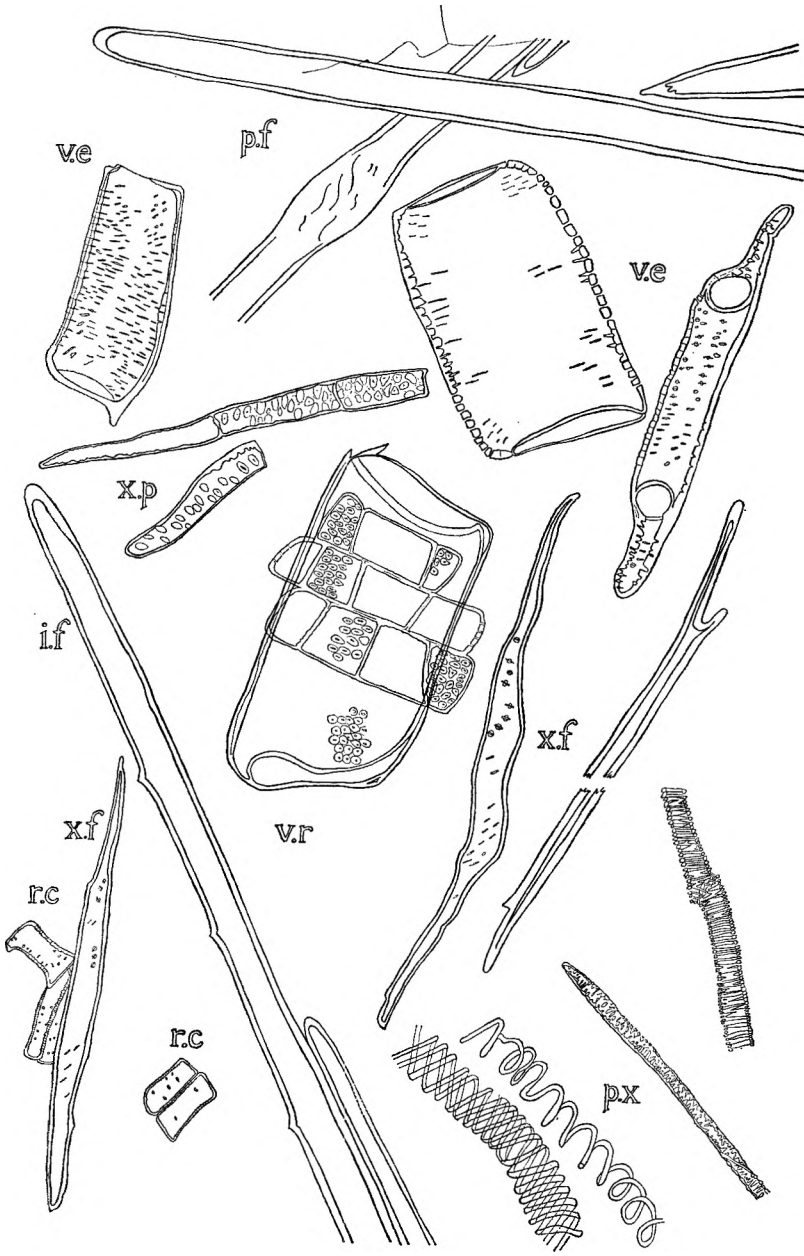

FIG. 3. *Datura leichhardtii* Muell. ex Benth. Isolated lignified elements of stem × 180. *i.f*, internal fibres; *p.f*, pericyclic fibres; *p.x*, primary xylem; *r.c*, medullary ray cells; *v.e*, vessel elements; *v.r*, vessel element with medullary ray cells attached; *x.f*, xylem fibre; *x.p*, xylem parenchyma.

ray may possess a scalloped outline and a few have bifurcated ends (Fig. 3, x.f.).

In young stems the medullary rays are mainly uniseriate in transverse section and, up to about 60 cells deep (Fig. 2, C; 4, A); similar sections of older stems, particularly from material grown in Australia and raised in the field in England, show rays 1 to **2** to **3** to 4 cells wide and 3 to **5** to **10** to 13 cells deep (Fig. 4, B). For individual cells, R = 6 to **15** to **30** to 42 μ, T = 3 to **6** to **24** to 36 μ and L = 15 to **20** to **60** to 81 μ but for some material that was grown under glass, the uniseriate rays were difficult to distinguish from wood parenchyma cells because of their often increased length, L = 24 to **30** to **180** to 195 μ (Fig. 2, F). The cells of the medullary rays contain small starch granules (2 μ) and the walls may have simple or bordered pits according to the nature of the adjacent cell; xylem parenchyma is scanty, apotracheal and diffuse and contains abundant starch granules about 2 μ in diameter; the cells measure R and T = 9 to **12** to 16 μ, L = 28 to **90** to **196** to 250 μ (Fig. 2, C; Fig. 3, x.p.).

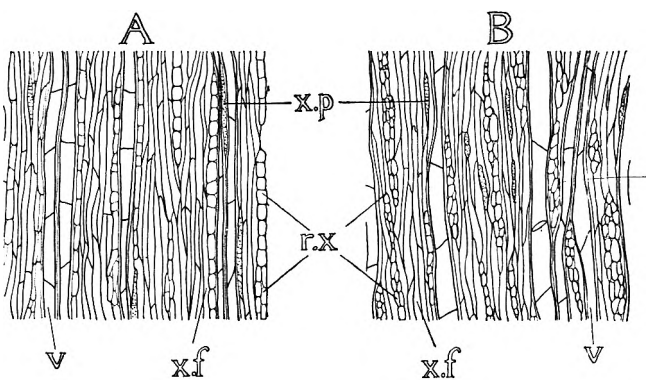

FIG. 4. *Datura leichhardtii* Muell. ex Benth. Tangential longitudinal sections of the secondary xylem. A, Nottingham greenhouse plant. B, Australian plant. Both × 45. *r.x*, medullary rays; *x.f*, xylem fibres; *x.p*, xylem parenchyma; *v*, vessel.

Groups of primary xylem contain radially arranged vessels with spiral or scalariform thickenings (Fig. 2, D; 3, p.x.). Approximately four cells interior to the primary xylem is a ring of compact groups of small thin-walled cells constituting the internal phloem (Fig. 2, E). Adjacent to the inner phloem are single, or small groups of, fibres having highly refractive cell walls which stain a pale pink with phloroglucinol and hydrochloric acid. They may be isolated from a 5 per cent potassium hydroxide maceration (Fig. 2, E; 3, i.f.); length 1·6 to at least 8·5 mm., lumen diameter 18 to 33 μ, thickness of walls 3 to 6 μ.

The medulla is composed of a relatively large-celled parenchyma, some cells of which contain starch granules similar to those of the cortex and others, sandy crystals of calcium oxalate. In longitudinal sections the cells often appear to be arranged in vertical columns. R and T = 165 to **200** to 270 μ and L approximately 120 μ.

LEAVES

Lamina, Interneural Region

The lamina consists of an upper and lower epidermis with stomata and trichomes, a single layer of palisade cells, collecting cells containing calcium oxalate crystals and about 3 to 4 layers of spongy mesophyll (Fig. 5, B). It measures about 175 to 275 μ in thickness.

The upper epidermis consists of cuticularised cells with almost straight anticlinal walls, length 18 to **30** to **70** to 105 μ, breadth 12 to **20** to **50** to 60 μ and depth 15 to **18** to **24** to 30 μ. Stomata are mainly of the anisocytic type, elliptical in outline, length 27 to 36 μ and width 8 to 12 μ (Fig. 5, E). The stomatal index is 10·4 to **15** to 19 (English samples) and 19·5 to **21·3** to 24 (Australian samples). The lower epidermis has a smooth, thin cuticle and the anticlinal walls of individual cells are wavy, in outline, length 18 to **30** to **80** to 105 μ, breadth 9 to **20** to **40** to 60 μ, depth **9** to **15** to 21 μ. The stomata resemble those of the upper epidermis. The stomatal index is 15 to **19** to 24 (English samples) and 22·7 to **23·5** to 24 (Australian samples), and the stomatal number ratio is 1·3 to **1·8** to 2·4.

Uniseriate clothing trichomes (Fig. 6, A) occur on both surfaces of the lamina, but particularly along the lower surface of the veins. They consist of 1 to **2** to **3** to 4 cells with warty walls, sometimes collapsed at right angles to one another even when examined from fresh material. Length of trichomes 69 to **125** to **200** to 300 μ and with the basal cell, length 35 to **63** to 98 μ, diameter 16·5 to **20** to **30** to 42 μ often funnel-shaped at the point of attachment to the epidermis and extending to a diameter of 20 to **40** to **50** to 75 μ. Yellow contents of the trichomes, especially of fresh material, stain with Tincture of Alkanna and contract to a small mass when treated overnight with ethanol and are virtually unaffected by ether. In choral hydrate preparations the trichomes are completely cleared and an occasional small crystal is evident. Small glandular trichomes possessing a unicellular stalk and usually about five cells in the head (Fig. 6, A) are found on both surfaces of the lamina, especially along the veins; their yellow contents respond to chemical reagents in a similar way as those of the clothing trichomes. For the unicellular stalk, length = 9 to **15** to **24** to 27 μ and breadth = 12 to **15** to 21 μ; for the head, length = 30 to **35** to **50** to 56 μ and breadth = 30 to **35** to **50** to 56 μ. Glandular trichomes, length 132 to **200** to **400** to 432 μ having single celled heads, diameter 12 to **15** to **25** to 32 μ and uniseriate multicellular stalks are occasionally present, especially near the leaf-margin and on the lower surface of the veins. The stalks, usually of three cells, are as warty as the covering trichomes but the glandular head is transparent. Similar trichomes were also found on seedlings of *D. stramonium* var. *tatula*.

Counts of trichomes (combined clothing and long glandular) were made for interneural regions of the lower epidermis together with similar counts for other species of *Datura*. For *D. leichhardtii*, *D. stramonium*, *D. ferox* and *D. metel* figures in the range 0 to 4/mm.^{2} were obtained whereas *D. innoxia* and *D. meteloides* gave figures of about 45/mm.^{2} and *D.*

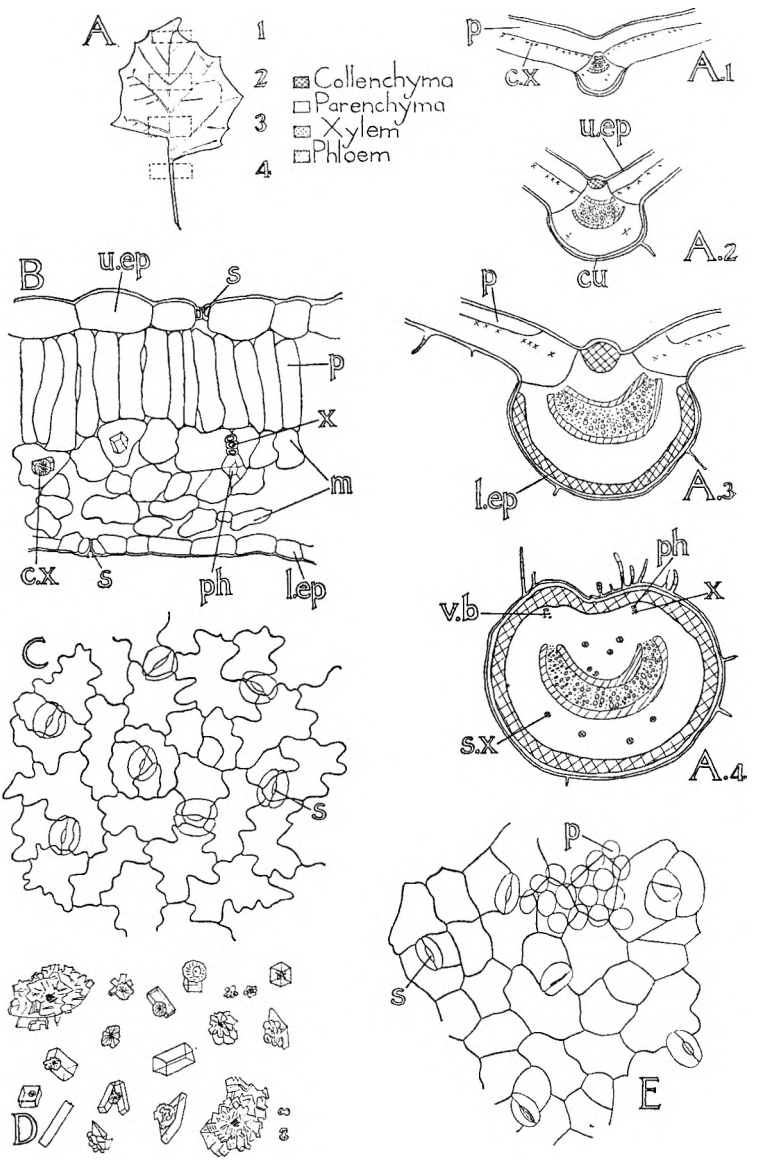

FIG. 5. Leaf of *Datura leichhardtii* Muell. ex Benth. A1 to A4, general diagrams, × 45, of transverse sections taken at positions 1 to 4 on A. B, transverse section of lamina. C, lamina, lower epidermis. D, calcium oxalate crystals from collecting layer of leaf. E, lamina, upper epidermis. B to E × 180. *cu*, cuticle; *c.x*, calcium oxalate crystal; *l.ep*, lower epidermis; *m*, spongy mesophyll; *p*, palisade layer; *ph*, phloem; *s*, stomata; *u.ep*, upper epidermis; *v.b*, vascular bundle; *x*, xylem.

discolor 11 and 40/mm.^{2} according to the sample. Verzár-Petri and Sárkány (1961) for the lower epidermis quote figures of 17/mm.^{2} for the long glandular trichomes of *D. innoxia* and 1/mm.^{2} for the clothing trichomes of various varieties of *D. metel.*

The palisade layer consists of a single layer of cells, height 60 to **70** to **135** to 144 μ and palisade ratio 2·3 to **4·8** to 7·5. A crystal layer of calcium oxalate occurs in the collecting cells, many of which contain independent crystal clusters, diameter 9 to **30** to **50** to 60 μ, prisms 9 to **12** to **25** to 42 μ long and 4 to **6** to **18** to 21 μ wide, conglomerates of clusters and prisms and crystal sand (Fig. 5, D). Large cluster crystals were predominant in the Australian sample, individual prisms with associated rosettes in greenhouse plants and, a mixture of crystal clusters and prisms in the English field plants. A small fragment of the leaf of the type specimen showed a mixture of small cluster crystals and prisms.

Midrib

The general structure of the midrib taken at various positions along the leaf is shown in Fig. 5, A1 to A3 and a more detailed illustration of a transverse section through the lower part of the midrib is given in Fig. 7, A. The upper epidermis consists of a layer of straight-walled cells elongated in the direction of the long axis of the leaf and bearing clavate and clothing trichomes similar to those on the lamina. The lower epidermis is similar. Towards the base of the leaf, the hypodermal regions of both upper and lower surfaces are collenchymatous. For individual cells, R and T = 13 to **15** to **27** to 36 μ and L = 75 to **120** to **255** to 300 μ. The remainder of the cortex consists of parenchymatous cells, R and T = 28 to **33** to **60** to 75 μ and L = 84 to **120** to **250** to 360 μ; some cells contain small starch grains and others, especially towards the petiole, sandy crystals of calcium oxalate.

In transverse section the meristele is arcuate and composed of xylem bounded above and below by phloem. The latter consists of groups of thin walled tissue separated by medullary rays and the wood vessels, with annular, spiral and scalariform thickenings are radially arranged and measure chiefly about 20 to 30 μ in diameter. Unlignified medullary rays, one or two cells wide, pass through the xylem.

Petiole

The distribution of tissues in the petiole is indicated in Fig. 5, A4 and Fig. 7, B. In surface view the epidermal cells of both the adaxial and abaxial surfaces are similar, being elongated with respect to the long axis and having unevenly thickened anticlinal walls, R and T = 24 to **30** to **40** to 48 μ and L = 24 to **40** to **120** to 180 μ. The cuticle of the epidermis is occasionally striated. Stomata, similar to those found on the lamina, are present on the upper epidermis but rarely on the lower and trichomes of the usual types are numerous, particularly along the groove on the adaxial surface. The hypodermal collenchyma 4 to 5 cells deep is continuous beneath the epidermis; its cells contain small starch granules about 2 μ in

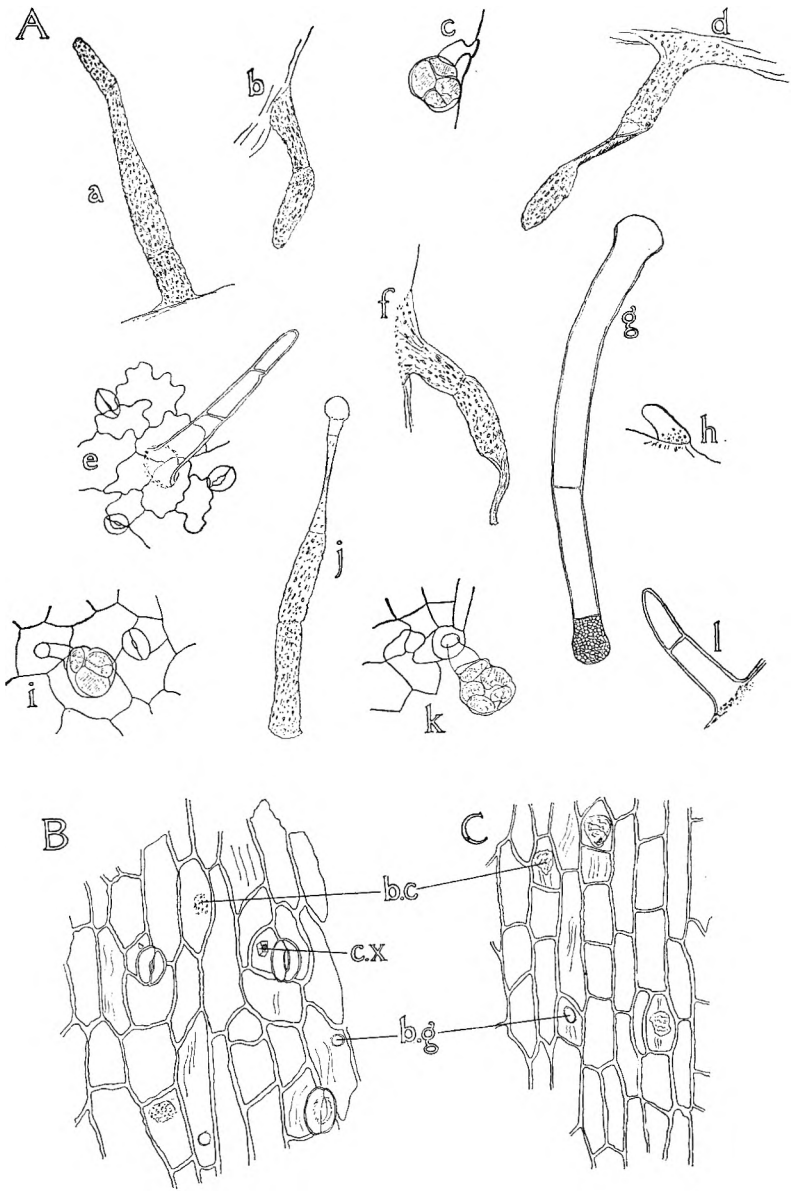

FIG. 6. *Datura leichhardtii* Muell. ex Benth. A, trichomes of stems and leaves. B. petiole, upper epidermis. C, petiole, lower epidermis. All × 180. a, b, d, e, f, h, l, clothing trichomes; c, i, k, glandular trichomes with unicellular stalk. g, glandular trichome of stem with multicellular stalk; j, ditto of petiole. *b.c*, scar of attachment of clothing trichome; *b.g*, ditto small glandular trichome; *c.x*, calcium oxalate crystal.

diameter. Both the collenchymatous cells and the underlying parenchyma are longitudinally elongated. Two small ridge bundles are embedded in the parenchyma of the ridges on the adaxial surface. Scattered idioblasts containing sandy crystals of calcium oxalate occur throughout the cortical parenchyma, other cells contain single starch grains of diameter 1 to 5 to 7 μ, compound granules each composed of 2 to 3 to 6 components and complex masses of starch granules up to 30 μ in diameter. The meristele resembles that of the midrib; annual and spiral vessels constitute the protoxylem with scalariform thickened vessels of the metaxylem located towards the abaxial perimeter of the xylem.

Discussion

A complete key for the classification of the herbaceous *Datura* species, based on microscopical characters, cannot be attempted at present owing to the paucity or lack of information on some species. Morphologically the species can be divided into those with pubescent leaves and those having few trichomes on the leaf surfaces and appearing almost glabrous. Although the actual number of trichomes per mm.^{2} of leaf surface probably varies considerably for any one species, our limited investigations suggest that it can be used to differentiate between the two groups by microscopical means. In this way *D. leichhardtii* can be distinguished from other members of the Section Dutra, with the exception of *D. metel* and its varieties. The warty-walled clothing trichomes of *D. leichhardtii*, often with their characteristic flattened cells, are generally shorter than those of *D. stramonium* (Timmermann, 1927*a*), *D. innoxia* (Timmermann, 1927*a*; Košová and Chládek, 1957) but resemble those of *D. meteloides* and *D. discolor* (Kalemkiarian and Miller, 1957); they may be shorter than (Timmermann, 1927*a*; Santos, 1927; Košová and Chládek, 1957) or about the same length as (Verzár-Petri and Sárkány, 1961) those of *D. metel*. Glandular trichomes with a single celled head and a uniseriate stalk, which are of limited occurrence on the leaves and petioles of *D. leichhardtii* have previously only been reported on the leaves of *D. innoxia* (Timmermann, 1927*a*; Haller, 1946; Karkoszka, Krasowska and Rogoyska, 1957; Verzár-Petri and Sárkány, 1961) but they are common on corollas (Santos, 1927; Wallis and Rohatgi, 1952) and we have observed them on young seedlings of *D. stramonium*. The stomatal index of the leaves of *D. leichhardtii*, when compared with published values for *D. stramonium*, *D. innoxia* and *D. metel* (Rowson, 1946; Košová and Chládek, 1957; Verzár-Petri and Sárkány, 1961) would seem to be of little value for differentiation. Similarly the palisade ratio is within the general range for the genus and the stomatal number ratio, although different from *D. innoxia* is similar to that of *D. stramonium* (Timmermann, 1927*b*). In thickness the lamina is similar to *D. stramonium* and *D. metel* but thinner than *D. innoxia* (Timmermann, 1927*a*). A tendency towards the formation of prismatic crystals of calcium oxalate in the collecting layer of leaves of *D. leichhardtii* can serve as a useful distinguishing feature but this characteristic is variable and cluster crystals may predominate. The only other species of *Datura* in which prismatic crystals

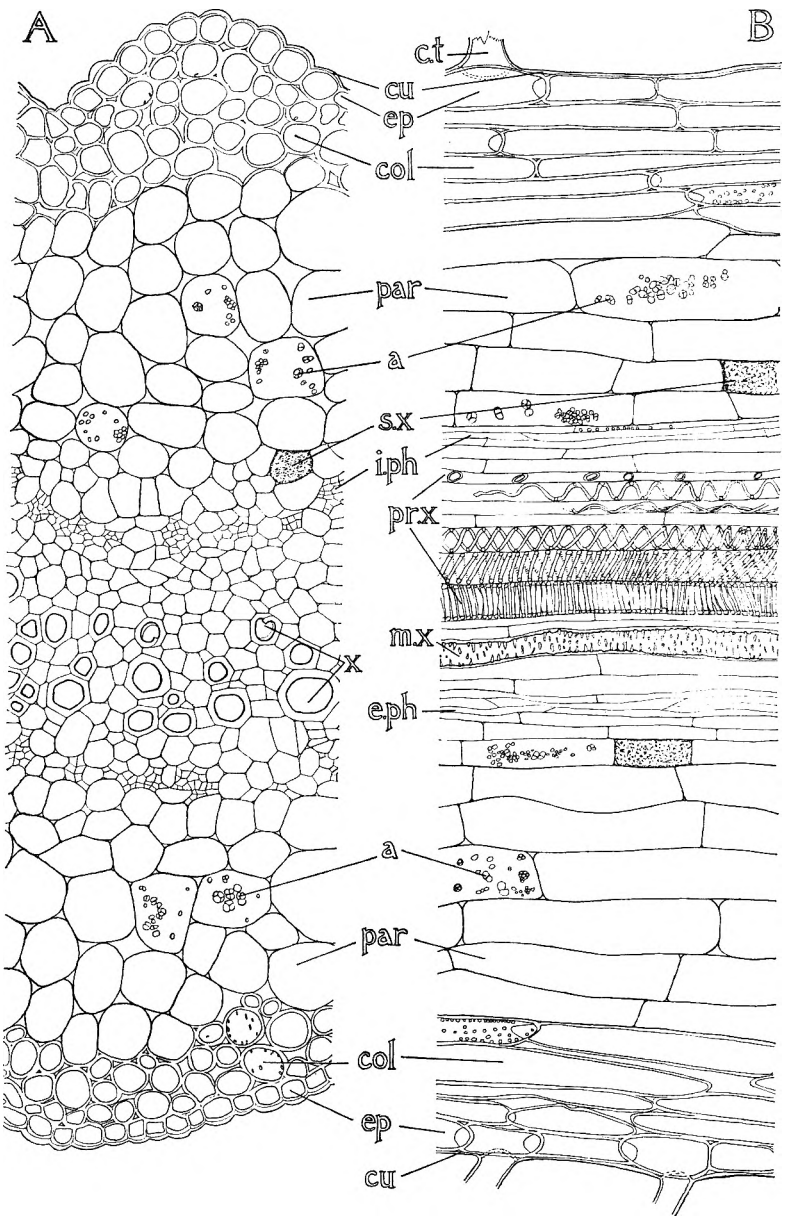

FIG. 7. *Datura leichhardtii* Muell. ex Benth. A, transverse section of midrib at location A3, Fig. 5. B, longitudinal section through petiole at right angles to the plane of symmetry. Both × 180. *a*, starch; *col*, collenchyma; *c.t*, clothing trichome; *cu*, cuticle; *ep*, epidermis; *e.ph*, external phloem; *i.ph*, internal phloem *m.x*, metaxylem; *par*, parenchyma; *pr.x*, protoxylem; *s.x*. sandy crystals of calcium oxalate; *x*, xylem.

of oxalate have been cited as the main type is *D. discolor* (Kalemkiarian and Miller, 1957) but leaves of this species, raised in Nottingham, have consistently contained cluster crystals.

The structure of the transverse section of the stem closely resembles that of *D. stramonium* (Fischer, 1937).

Summary of Microscopical Characters

The principal features of the microscopy of the leaves and stems of *D. leichhardtii* are:

1. An upper leaf epidermis having cells with almost straight anticlinal walls and possessing mainly anisocytic stomata. A lower epidermis with wavy anticlinal walls and about twice as many stomata. The cuticle of neither epidermis is striated.
2. Leaf trichomes are of three types: small clavate glandular trichomes of the type found in all the examined species of the genus; a limited number of glandular trichomes possessing a uniseriate stalk and a single-celled head; covering trichomes, uniseriate and 1 to 4 celled, with warty walls and commonly 125 to 200 μ in length.
3. A collecting layer of cells in the leaf lamina containing prismatic or cluster crystals of calcium oxalate or both.
4. The structure of the midrib resembles that of other members of the genus as does that of the stem. The lignified wood elements of the latter include vessels, fibres, wood parenchyma and medullary rays. Internal and external phloem are present; fibres are associated with the internal phloem and slightly lignified fibres indicate the pericycle.
5. Sandy crystals of calcium oxalate occur as idioblasts in the parenchyma of the stem, petiole and midrib.
6. Palisade ratio, stomatal index and stomatal ratio are of little value for distinguishing *D. leichhardtii* from other members of the genus.

The similarity of these characters to those of some other species of *Datura* renders the identification of *D. leichhardtii* in the powdered form difficult. The anatomical features of the leaves which collectively serve to distinguish the species are: the occurrence in some samples of many, and often a predominance of, prismatic crystals in the collecting layer of the leaves, relatively few clothing trichomes on the leaf surface compared with most other members of the Section Dutra, and the presence of glandular trichomes with a uniseriate stalk and a single celled head.

Acknowledgements. We are indebted to the late Mr. H. A. Berens for obtaining the Australian sample of *D. leichhardtii* and also to the Director, Royal Botanic Gardens, Kew, for the facilities accorded us in examining herbarium specimens.

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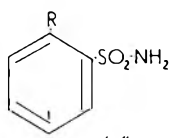
STUDIES IN THE FIELD OF DIURETIC AGENTS. PART VI
SOME SULPHAMOYLBENZOIC ACIDS

By G. B. Jackman, V. Petrow, O. Stephenson and A. M. Wild
From the British Drug Houses Ltd., Graham Street, London, N.1

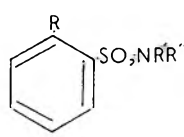
Received January 25, 1962

The requirements for diuretic activity in the sulphamoylbenzoic acid series have been studied. Optimal activity is shown by 4-halogeno-3-sulphamoylbenzoic acids.

In Part V (Petrow, Stephenson and Wild, 1960) we studied the relationship between structure and diuretic activity in *N*-alkyl- and *NN*-dialkylbenzene-1,3-disulphonamides of types (I) and (II). Our results showed clearly the outstanding superiority of structure (I) over its isomer (II). In extending these observations we decided initially to retain intact the substituents present in positions 3 and 4 in (I) and to vary the electronegative group (X) at position 1 as in (III).



I



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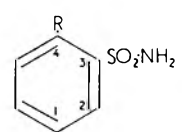


III

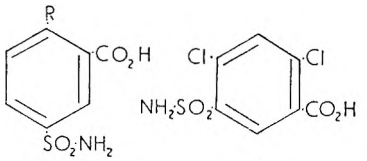
4-Nitrotoluene-2-sulphonamide (III; R = Me, X = NO_2) and 2-chloro-5-nitrobenzenesulphonamide (III; R = Cl, X = NO_2) (Petrow, Stephenson and Wild, 1960), though unlikely to be of any clinical interest, were tested biologically and found to possess some oral diuretic activity, albeit only at doses of 90 mg./kg. (in the saline-loaded rat). Methyl 4-methyl(or chloro)-3-sulphamoylphenyl sulphones (III; R = Me or Cl, X = SO_2Me), prepared directly from the appropriate sulphones, were active oral diuretics at the 30 mg./kg. dose level. The trifluoromethyl analogue (III; R = Cl, X = CF_3) was found to be inactive at this dosage.

Attention was next directed to substituted *m*-sulphamoylbenzoic acids (III; X = CO_2H), a class of readily available compounds which has been virtually neglected hitherto.

m-Sulphamoylbenzoic acid (Smiles and Stewart, 1921) and its methyl ester were both devoid of oral diuretic activity. This was expected since we had already shown (Petrow, Stephenson and Wild, 1960) that disulphamoyl compounds of type (I; R = H) were inactive or possessed only low activity. The corresponding toluic acid derivative (III; R = Me, X = CO_2H) was of some interest and although only a very weak inhibitor of carbonic anhydrase (*ca.* 1/250th of acetazolamide), it proved to be an effective oral diuretic at doses of 8 mg./kg. in the saline-loaded rat. This compound, prepared previously by the oxidation of *p*-xylenesulphonamide (Iles and Remsen, 1878) or of cymene-2-sulphonamide (Hall and Remsen,

1879) with chromic acid, was more readily obtained by us by direct chlorosulphonation of *p*-toluic acid followed by reaction of the sulphonyl chloride with ammonia. Its methyl, ethyl and butyl esters were all less effective than the parent acid, a fact which again emphasised the lack of a direct relation between diuretic potency and carbonic anhydrase inhibitory activity, since the butyl ester was twenty times as active as the acid as an inhibitor of the enzyme.

Inter alia we examined the isomeric 2-methyl-5-sulphamoylbenzoic acid (IV; R = Me). This compound was prepared originally (Jacobsen, 1881) by oxidation of *o*-xylenesulphonamide and fractional crystallisation of the resultant mixed product. It was obtained by us directly from *o*-toluic acid, and proved to be without diuretic activity. Its methyl ester was also inactive.



Attention was next directed to the halogenated analogues of the potent 4-methyl-3-sulphamoylbenzoic acid. Three 4-halogeno-3-sulphamoylbenzoic acids (III; R = F, Cl or Br; X = CO_2H) were prepared by direct chlorosulphonation of the appropriate halogenobenzoic acid followed by reaction of the resultant sulphonyl chlorides with ammonia. The diuretic activity of these acids increased in the order—bromo>chloro> fluoro, the bromo-compound being approximately eight times as active as 4-methyl-3-sulphamoylbenzoic acid. On the other hand, the carbonic anhydrase inhibitory activity of the acids was chloro (0:1), fluoro and bromo (<0·01) (compared with acetazolamide = 1·0), so that there was again no direct correlation between the two parameters.

One isomeric acid, 2-chloro-5-sulphamoylbenzoic acid (IV; R = Cl), prepared earlier (Basu and Das Gupta, 1939) by oxidation of the corresponding toluene derivative and obtained by us *via* direct chlorosulphonation of *o*-chlorobenzoic acid, resembled the corresponding *o*-toluic acid derivatives in being devoid of diuretic activity. Introduction of a halogen substituent into 4-chloro-3-sulphamoylbenzoic acid (III; R = Cl. X = CO_2H) to give 2,4-dichloro-5-sulphamoylbenzoic acid (V) (prepared from 2,4-dichlorobenzoic acid), was likewise accompanied by loss of biological potency.

In further attempts to define the structural requirements necessary for diuretic activity in the sulphamoylbenzoic acids series, the preparation was undertaken of the isomeric 3-bromo- and 3-chloro-4-sulphamoylbenzoic acids (VI) in which the halogen-group and sulphamoyl group of 4-bromo- or 4-chloro-3-sulphamoylbenzoic acid (III; R = Br or Cl, X = CO_2H) are interchanged. These compounds were obtained *via* the 4-amino-3-halogenotoluenes (VII; R = NH_2) which were converted

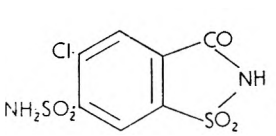
into the sulphonyl chlorides (VII; R = SO_2Cl) by diazotisation and reaction of the diazonium chloride with a saturated solution of sulphur dioxide in acetic acid in the presence of cuprous salts (cf. Petrow and others, 1960). The sulphonamides (VII; R = SO_2NH_2) were obtained in the normal way and furnished the required products (VI) by oxidation with aqueous potassium permanganate.

The compounds (VI; Hal = Cl or Br) had no diuretic activity at doses of 10 mg./kg. in saline-loaded rats.

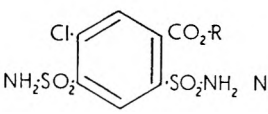
Finally, we examined the oxidation of 4-methyl-3-sulphamoylbenzoic acid, 5-chlorotoluene-2,4-disulphonamide (disulphamide) and 2-chlorotoluene-3,5-disulphonamide. The latter two compounds described earlier (Boggiano and others, 1960) had both been found to possess noteworthy diuretic activity.

Oxidation of the first compound with potassium permanganate in aqueous alkaline solution yielded a crude mixture of acid (III; R = X = CO_2H) and the derived saccharin, which, without separation, was converted directly into methyl 2-sulphamoylterephthalate (III; R = X = CO_2Me) by heating with methanolic hydrogen chloride.

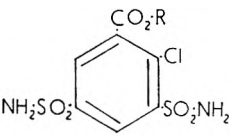
Oxidation of disulphamide with aqueous alkaline potassium permanganate below 40° yielded a mixture of the saccharin (VIII) (de Stevens, Halamandaris, Ricca and Werner, 1959) and the acid (IX; R = H) which were readily separated by graded acidification of the reaction mixture. Both compounds retained some diuretic activity,



VIII



IX



X

though neither was superior to the original disulphamide. The derived methyl (IX; R = Me) and butyl (IX; R = Bu) esters were prepared, most conveniently from crude mixtures of acid and saccharin. The latter ester had almost the same diuretic potency as disulphamide and more than five times the carbonic anhydrase inhibitory activity of acetazolamide (cf. Beasley, Overell, Petrow and Stephenson, 1958).

Similar oxidation of 2-chlorotoluene-3,5-disulphonamide yielded the acid (X; R = H), which was converted into the butyl ester (X; R = Bu), but both compounds were markedly inferior to the original disulphonamide as diuretic agents.

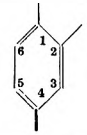
Experimental

The early experiments illustrate the methods used for the preparation of compounds listed in Table I, which also contains the analyses.

Methyl 4-methyl-3-sulphamoylphenyl sulphone. Methyl *p*-tolyl sulphone (30 g.) was dissolved in chlorosulphonic acid (120 g.) and the mixture heated on the steam bath for 1 hr. It was then cooled, poured on to ice, the solids collected and washed with cold water. Crystallisation from

TABLE I

SULPHAMOYLBENZOIC ACIDS



Substituent at position			m.p. °C.	Formula	Found					Required				
1	2	4			C	H	Cl	N	S	C	H	Cl	N	S
H	SO_2NH_2	CO_2H	246	$C_7H_7NO_4S$										
H	SO_2NH_2	CO_2Me	131–133	$C_8H_9NO_4S$	44·5	4·2		6·2		44·7	4·2		6·5	
Me	SO_2NH_2	CO_2H	267–269	$C_8H_9NO_4S$	44·7	4·5		6·3		44·7	4·2		6·5	
Me	SO_2NH_2	CO_2Me	119–120	$C_9H_{11}NO_4S$	47·4	4·8		6·0	14·1	47·1	4·8		6·1	14·0
Me	SO_2NH_2	CO_2Et	106–107	$C_{10}H_{13}NO_4S$	49·3	5·3		5·8	13·0	49·4	5·4		5·8	13·2
Me	SO_2NH_2	CO_2Bu	96–98	$C_{12}H_{17}NO_4S$	53·1	6·1		5·0	12·0	53·1	6·3		5·2	11·8
Me	SO_2NHMe	CO_2H	210–212	$C_9H_{11}NO_4S$	47·3	4·8		6·1	13·6	47·1	4·8		6·1	14·0
Me	CO_2H	SO_2NH_2	250	$C_8H_9NO_4S$	44·4	4·1		6·6	14·7	44·7	4·2		6·5	14·9
Me	CO_2Me	SO_2NH_2	148–149	$C_9H_{11}NO_4S$	46·8	4·5		6·5		47·1	4·8		6·1	
Me	SO_2NH_2	SO_2Me	202–204	$C_8H_{11}NO_4S_2$	38·5	4·3		5·8	25·7	38·6	4·5		5·6	25·7
F	SO_2NH_2	CO_2H	239–241	$C_7H_6FNO_4S$	38·7	3·0		6·0		38·4	2·8		6·4	
F	CO_2H	CO_2H	297–298	$C_8H_5FO_4$	52·6	2·6				52·2	2·7			
Cl	SO_2NH_2	CO_2H	258–260	$C_7H_6ClNO_4S$	36·0	2·6	14·9	6·3	13·5	35·7	2·6	15·0	5·9	13·6
Cl	SO_2NH_2	CO_2Me	124–125	$C_8H_8ClNO_4S$	38·3	3·1	14·4	5·5		38·5	3·2	14·2	5·6	
Cl	SO_2NH_2	CO_2Et	139–140	$C_9H_{10}ClNO_4S$	41·1	3·9	13·6	5·5		41·0	3·8	13·5	5·3	
Cl	SO_2NHMe	CO_2H	236–238	$C_8H_8ClNO_4S$	38·7	3·0		5·6		38·5	3·2		5·6	
Cl	SO_2NMe_2	CO_2H	247–248	$C_9H_{10}ClNO_4S$	41·5	4·0	13·4	5·4	12·0	41·0	3·8	13·5	5·3	12·2
Cl	SO_2NH_2	CF_3	161–163	$C_7H_5ClF_3NO_2S$	32·7	2·3		5·1		32·4	1·9		5·4	
Cl	CO_2H	SO_2NH_2	220–222	$C_7H_6ClNO_4S$	35·8	2·2	15·2	5·9	13·9	35·7	2·6	15·0	5·9	13·6
Cl	CO_2Et	SO_2NH_2	128–130	$C_9H_{10}ClNO_4S$	41·3	3·7	13·5	5·8	12·4	41·0	3·8	13·5	5·3	12·2
Cl	SO_2NH_2	SO_2Me	227–229	$C_7H_6ClNO_4S_2$	31·4	3·3	13·1	5·1	23·9	31·2	3·0	13·1	5·2	23·8
Br	SO_2NH_2	CO_2H	273–275	$C_7H_6BrNO_4S$	30·0	2·4	28·5*	5·1	11·5	30·0	2·2	28·5*	5·0	11·4
CO_2Me	SO_2NH_2	CO_2Me	172–173	$C_{10}H_{11}NO_6S$	43·9	4·1		5·1	11·3	43·9	4·1		5·1	11·7
SO_2NH_2	Cl	CO_2H	235–237	$C_7H_6ClNO_4S$	35·8	2·7	15·3	6·0	13·6	35·7	2·6	15·1	5·9	13·6
SO_2NH_2	Br	CO_2H	259–260	$C_7H_6BrNO_4S$	30·0	2·0	29·0*	5·2		30·0	2·2	28·5*	5·0	
SO_2NH_2	Br	CO_2Me	154–155	$C_8H_8BrNO_4S$	32·7	2·6	27·4*	4·9		32·7	2·7	27·2*	4·8	

* Bromine

1,2-dichloroethane-light petroleum (b.p. 60–80°) furnished 3-*chlorosulphonyl*-4-*methylphenylmethyl sulphone*, m.p. 145–147°. Found: C, 35·6; H, 3·3; S, 23·8; Cl, 13·4. $C_8H_9ClO_4S_2$ requires C, 35·8; H, 3·4; S, 23·9; Cl, 13·2 per cent. The sulphonyl chloride was added with stirring at room temperature to a mixture of aqueous ammonia (500 ml.; d = 0·880) and chloroform (200 ml.). Stirring was continued for 30 min. when excess of ammonia and chloroform were boiled off. The residual liquid was cooled and acidified with hydrochloric acid to yield the *product*, which had m.p. 202–204° after crystallisation from ethanol.

4-*Chloro*-3-*sulphamoylphenylmethyl sulphone*. The preparation of 4-*chloro*-3-*chlorosulphonylphenylmethyl sulphone* from *p*-chlorophenyl methyl sulphone was carried out as described in the preceding experiment. It had m.p. 145–147° after crystallisation from 1,2-dichloroethane. Found: C, 29·1; H, 2·3; S, 22·4; Cl, 24·2. $C_7H_6Cl_2O_4S_2$ requires C, 29·1; H, 2·1; S, 22·2; Cl, 24·5 per cent. It reacted with aqueous ammonia-chloroform to yield the *product*, m.p. 227–229° (from ethanol).

4-*Methyl*-3-*sulphamoylbenzoic acid*. *p*-Toluic acid (34 g.) was added in portions with stirring and cooling below 20° to chlorosulphonic acid (100 ml.) when the mixture was heated to 120° for 7 hr. It was cooled, poured on to ice and the crude sulphonyl chloride extracted with chloroform. The extract was washed with ice-water, dried with anhydrous sodium sulphate and concentrated to *ca* 300 ml. 3-*Chlorosulphonyl*-4-*methylbenzoic acid* separated and had m.p. 173° after crystallisation from chloroform. Found: C, 41·4; H, 3·0; S, 13·4. $C_8H_7ClO_4S$ requires C, 41·0; H, 3·0; S, 13·7 per cent. The sulphonyl chloride (23·5 g.) was added in portions with stirring to liquid ammonia (200 ml.) and the ammonia was then allowed to evaporate. The residue was dissolved in water and acidified with hydrochloric acid to yield the *product* which had m.p. 267–269° after crystallisation from water.

Butyl 4-*methyl*-3-*sulphamoylbenzoate*. A solution of the foregoing acid (12·8 g.) in butanol (120 ml.) containing hydrogen chloride (2 g.) was heated under reflux for 4 hr. when excess of solvent was distilled off at reduced pressure. Crystallisation of the residue from ether-light petroleum (b.p. 40–60°) furnished the *product*, m.p. 96–98°. It was occasionally obtained in a metastable form, m.p. 69–71°.

4-*Chloro*-3-*sulphamoylbenzoic acid*. A mixture of *p*-chlorobenzoic acid (410 g.), chlorosulphonic acid (1232 g.) and pentachloroethane (315 ml.) was heated at reflux temperature (125–140°) for 3 hr. The *sulphonyl chloride*, isolated in the usual manner, had m.p. 168–170° after crystallisation from 1,2-dichloroethane. Found: C, 33·2; H, 1·6; S, 12·4; Cl, 28·0. $C_7H_4Cl_2O_4S$ requires C, 33·0; H, 1·6; S, 12·6; Cl, 27·8 per cent. Reaction with liquid ammonia as described earlier yielded the *product* which had m.p. 258–260° after crystallisation from aqueous ethanol.

4-*Fluoro*-3-*sulphamoylbenzoic acid*. The *sulphonyl chloride* prepared by heating *p*-fluorobenzoic acid (30 g.) with chlorosulphonic acid (70 ml.) at 120° for 8 hr. had m.p. 147–148° after crystallisation from 1,2-dichloroethane-light petroleum (b.p. 60–80°). Found: C, 34·7; H, 1·7; S, 13·1;

Cl, 14·9. $C_7H_4ClFO_4S$ requires C, 35·2; H, 1·7; S, 13·4; Cl, 14·9 per cent. Reaction of the sulphonyl chloride with liquid ammonia furnished the *product* which had m.p. 239–241° after crystallisation from water.

4-*Bromo*-3-*chlorosulphonylbenzoic acid*, prepared as described for the 4-chloro analogue, had m.p. 203–205° after crystallisation from 1,2-dichloroethane. Found: C, 28·3; H, 1·5; S, 10·9. $C_7H_4BrClO_4S$ requires C, 27·9; H, 1·3; S, 10·6 per cent.

2,4-*Dichloro*-5-*sulphamoylbenzoic acid* was prepared by chlorosulphonation of 2,4-dichlorobenzoic acid at 140° for 8 hr. followed by reaction of the resultant sulphonyl chloride with liquid ammonia. It had m.p. 235–236° after crystallisation from aqueous ethanol. Found: C, 31·2; H, 2·1; N, 5·3; S, 12·0; Cl, 25·9. $C_7H_5Cl_2NO_4S$ requires C, 31·1; H, 1·9; N, 5·2; S, 11·9; Cl, 26·3 per cent.

3-*Bromo*-4-*sulphamoylbenzoic acid.* (*a*) 3-*Bromo*-4-*chlorosulphonyltoluene.* 4-Amino-3-bromotoluene (37·2 g.) was added with stirring to 24 per cent hydrochloric acid and the mixture diazotised at 0–5° by the slow addition of a solution of sodium nitrite (15·2 g.) in water (30 ml.). The diazo-solution was added over 5 min. with stirring to a saturated solution of sulphur dioxide in acetic acid (320 ml.) containing cuprous chloride dihydrate (14 g.) in water (10 ml.) at 15°. The temperature of the mixture rose to 27° and reaction was completed by heating to 40°. The mixture was cooled and crushed ice added to complete precipitation of the sulphonyl chloride. The crude product was used for the next stage in the reaction. A portion, crystallised from light petroleum (b.p. 60–80°) had m.p. 70–71°. Found: C, 31·5; H, 2·3; S, 12·2; total halogen 43·2. $C_7H_6BrClO_2S$ requires C, 31·2; H, 2·2; S, 11·9; total halogen, 42·8 per cent.

(*b*) 3-*Bromotoluene*-4-*sulphonamide.* A solution of the foregoing sulphonyl chloride in chloroform (150 ml.) was added with stirring to aqueous ammonia prepared from ammonia solution (150 ml., d = 0·880) and water (150 ml.). Stirring was continued for 1 hr. The solids (35·4 g., m.p. 188–190°) were collected. Concentration of the filtrate followed by acidification with hydrochloric acid furnished a second crop of material (8·5 g., m.p. 186–188°). A portion of the product had m.p. 188–190° after crystallisation from aqueous ethanol. Found: C, 33·9; H, 3·2; N, 5·4; S, 12·5. $C_7H_8BrNO_2S$ requires C, 33·6; H, 3·2; N, 5·6; S, 12·8 per cent.

(*c*) 3-*Bromo*-4-*sulphamoylbenzoic acid.* A solution of the foregoing sulphonamide (10 g.) in water (60 ml.) containing sodium hydroxide (1·6 g.) was heated on the steam bath to about 60° and treated in portions with a slurry of potassium permanganate (12·7 g.) in water (50 ml.). When the reaction was complete the mixture was decolourised with sulphur dioxide and the *product* collected, washed with water and dried (yield = 10·0 g.). A portion, crystallised from aqueous methanol had m.p. 259–260°.

3-*Chloro*-4-*sulphamoylbenzoic acid.* (*a*) 3-*Chlorotoluene*-4-*sulphonamide* was prepared from 4-amino-3-chlorotoluene as described for the preceding bromo-analogue. The sulphonyl chloride, obtained as an

oil from the diazo-reaction was dissolved in chloroform and condensed directly with ammonia solution (d = 0·880). The *product* had m.p. 184–186° after crystallisation from aqueous ethanol. Found: C, 40·8; H, 4·1; N, 6·8; S, 15·3; Cl, 17·3. Calc. for $C_7H_8ClNO_2S$: C, 40·9; H, 3·9; N, 6·8; S, 15·6; Cl, 17·2 per cent.

(*b*) 3-*Chloro*-4-*sulphamoylbenzoic acid* was prepared by oxidation of the foregoing sulphonamide with alkaline permanganate solution at 40–45° and finally at 60–70°. It had m.p. 235–237° after crystallisation from water.

Methyl 2-*sulphamoylterephthalate*. A solution of 4-methyl-3-sulphamoylbenzoic acid in water (500 ml.) containing anhydrous sodium carbonate (10·6 g.) was stirred and treated with powdered potassium permanganate (63·2 g.) added over 20 hr. at 45°. The precipitated manganese dioxide was filtered off and washed with hot water (100 ml.). The filtrate and washings were concentrated to half bulk and neutralised with hydrochloric acid. The solids (10·6 g.) which separated (unchanged starting material), were filtered off. The filtrate, acidified to pH 4 with hydrochloric acid furnished solids (19·2 g.) m.p. 312–314° (decomp.) after crystallisation from water. These were suspended in methanol (180 ml.) and hydrogen chloride (8 g.) was passed into the mixture which was heated under reflux for 6 hr. Slight dilution with water furnished the *dimethyl ester* (10 g.) which had m.p. 172–173° after crystallisation from methanol.

Oxidation of 5-*chlorotoluene*-2,4-*disulphonamide* (with Dr. B. G. Boggiano). A solution of disulphamide (56·8 g.) in water (800 ml.) containing sodium hydroxide (12 g.) was heated at 40–45° with stirring and treated with powdered potassium permanganate (63·2 g.) added in portions over 6 hr. When reaction was complete the mixture was filtered hot and the filtrate concentrated to 300 ml. at reduced pressure. The solution was cooled and the pH adjusted to 7·5 when unchanged disulphamide (13·8 g.) separated out and was collected. Acidification of the filtrate to pH 6 furnished 5-*chloro*-6-*sulphamoylsaccharin* (11 g.) which had m.p. 276–278° after crystallisation from water. Found: C, 28·2; H, 2·1; N, 9·6; S, 21·6; Cl, 11·5. $C_7H_5ClN_2O_5S_2$ requires C, 28·3; H, 1·7; N, 9·4; S, 21·6; Cl, 12·0 per cent. Strong acidification of the filtrate with concentrated hydrochloric acid yielded 5-*chloro*-2,4-*disulphamoylbenzoic acid* m.p. 333° (decomp.). Found: C, 27·2; H, 1·5; N, 9·1; S, 20·8; Cl, 11·5. $C_7H_7ClN_2O_6S_2$ requires C, 26·7; H, 2·2; N, 8·9; S, 20·4; Cl, 11·3 per cent. A solution of the saccharin (3 g.) in butanol (40 ml.) containing hydrogen chloride (0·5 g.) was heated under reflux for 2 hr. Concentration of the solution followed by crystallisation from water yielded *butyl* 5-*chloro*-2,4-*disulphamoylbenzoate*, m.p. 159°. Found: C, 35·7; H, 4·0; N, 7·7; Cl, 9·9. $C_{11}H_{15}ClN_2O_6S_2$ requires C, 35·6; H, 4·1; N, 7·5; Cl, 9·6 per cent.

Methyl 5-*chloro*-2,4-*disulphamoylbenzoate* had m.p. 218–219° after crystallisation from aqueous methanol. Found: C, 29·5; H, 2·7; N, 8·6; Cl, 10·5. $C_8H_9ClN_2O_6S_2$ requires C, 29·2; H, 2·8; N, 8·5; Cl, 10·8 per cent.

2-*Chloro*-3,5-*disulphamoylbenzoic acid* was prepared by the oxidation of 2-chlorotoluene-3,5-disulphonamide (28·5 g.) with potassium permanganate (47 g.) in water (500 ml.) containing sodium hydroxide (5 g.) at 55–75°. It had m.p. 260–262° after crystallisation from water. Found: C, 27·2; H, 2·6; N, 9·1; Cl, 11·0. $C_7H_7ClN_2O_6S_2$ requires C, 26·7; H, 2·3; N, 8·9; Cl, 11·3 per cent. The *butyl ester* had m.p. 151–153° after crystallisation from water. Found: C, 35·8; H, 4·2; N, 7·3. $C_{11}H_{15}N_2O_6S_2$ requires C, 35·6; H, 4·1; N, 7·6 per cent.

Carbonic anhydrase inhibitory activities were determined with Mr. B. G. Overell, M.Sc. We are indebted to Dr. A. David and his colleagues for biological data.

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THE EFFECT OF THE ADMINISTRATION OF WATER OR ISOTONIC NaCl SOLUTION ON THE URINARY EXCRETION OF 5-HYDROXYINDOLEACETIC ACID IN THE RAT

By G. Bertaccini and V. Erspamer

From the Institute of Pharmacology, University of Parma, Italy

Received April 6, 1962

Administration of tap water by stomach tube or of isotonic NaCl solution orally or subcutaneously produces a conspicuous increase in the urinary excretion of 5-hydroxyindoleacetic acid (5-HIAA) in the rat. The same increase can be observed after administration of water plus posterior-pituitary antidiuretic hormone (ADH). Tap water is less effective than physiological saline; the latter, in its turn, is more effective by the subcutaneous route than by mouth. Increase of 5-HIAA may be, for short periods, as high as 3 times normal. The minimum oral dose of tap water causing a significant increase in urinary 5-HIAA is 2 ml./100 g., that of physiological saline 1 ml./100 g. Repeated doses of both tap water and physiological saline produce a more intense and long-lasting increase of 5-HIAA output than single doses. Generally, excess 5-HIAA coincides with excess urine elimination, but there is no obligatory correlation of the intensity of the two phenomena. Recovery, as urinary 5-HIAA, of exogenous 5-HIAA, 5-hydroxytryptamine (5-HT) and 5-hydroxytryptophan (5-HTP) is the same in control, non-hydrated rats and in rats given single or repeated doses of tap water, tap water plus ADH, or isotonic saline solution. The mechanism by which water or saline administration produces increased urinary excretion of 5-HIAA is discussed.

In the course of other investigations it was observed that rats given a water load by stomach tube excreted more 5-hydroxyindoleacetic acid (5-HIAA) during the period of water diuresis than did control rats given no water.

This paper describes the results of a more complete investigation on the influence of administration of water and of physiological NaCl solution on urinary 5-HIAA excretion in rats.

Experimental

Experimental animals. Approximately 300 Wistar rats, of both sexes, weighing 180–240 g., were used. The same rats were given water or saline loads at intervals of 10–15 days. The diet was the standard laboratory diet.

Urine collection. Urine was collected in graduated cylinders from groups of four rats kept in diuresis cages.

Estimation of urinary 5-HIAA. 5-HIAA was estimated by the method of Macfarlane, Dalgliesh, Dutton, Lennox, Nyhus and Smith (1956) usually immediately after urine collection; if this was not possible the urine was treated with a few drops of acetic acid and the same volume of acetone and then stored in the refrigerator. When large volumes of urine were excreted they were concentrated at 40 to 50° under reduced

pressure. In recovery experiments 80 to 100 per cent of added 5-HIAA was found in urine after its concentration.

Estimation of 5-HT *in tissues.* Tissues and serum were extracted twice with 4 parts (4 ml./g. or 4 ml./ml.) of acetone. Bioassay of 5-HT was by the rat uterus preparation, as described previously (Erspamer, 1956).

Compounds. 5-Hydroxyindoleacetic acid, 5-hydroxytryptamine creatinine sulphate and DL-5-hydroxytryptophan were kindly supplied by the Farmitalia S.p.A., Milan. The posterior pituitary preparation used was Pitressin, Parke, Davis & Co.

RESULTS

The Effect of Single Oral Doses of Tap Water

Administration of 5 ml./100 g. of tepid tap water by stomach tube was followed by diuresis. This diluted urine contained about twice the 5-HIAA content of the urine of control rats. The increase in 5-HIAA output lasted only 2 hr. and ceased at the same time as did the water diuresis (Table I).

TABLE I

URINE VOLUME AND URINARY 5-HIAA EXCRETION (IN ML./KG. AND μG./KG. ± S.E.) AFTER ORAL ADMINISTRATION OF A SINGLE DOSE OF TAP WATER. IN PARENTHESES THE NUMBER OF GROUPS* OF RATS

Dose of administered water	(A) Unit volume (ml./kg.) (B) 5-HIAA(μg./kg.)	Urine collection period hr.			
		0–2	2–6	6–24	0–24
No water (24)	A	2·6 ± 0·32	3·7 ± 0·22	16·8 ± 0·77	22·4
	B	9·8 ± 0·59	17·6 ± 0·41	85·9 ± 0·71	113·3
5 ml./100 g. (24)	A	37·5 ± 1·73	11·9 ± 0·57	15·5 ± 0·61	64·9
	B	22·4 ± 0·86	19·7 ± 1·02	86·7 ± 2·0	128·8
3 ml./100 g. (6)	A	18·0 ± 0·29	9·0 ± 0·58	12·0 ± 0·58	39·0
	B	15·3 ± 1·16	22·6 ± 1·16	91·0 ± 2·90	129·0
No water (8)	A	6·4 ± 0·7	6·0 ± 0·18	—	—
	B	7·5 ± 0·49	21·2 ± 0·42	—	—
2 ml./100 g. (8)	A	18·0 ± 1·1	6·7 ± 0·6	—	—
	B	10·5 ± 0·57	21·2 ± 1·0	—	—
1 ml./100 g. (8)	A	12·1 ± 1·0	7·2 ± 0·7	—	—
	B	8·1 ± 0·67	25·0 ± 1·87	—	—

*4 in each group.

The effect of a water load of 3 ml./100 g. was similar to that produced by 5 ml./100 g.; 2 ml./100 g. still produced a significant increase in 5-HIAA excretion ($P < 0.01$); 1 ml./100 g. caused no appreciable effect ($P > 0.4$). 1 ml. tap water plus 4 ml./100 g. paraffin oil provoked the same urinary excretion of 5-HIAA as 1 ml. tap water alone (Table II).

The Effect of Single Oral or Subcutaneous Doses of Isotonic and Hypertonic Solution of NaCl

Table III shows that both isotonic (0·9 per cent) and hypertonic (1·8 per cent) solution of NaCl given by mouth in amounts of 5 ml./100 g. produced an increase in the urinary output of 5-HIAA similar to that produced by tap water. Isotonic saline was more effective than, and hypertonic saline

TABLE II

URINE VOLUME AND URINARY 5-HIAA EXCRETION (IN ML./KG. AND μG./KG. ± S.E.) AFTER THE ORAL ADMINISTRATION OF PARAFFIN OIL. IN PARENTHESES THE NUMBER OF GROUPS* OF RATS

Treatment	(A) Urine volume (ml./kg.) (B) 5-HIAA(μg./kg.)	Urine collection period hr. 0–2	2–6	6–24	0–24
Tap water 1 ml./100 g. (12)	A	8·5 ± 0·57	6·3 ± 0·86	13·2 ± 0·29	27·7
	B	10·9 ± 0·73	22·8 ± 1·32	108·5 ± 2·35	141·4
Tap water 1 ml./100 g. + paraffin oil 4 ml./100 g. (12)	A	9·3 ± 1·17	5·1 ± 0·57	13·9 ± 1·17	28·3
	B	11·0 ± 0·29	23·3 ± 1·47	107·0 ± 2·64	141·0

* 4 in each group.

approximately as effective as, tap water. With isotonic saline and hypertonic saline the excess urinary excretion of 5-HIAA lasted at least for 6 hr. The threshold dose of oral physiological saline causing a significant increase in urinary 5-HIAA during the first 2-hr. period was as low as 1 ml./100 g. ($P < 0·01$).

Subcutaneous isotonic saline caused a higher urinary output of 5-HIAA than oral isotonic saline. Maximum excretion of 5-HIAA occurred in the 6–11 hr. period, concomitantly with maximum diuresis.

TABLE III

URINE VOLUME AND URINARY 5-HIAA EXCRETION (IN ML./KG. AND μG./KG. ± S.E.) AFTER ORAL OR SUBCUTANEOUS ADMINISTRATION OF SINGLE DOSES OF TAP WATER, ISOTONIC NaCl SOLUTION (0·9 PER CENT), AND HYPERTONIC NaCl SOLUTION (1·8 PER CENT). IN PARENTHESES THE NUMBER OF GROUPS* OF RATS

Treatment	(A) Urine volume (ml./kg.) (B) 5-HIAA(μg./kg.)	Urine collection period hr. 0–2	2–6	6–11	11–24
Controls (12)	A	4·9 ± 0·69	5·5 ± 0·52	5·0 ± 0·59	7·0 ± 0·59
	B	7·6 ± 1·1	16·7 ± 0·87	20·0 ± 1·15	65·5 ± 1·73
Tap water by mouth 5 ml./100 g. (14)	A	33·0 ± 1·6	12·4 ± 0·56	6·1 ± 0·56	9·2 ± 0·67
	B	20·5 ± 1·1	16·5 ± 1·26	21·7 ± 2·68	69·0 ± 2·46
Isotonic NaCl by mouth 5 ml./100 g. (16)	A	9·2 ± 0·62	14·1 ± 0·82	11·5 ± 0·75	15·8 ± 0·60
	B	20·2 ± 0·87	21·5 ± 0·69	29·4 ± 0·81	78·0 ± 1·12
Isotonic NaCl by mouth 1 ml./100 g. (6)	A	11·5 ± 0·90	19·6 ± 1·22	—	—
	B	11·5 ± 0·65	23·1 ± 2·45	—	—
Isotonic NaCl by s.c. route 5 ml./100 g. (6)	A	5·8 ± 0·81	11·6 ± 0·81	23·0 ± 1·44	13·8 ± 1·46
	B	12·0 ± 1·44	25·0 ± 1·22	58·0 ± 3·26	71·5 ± 4·07
Hypertonic NaCl by mouth 5 ml./100 g. (6)	A	21·6 ± 2·04	7·2 ± 1·02	5·5 ± 0·94	10·0 ± 1·02
	B	17·0 ± 1·28	23·6 ± 0·91	20·0 ± 1·83	68·0 ± 2·73

* 4 in each group.

The Effect of Repeated Doses of Tap Water and Isotonic Saline

Fig. 1 shows the effect on urinary 5-HIAA excretion and urine volume of tap water given by mouth, and of isotonic saline, given by mouth or subcutaneously on three occasions at 2-hr. intervals.

In another experiment, isotonic saline was given 6 times by mouth at 2-hr. intervals. During the 0–6, 6–12, 12–24 and 24–36 hr. periods the four groups of rats excreted 12·5, 13·6, 6·5 and 4 μg. 5-HIAA per kg./hr., respectively, and eliminated 15, 26, 4 and 1·7 ml./kg./hr. of urine. Total

excretion of 5-HIAA in the first 24-hr. period was 235 μg. compared with 136 μg. of the controls.

Repeated doses of tap water are seen to produce urinary excretion of 5-HIAA throughout the whole period of water diuresis (6 hr.); the amount of 5-HIAA was up to 3–4 times the control value. Isotonic saline given by mouth had a similar effect. Repeated subcutaneous doses of isotonic saline caused a more delayed diuresis and a conspicuous increase in urinary

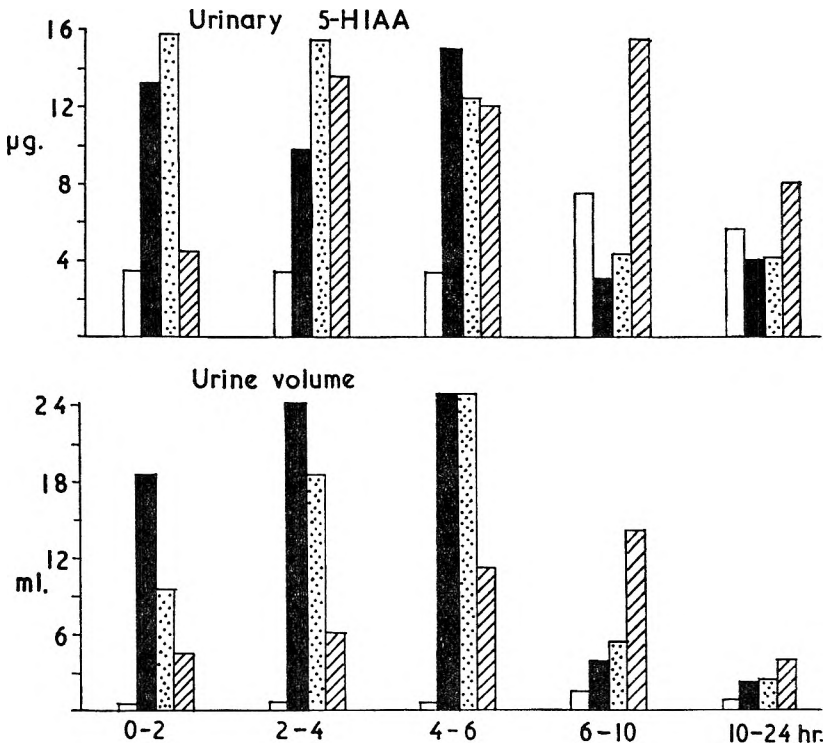

FIG. 1. Urinary 5-HIAA excretion (in μg./kg./hr.) and urine volume (in ml./kg./hr.) in control rats (unshaded columns), in rats given three doses of 5 ml./100 g. tap water by mouth (black columns), in rats given three doses of 5 ml./100 g. isotonic NaCl solution by mouth (dotted columns), and in rats given the same amount of isotonic saline by subcutaneous injection (hatched columns). Water and saline were given at 2-hour intervals, beginning at 0 time. Each value refers to the pool of urine obtained from 16 rats.

excretion of 5-HIAA lasting over 12 hr. During the whole period the injected rats excreted twice as much 5-HIAA as did the control rats.

Repeated doses of subcutaneous saline caused more 5-HIAA excretion than did repeated doses of oral saline.

Although during the first 6 hr. rats given tap water excreted almost 4 times more 5-HIAA than control rats (76 μg. compared with 20·5 μg./kg.), the 24-hr. output of 5-HIAA was the same in the two groups of rats (140 μg. compared with 136 μg./kg.). This is due to the less intense

excretion of 5-HIAA shown by hydrated rats in the 10–24 hr. and, still more so, in the 6–10 hr. periods. Yet, during these periods hydrated rats excreted a half to twice as much more urine than control rats. This affords a striking example of the lack of a clear-cut and constant relation between diuresis and 5-HIAA excretion.

The Effect of Single or Repeated Oral Doses of Tap Water Plus Antidiuretic Hormone of the Posterior Pituitary (ADH)

The effect on 5-HIAA excretion of single oral doses of 5 ml./100 g. of tap water given at the same time with a subcutaneous injection of 0·1 or 1 unit/kg. of ADH (in 0·5 ml. distilled water/100 g.) is shown in Fig. 2.

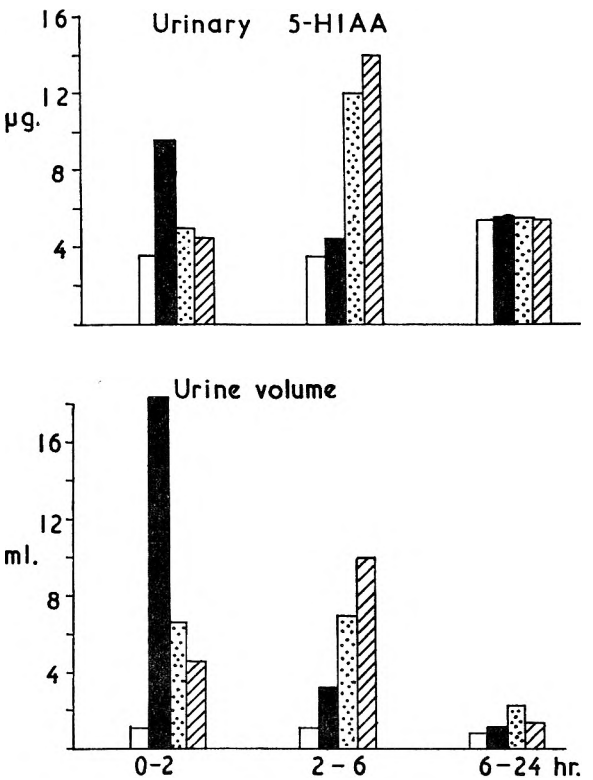

FIG. 2. Urinary 5-HIAA excretion (in μg./kg./hr.) and urine volume (in ml./kg./hr.) in control rats (unshaded columns), in rats given 5ml./100 g. tap water by mouth (black columns), and in rats given the same amount of tap water plus 0.1 unit/kg. (dotted columns) or 1 unit/kg. (hatched columns) of posterior-pituitary antidiuretic hormone, subcutaneously. Each value refers to the pool of urine obtained from 12 rats.

In another experiment, two water loads were given at 2-hr. intervals, simultaneously with two subcutaneous injections of 1 unit/kg. ADH. After 4, 6 and 8 hr., three other injections of ADH were given without any further water load. During the 0–6, 6–12, 12–24 and 24–36 hr.

periods the four groups of rats given water plus ADH excreted 5·1, 6·3, 10·8 and 4·2 μg. 5-HIAA per kg./hr., respectively, and eliminated 3, 2·5, 9 and 1·6 ml./kg./hr. of urine; four groups of untreated control rats excreted 3·4, 7·8, 5·7 and 4 μg./kg./hr. of 5-HIAA, and eliminated 0·8, 1·5, 1 and 1 ml./kg./hr. of urine, respectively.

It seems that after the administration of ADH both water diuresis and maximum excretion of 5-HIAA were much delayed. After a single dose of ADH, maximum diuresis and 5-HIAA excretion occurred in the 2–6 hr. period; after repeated doses of ADH, in the 12–24 hr. period, or more than 10 hr. after the water loads. In the first 12-hr. period the amount of 5-HIAA excreted by rats given six injections of ADH did not exceed that of the control rats, in spite of excreting more than twice the volume of urine.

Single or repeated injections of ADH did not show an appreciable effect on the urinary excretion of 5-HIAA in non-hydrated, control rats.

The Recovery, as Urinary 5-HIAA, *of Exogenous* 5-HTP, 5-HT *and* 5-HIAA *in Control Rats and in Rats Given Isotonic Saline or Tap Water plus* ADH

Numerous experiments were made to check the influence of repeated oral doses of physiological saline or of tap water plus ADH on the urinary excretion of strictly exogenous 5-HIAA and also of the 5-HIAA originating in the tissues from exogenous 5-HTP or 5-HT.

TABLE IV

RECOVERY, AS URINARY 5-HIAA, OF 5-HT AND 5-HIAA ADMINISTERED TO CONTROL RATS, TO RATS GIVEN TAP WATER AND TO RATS GIVEN TAP WATER PLUS ADH. WATER, 5-HT AND 5-HIAA WERE ADMINISTERED BY MOUTH, ADH BY SUBCUTANEOUS ROUTE IN SINGLE OR REPEATED DOSES, AT 2-HR. INTERVALS. IN PARENTHESES THE NUMBER OF GROUPS* OF RATS

Administered water	Dose/kg. of 5-HT or 5-HIAA	5-HIAA excretion (μg./kg. ± s.e.)		
		0–6 hr.	6–12 hr.	12–24 hr.
Nil (4)	5-HT, 1 mg. × 2	92·2 ± 3·92	48·2 ± 3·12	57·5 ± 5·03
5 ml./100 g. × 2 (4)	5-HT, 1 mg. × 2	108·5 ± 2·82	39·0 ± 2·61	61·0 ± 2·43
5 ml./100 g. × 2 + ADH, 1 unit/kg. × 3 (4)	5-HT, 1 mg. × 2	87·7 ± 4·17	69·7 ± 7·01	57·0 ± 1·8
Nil (4)	5-HIAA, 1 mg. × 2	365·5 ± 16·0	164·7 ± 15·3	65·0 ± 8·7
5 ml./100 g. × 2 (4)	5-HIAA, 1 mg. × 2	307·5 ± 10·1	95·0 ± 2·1	76·5 ± 5·2
5 ml./100 g. × 2 + ADH, 1 unit/kg. × 3 (4)	5-HIAA, 1 mg. × 2	229·5 ± 11·3	137·5 ± 9·7	91·2 ± 8·6
Nil (3)	Nil	18·0 ± 1·15	35·3 ± 2·6	—
Nil (3)	5-HIAA, 1 mg.	223·2 ± 9·38	69·3 ± 12·7	—
5 ml./100 g. (3)	Nil	26·3 ± 2·18	36·0 ± 2·83	—
5 ml./100 g. (3)	5-HIAA, 1 mg.	246·7 ± 3·34	61·0 ± 2·33	—
Nil (3)	5-HIAA, 1 mg.	146·6 ± 26·1	—	—
5 ml./100 g. (3)	5-HIAA, 1 mg.	132·6 ± 9·1	—	—

* 4 in each group.

To avoid the pharmacological actions of 5-HTP the dose was restricted to 1 mg./kg. since Erspamer and Bertaccini (1962) showed the minimum antidiuretic dose of subcutaneous DL-5-HTP to be 10–20 mg./kg. The dose was chosen so that the amount of 5-HT produced would be similar to that produced by endogenous 5-HTP. For the same reasons 5-HT was

administered only by mouth at a dose which proved to be completely ineffective on diuresis (Erspamer and Ottolenghi, 1953).

The most important results obtained in these experiments are shown in Tables IV and V.

TABLE V

RECOVERY, AS URINARY 5-HIAA, OF DL-5-HTP ADMINISTERED BY SINGLE OR REPEATED SUBCUTANEOUS OR INTRAPERITONEAL INJECTIONS TO CONTROL RATS AND TO RATS GIVEN SINGLE OR REPEATED ORAL LOADS OF WATER (WITH OR WITHOUT ADH) OR ISOTONIC SALINE. ADH WAS GIVEN BY SUBCUTANEOUS ROUTE. IN PARENTHESES THE NUMBER OF GROUPS* OF RATS

Water or saline load	Dose of DL-5-HTP	5-HIAA excretion (in μg./kg. ± s.e.)		
		0–6 hr.	6–12 hr.	12–24 hr.
1. Nil (4)	1 mg./kg. s.c. × 2	124·5 ± 3·8	68·2 ± 8·16	48·75 ± 2·13
Water 5 ml./100 g. × 2 (4)	1 mg./kg. s.c. × 2	160·0 ± 4·3	46·2 ± 2·4	41·75 ± 1·5
Water 5 ml./100 g. × 2 + ADH, 1 unit/kg. × 3 (4)	1 mg./kg. s.c. × 2	131·5 ± 3·8	79·5 ± 7·0	48·5 ± 3·4
2. Nil (3)	Nil	19·3 ± 1·3	41·0 ± 2·08	—
Nil (3)	1 mg./kg. s.c. × 2	103·3 ± 8·83	71·0 ± 0·58	—
Saline 5 ml./100 g. × 2 (3)	Nil	61·6 ± 4·48	37·0 ± 4·93	—
Saline 5 ml./100 g. × 2 (3)	1 mg./kg. s.c. × 2	184·3 ± 2·26	30·0 ± 1·73	—
3. Nil (3)	1 mg./kg. i.p.	106·7 ± 5·95	60·0 ± 9·75	77·3 ± 3·6
Water 5 ml./100 g. (3)	1 mg./kg. i.p.	147·0 ± 7·23	43·3 ± 6·20	72·0 ± 8·2
ADH, 1 unit/kg. × 3, no water (3)	1 mg./kg. i.p.	108·3 ± 4·4	78·3 ± 9·3	73·7 ± 6·87
Water 5 ml./100 g. + ADH, 1 unit/kg. × 3 (3)	1 mg./kg. i.p.	120·0 ± 11·6	43·0 ± 3·6	57·0 ± 4·04
4. Nil (6)	Nil	19·8 ± 1·39	40·8 ± 1·26	—
Nil (6)	1 mg./kg. s.c. × 2	72·2 ± 4·86	54·5 ± 1·55	—
Saline 5 ml./100 g. × 2 (3)	Nil	40·0 ± 3·5	31·7 ± 1·55	—
Saline 5 ml./100 g. × 2 (3)	1 mg./kg. s.c. × 2	94·3 ± 3·02	27·0 ± 1·8	—
ADH, 1 unit/kg. × 3, no water (3)	1 mg./kg. s.c. × 2	73·7 ± 5·06	54·7 ± 1·22	—
Water 5 ml./100 g. × 2 + ADH, 1 unit/kg. × 3 (3)	1 mg./kg. s.c. × 2	90·0 ± 0·81	34·3 ± 1·63	—
5. Nil (3)	Nil	47·0 ± 0·41		61·7 ± 1·63
Nil (6)	1 mg./kg. s.c. × 3	208·0 ± 14·7		84·3 ± 2·92
Saline 5 ml./100 g. × 2 (6)	Nil	74·5 ± 4·74		58·5 ± 5·6
Saline 5 ml./100 g. × 2 (6)	1 mg./kg. s.c. × 3	198·0 ± 8·17		59·5 ± 4·9
Water 5 ml./100 g. × 2 + ADH, 1 unit/kg. × 3 (3)	1 mg./kg. s.c. × 3	203·3 ± 17·3		44·7 ± 4·49

* 4 in each group.

The following conclusions are valid. Total recovery of 5-HTP, as urinary 5-HIAA, in rats given repeated doses of physiological saline or of tap water plus ADH was the same as, or lower than that observed in control rats. ADH administration to non-hydrated rats given 5-HTP did not produce any change in the urinary excretion of 5-HIAA.

Similar results were obtained after administration of exogenous 5-HT and 5-HIAA. In no instance was recovery of 5-HIAA greater after water loads or water plus ADH.

In experiments 1 and 5 of Table V excess 5-HIAA produced by the precursor amino-acid was completely eliminated within the first 6-hr. period by hydrated rats, whereas in non-hydrated rats it appeared also in the urine of the second 6-hr. period. This fact may be interpreted to be due to a more thorough washing out of the urinary bladder in hydrated rats. In control rats some of the small amount of urine collected in the bladder during the first 6-hr. period was probably evacuated during the second period, because of a lack of stimulus to urinate.

The Effect of Repeated Oral Administration of Physiological Saline or of Tap Water plus ADH *on the* 5-HT *Content of the Gastrointestinal Mucosa and other Tissues*

Four groups of two rats each of 180–250 g. were given 6 doses of physiological saline by mouth (5 ml./kg.) at 2-hr. intervals, and then killed by bleeding 2 hr. after the last dose. Four other groups were given 2 doses of tap water at 2-hr. intervals and at the same time two subcutaneous injections of 1 unit/kg. of ADH. ADH was given on four further occasions at 2-hr. intervals. Finally, four groups of rats served as controls. The results of 5-HT estimation are summarised in Table VI.

TABLE VI

THE 5-HT CONTENT IN DIFFERENT TISSUES OF CONTROL RATS, RATS GIVEN REPEATED ORAL DOSES OF ISOTONIC SALINE AND RATS GIVEN TAP WATER PLUS ADH. IN PARENTHESIS THE NUMBER OF GROUPS* OF RATS. FIGURES WITHOUT S.E. REFER TO A POOL OBTAINED FROM 8 RATS

Tissue	5-HT content (in μg./kg. or μg./ml. ± s.e.)		
	Control rats	Saline rats	ADH rats
Stomach (4)	1·86 ± 0·15	1·70 ± 0·24	1·41 ± 0·16
Small intestine, first quarter (4)	2·30 ± 0·13	1·86 ± 0·06	1·59 ± 0·14
Small intestine, last three quarters (4)	2·57 ± 0·16	1·71 ± 0·15	1·70 ± 0·16
Large intestine (4)	6·32 ± 0·5	5·65 ± 0·5	5·40 ± 0·3
Lung	2·0	1·5	1·55
Spleen	2·15	2·0	2·15
Serum	0·65	0·60	0·62
Brain	0·48	0·30	0·26
Gastrointestinal tract	1·87	1·31	1·22
Lung	0·84	0·66	0·64
Spleen	1·65	1·65	1·60
Serum	0·52	0·54	0·60

*2 in each group.

In a second experiment three additional groups of 8 rats, each of 130–160 g. were treated exactly as above, with the results shown in the lower part of the Table.

The urine volume and the total 5-HIAA excretion during the 12-hr. period elapsing from the first water or saline load until death were in the first experiment: Control rats 20 ml./kg.; 71 μg./kg.; isotonic saline rats 262 ml./kg.; 210 μg./kg., and ADH rats 38 ml./kg.; 78 μg./kg.

And in the second experiment: Control rats 19 ml./kg.; 64 μg./kg., isotonic saline rats 266 ml./kg.; 221 μg./kg., and ADH rats 33 ml./kg.; 57 μg./kg.

It may be seen that at the time of death the rats treated with physiological saline had eliminated most of the administered liquid and as much as 3 to 3·5 times more 5-HIAA than the controls. Rats treated with ADH, on the contrary, still retained enormous amounts of water (in some instances there was haemoglobin in urine, indicating an incipient haemolysis) and the excreted amount of 5-HIAA did not exceed that of controls.

In the first experiment the weight of the intestines was 50·7 g./kg. in control rats, 53·2 g. in isotonic saline rats, and 50·3 g./kg. in ADH rats; in the second experiment 52·2, 55·5 and 51·8 g./kg., respectively. Thus the decrease in the 5-HT content of the intestines cannot be ascribed,

except to an insignificant extent and this only in isotonic saline rats, to an increase in the weight of the tissue produced by water or saline loads.

In a third experiment, 100 rats served as controls and 20 were treated, without any water load, with 5 subcutaneous doses of 1 unit/kg. ADH in 0·25 ml./100 g. physiological saline. At the end of the 12-hr. observation period 5-HIAA excretion was 49 μg./kg. in control rats and 47 μg./kg. in ADH rats. The 5-HT content of the gastrointestinal tract was 2·8 and 2·45 μg./g., respectively; that of the brain 0·41 and 0·38 μg./g., respectively. It is evident that ADH alone does not interfere either in the storage or metabolism of 5-HT.

The above estimations of 5-HT in tissues must be considered as preliminary to a more thorough investigation, and more evidence is needed before it may be concluded that excess urinary excretion of 5-HIAA produced by isotonic saline or water plus ADH is accompanied by a moderate reduction in the 5-HT content of the gastrointestinal tract, lung and brain. The only thing which seems certain is that the 5-HT content of serum and spleen remains unchanged.

Discussion

Administration of water or isotonic saline produced in rats a variable but always conspicuous increase in the urinary excretion of 5-HIAA. This fact seems to be firmly established, but the interpretation of the phenomenon appears to be extremely difficult, owing to the existence of at least three possible explanations.

The first which may be put forward is that oral water or saline increases 5-HIAA output by stimulating the release of 5-HT from the gastrointestinal mucosa through increase of the intraluminal pressure, particularly in the stomach and upper part of the small intestine. Attention on this possibility has been called especially by Bülbring and Lin (1958) and Bülbring and Crema (1959), who showed that, in the guinea-pig, increase of intraluminal pressure in an isolated intestinal loop produced the release into the lumen of measurable amounts of 5-HT.

In the present experiments the participation of intestinal distension in producing excess urinary excretion of 5-HIAA can be excluded by the observation that subcutaneous saline was more effective than oral saline, and that paraffin oil (40 ml./kg.), provoking a distension of the entire gastrointestinal tract, failed to cause any increase in urinary 5-HIAA. The latter observation does not favour the hypothesis that the physiological stimulus for release of 5-HT by the intestinal mucosa, at least for the release into the blood, is represented by increased intraluminal pressure.

The second possibility is that water or saline administration causes increased 5-HT release from the gastrointestinal tract and possibly from other tissues, and hence increased 5-HIAA excretion in urine, as a consequence of the expansion of blood volume or hydraemia it produces or both. Finally, a third possibility is that increased urinary excretion of 5-HIAA is of merely renal origin, being due to a facilitated excretion by

the tubules of the circulating 5-HIAA or to a defect in the tubular reabsorption of the filtered or excreted 5-HIAA.

On the whole, the third hypothesis seems to be most plausible, as it gives a satifactory explanation of the fact that increased 5-HIAA excretion generally paralleled increased urine elimination and that following administration of oral water plus ADH, maximum 5-HIAA excretion was delayed until cessation of block of diuresis produced by ADH.

However, the following experimental observations are difficult to explain and to reconcile with the hypothesis that water or saline loads simply act through renal mechanisms. Although, as stated above, increased 5-HIAA excretion generally coincided with increased urine elimination, there was no constant and obligatory correlation of the two phenomena. For example, repeated subcutaneous doses of isotonic saline produced during the second 2-hr. observation period the excretion of 6 ml./kg./hr. urine and 13·6 μg./kg./hr. 5-HIAA, whereas repeated oral doses of saline caused the excretion of 18·2 ml./kg./hr. urine and 15·6 μg./kg./hr. 5-HIAA (Fig. 1); single oral doses of water caused, during the first 2-hr. period, the excretion of three times more urine than single doses of isotonic saline, yet in both instances urine contained the same absolute amount of 5-HIAA (Table III).

In some experiments, hyperexcretion of 5-HIAA by hydrated rats was followed by a period of reduced excretion, in spite of the urine volume being greater than that of the controls (Fig. 1).

Urinary recovery, as 5-HIAA, of exogenous 5-HTP was satisfactorily the same in hydrated rats (with and without ADH) and in control rats and, at any rate, never larger. The same was true of urinary recovery, as 5-HIAA, of exogenous 5-HT and 5-HIAA. This signifies that saline or water loads (with or without ADH) do not interfere either with the formation of 5-HIAA, via 5-HT, or with the renal excretion of 5-HIAA. Moreover, whereas in rats given water plus ADH, the excretion of 5-HIAA originating from exogenous 5-HTP, 5-HT or 5-HIAA was maximum in the first 6-hr. period, i.e. during ADH antidiuresis, maximum excretion of strictly endogenous 5-HIAA occurred only in the second 6-hr. period, in which as much as 70 to 80 per cent of administered water was eliminated.

Our preliminary studies on the 5-HT content of rat tissues after repeated doses of isotonic saline or of tap water plus ADH have not given conclusive results. At any rate the release of as little as 20 to 30 per cent of the 5-HT contained in the gut, lung and other tissues would certainly not be sufficient to explain the observed huge increase in the urinary 5-HIAA excretion. However, it should be emphasised that static measurements of the 5-HT content of tissues do not reflect the rate of 5-HT formation and metabolism. The 5-HT found at a given moment in tissues corresponds merely to the algebraic sum of the 5-HT formed and that released, and it may well be that administration of water or physiological saline produced not only a moderate reduction of the storing capacity of the tissues, but also an accelerated biosynthesis of 5-HT.

The problem will be further investigated by the administration of L-tryptophan, the remote precursor of 5-HT, α-methyldopa, an inhibitor of the biosynthesis of 5-HT, and reserpine, a powerful 5-HT liberator.

It has been recently claimed that the renal excretion rate of weak organic acids and bases, such as indoleacetic acid, 5-HIAA and 5-HT may depend on the pH of urine. The interference of the urine reaction in the results of present experiments can be excluded, since the pH of urine was practically the same in control rats, in rats given water and in rats given saline, and varied in different experiments only within a small range (6·2–6·9). Moreover, Milne, Crawford, Girão and Loughridge (1960) were unable to find any significant alteration in urinary excretion of exogenous 5-HIAA in the rat by changing the pH, and any alteration in excretion of endogenous 5-HIAA in man; and similarly Sandler and Spector (1961) observed no change in the urinary elimination of 5-HIAA after administration of 5-HT in "acid" rats (pH of urine 5·5–6) and in "alkaline" rats (pH of urine 8–8·5).

Summing up, it is clear that the problem of the origin of excess urinary 5-HIAA after water or isotonic NaCl administration cannot be considered to be solved. Further extensive investigation must be made not only in rats, but also in other animal species. Preliminary experiments in man seem to confirm the data obtained in rats.

Acknowledgement. This work was supported by a grant from the Rockefeller Foundation, New York.

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LETTERS TO THE EDITOR

A Sex Difference in Sensitivity of GFF Mice to an Anaesthetic Steroid

SIR,—The sex difference in sensitivity of mice to certain barbiturates is well known (Hurst, 1958). Here we report a similar finding with the anaesthetic steroid hydroxydione (21-hydroxy-5β-pregnane-3,20-dione 21-hemisuccinate sodium).

Hydroxydione, in 1 per cent aqueous solution, was injected into the tail veins of fawn mice (GFF strain, 19·5 to 22·5 g.). Groups of 12 males received doses of 50 or 100 mg./kg. weight and groups of 12 females received 25 or 50 mg./kg. The times between the loss and recovery of the righting reflex were recorded ("sleep time"); during sleep, the mice were kept in a cabinet at 35°.

Given doses of 50 or 100 mg./kg., male mice slept for 31·1 min. $\pm$ 2·1 (group mean $\pm$ S.E.) and 52·1 min. $\pm$ 2·1, respectively. The females on 25 mg./kg. slept for 18·2 min. $\pm$ 1·5 and on 50 mg./kg. for 46·3 min. $\pm$ 2·9. The results have been statistically analysed; the sleep times and the logarithms of the doses were used as metameters, and validity criteria were applied (Emmens, 1948). The sensitivity of the female mice to hydroxydione was 1·5 times that of the males (fiducial limits, P = 0·95, 1·3 and 1·7).

Twelve male and 12 female mice of the same strain and weight range were injected intravenously with 80 mg./kg. of another anaesthetic steroid, 3α-hydroxy-5β-pregnane-11,20-dione 3-phosphate disodium. The males slept for 35·6 min. $\pm$ 3·6 and the females for 38·8 min. $\pm$ 3·0; the difference was not significant ($t = 0{\cdot}68$; $P > 0{\cdot}5$). Thus the sex difference applies to one anaesthetic steroid but not to another. It may indicate a difference in the rates or pathways of metabolism of hydroxydione in the two sexes. The sex difference for hydroxydione is not found in all strains of mice. P'An and others (1955), using the Rockland Farm Strain of albino Swiss mice, measured the AD50 of hydroxydione for both sexes. (AD50 is the dose causing half the mice to lose their righting reflex.) They found no significant difference between the two sexes after administration by either the intravenous or the oral route.

Glaxo Research Ltd.,
Greenford, Middlesex.
August 27, 1962

R. M. ATKINSON.
M. A. PRATT.
E. G. TOMICH.

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Dilutions of Sulphuric Acid

SIR,—In practice, dilutions of sulphuric acid are made in three ways, volume in volume, weight in weight and weight in volume. Of these three procedures, that of weight in volume is the most tedious, as the mixture has to be cooled before it is made up to volume. It is also impracticable for strong solutions as they have values greater than 100 per cent. Whilst weight in weight dilutions are the most precise, being unaffected by temperature variations, the volume in volume method, for which measured volumes of acid and water are mixed, is

the easiest and could well replace the other methods, the acid content being checked by a specific gravity determination where necessary.

Using the values in the literature (Hodgman), the products of the three methods of dilution at 20° were compared graphically (Fig. 1). This Figure enables dilutions obtained by one method to be converted to another. It reveals that the B.P. 14·0 per cent v/v acid is nearly identical with the B.P.C. 25 per cent w/v acid, and that the 60 per cent v/v and w/w dilutions are much more dissimilar

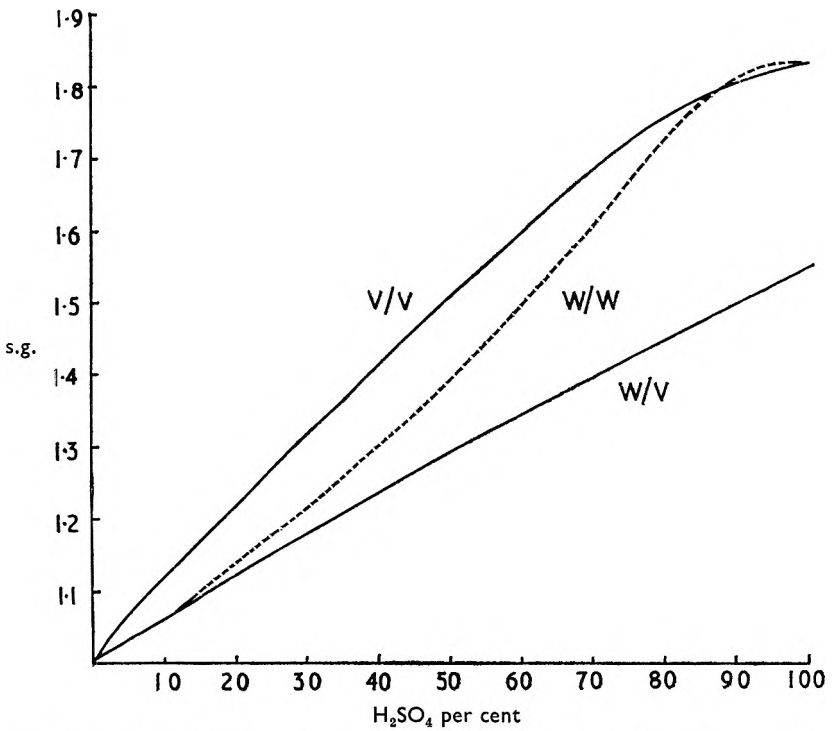

FIG. 1. Comparison of the products of the three methods of diluting H_2SO_4.

than the 80 per cent acids. The B.P. and the B.P.C. have eight dilutions of sulphuric acid, some prepared v/v, some w/w and one w/v. Using the v/v procedure, six dilutions would appear to be sufficient: 80 per cent (roughly equivalent to 80 per cent w/w B.P.C.); 50 per cent (roughly equivalent to 60 per cent w/w); 37·5 per cent (roughly equivalent to 50 per cent w/w B.P.); 20 per cent; 14 per cent (roughly equivalent to 25 per cent w/v B.P.C.); 5 per cent (roughly equivalent to Dilute Sulphuric Acid B.P., which is 10·4 per cent w/w).

Pharmacognosy Department, T. J. BETTS.
School of Pharmacy,
University of London,
29-39, Brunswick Square, London, W.C.1.
July 26, 1962

REFERENCE

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An Adrenergic Neurone Blocking Action of Dimethylphenylpiperazinium

SIR,—Recent work in this laboratory has attempted to localise the site of sympathetic inhibition in the isolated small intestine of the guinea-pig (Wilson, 1962) using transmurally stimulated (Paton, 1955) "Finkleman" (1930) preparations set up in Krebs's solution at 37°. With two sets of platinum electrodes and different stimulation parameters, the parasympathetic cholinergic nerve plexuses in the gut wall and the periarterial sympathetic adrenergic fibres may be stimulated both independently and simultaneously. A base-line of just submaximal transmural twitches is first obtained (stimuli of 1 to 4 V; 0·3 msec.; 5/min.) and these may then be abolished by the simultaneous maximal stimulation of the periarterial sympathetic nerves (stimuli of 15 to 20 V; 1 msec.; 25 to 50/sec. for 10 to 20 sec.) That the origin of the transmural contractions and the site of the sympathetic inhibition are both distal to the parasympathetic ganglia is shown by the persistence of these effects in the presence of ganglion blocking concentrations of hexamethonium bromide (5×10^{-5} to 1×10^{-4}), or of DMPP (1,1-dimethyl-4-phenylpiperazinium iodide; 5×10^{-6}) held in contact with the intestine for 10 min.

But, if the intestine is left in contact with this concentration of DMPP for 10 to 30 min., another action of the drug is seen which is separate from its known ganglion stimulating and ganglion blocking properties. The inhibitory effect of sympathetic nerve stimulation now becomes progressively smaller and is finally reversed to a potentiation of the transmural twitch; in spite of repeated washings, this total blockade of adrenergic nerve inhibition remains unchanged for several hours, although (–)-adrenaline added to the organ bath still gives a complete inhibition of the transmural contractions. For the most part these findings agree with the results of Bentley (1962), who has shown a similar action of DMPP on the rabbit Finkleman preparation and the hypogastric nerve-vas deferens preparation of the guinea-pig. The only difference is Bentley's observation that in the rabbit Finkleman preparation, sensitivity to the inhibitory action of added (–)-noradrenaline is increased in the presence of DMPP, whilst in my experiments with the guinea-pig, greater than normal amounts of added (–)-adrenaline are needed to suppress the transmural twitches.

The evidence from both sources appears to establish a postganglionic adrenergic blocking action of DMPP which interferes in some way with the eventual release of the sympathetic transmitter substance. This action is of interest because DMPP is chemically distinct from the known adrenergic neurone blocking agents, xylocholine (Exley, 1957), reserpine (Burn and Rand, 1958), bretylium (Boura and Green, 1959) and guanethidine (Maxwell and others, 1960).

Department of Pharmacology,
King's College, University of London,
Strand, W.C.2.
August 25, 1962

A. B. WILSON.

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