REVIEW ARTICLE

INFLAMMATION AND THE INFLAMMATORY MECHANISMS*

By Professor N. JANCSÓ, M.D., M.Hungarian Acad. Sci.

From the Pharmacological Institute of the Medical University of Szeged, Hungary

DURING the past decade research concerning the mechanism of inflammation was focused mainly on the so-called mediators of the inflammatory tissue reaction. Numerous studies have been carried out, especially on rats, in which oedema-producing agents were employed, and the aetiology as well as the pharmacology of the resultant inflammation has been extensively investigated. Mainly as a result of these experiments it is generally believed that under the influence of inflammatory agents, vasoactive substances like histamine, 5-hydroxytryptamine (5-HT) or polypeptides are released or produced in the tissues and that actually it is these substances that induce the vascular inflammatory reaction. Recently published reviews of Feldberg (1956), Paton (1957) and Spector (1958) reflect impressively the progress in the elucidation of inflammatory processes achieved through this line of research.

In the present review, however, I wish to discuss new points of view concerning this topic. In our experiments made in the past few years we detected close relations existing between inflammatory processes and blood coagulation. On the basis of the results obtained we are convinced that some kind of a clotting process is involved in the mechanism of inflammation, and plays a decisive rôle in the causation of the inflammatory symptoms.

Before I begin to describe our own findings and the new concept based on them I should like to describe the method used.

We have developed, and described (Jancsó, 1960) a novel method for the visualisation of inflammatory reactions in the tissues of rats. Immediately before or after application of inflammatory stimuli, colloidal silver is injected into the blood stream, whereby the parts of the body involved in the inflammation process turn brown. We employed colloidal silver (Pharmacopoea Hung. V) in 1 per cent solution rendered isotonic with 5 per cent glucose. In most experiments 10 ml./kg. of this solution was injected into the tail vein. Whereas, Evans blue and other dyes used in inflammation research produce only a diffuse patch of colour, with colloidal silver a brilliant microscopic picture can be obtained which reveals the finest details of localisation.

Let us first consider one or two examples which illustrate how the silver introduced into the circulation is deposited in the inflamed tissues. Two different types of localisation can be observed. They may be clearly distinguished in Fig. 1 which shows a part of the mesenterium. Before the silver was injected 0·4 ml. of a 6 per cent kaolin suspension was injected into the peritoneal cavity to induce a violent inflammatory

* Based upon a Special University of London lecture given at King's College, London, on May 18, 1961.

reaction. After the injection of the silver colloid a large quantity of silver is seen to be bound by the tissue which forms brown coloured coatings on the internal surface of the minute and intermediate veins. At other sites, for instance in the subepidermal layer of the ear the silver

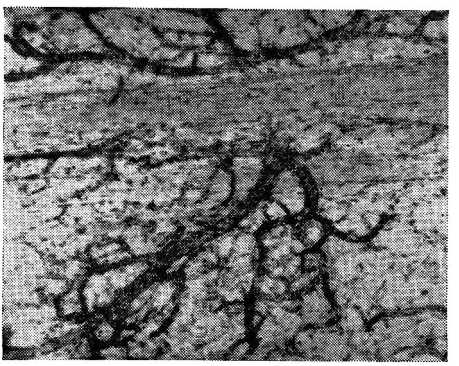


FIG. 1. Deposition of colloidal silver in the mesenterium of the rat subsequent to an intraperitoneal injection of 0·4 ml. of a 6 per cent kaolin suspension. Unstained stretch preparation.

coating may extend to the capillary walls, however, the site on which it mostly forms is the wall of the small venules. In the area of inflammation such coatings may develop within a few minutes.

In addition another form of localisation may also be distinguished on the photomicrograph. All histiocytes of the connective tissue are

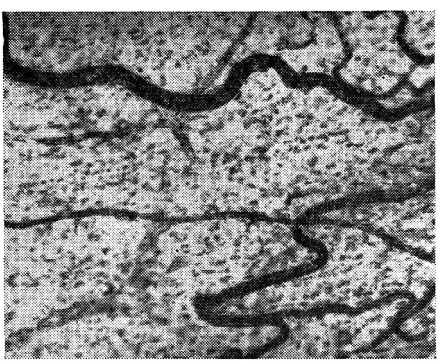


FIG. 2. Fixation of intravenous injected colloidal silver in the plantar skin of the rat paw after subplantar injection of 6 μg. of 5-HT. "Angiotaxis" and extravascular storage in histiocytes is seen.

crowded with silver granules proving that, owing to the inflammation, the vessel walls have become pervious to large colloidal particles, of about 200 Å diameter. In all probability this escape of the colloidal substance may be explained by the assumption that the inflammatory process opens large pores in the vessel wall through which the colloidal particles are driven out towards the surrounding tissue by hydrostatic forces. At

first only a diffuse brown imbibition could be observed in the tissue. It may last for 1–2 hr. till the histiocytes engulf the silver.

An essentially similar picture is obtained with other inflammatory substances also. Fig. 2 demonstrates the vascular and cellular silver deposition in the skin of the rat paw after subplantar injection of 6 μg. of 5-HT. The same picture is obtainable after local application of 10–20 μg. of compound 48/80. Fig. 3 shows the effect of 6 μg. of the venom of *Vipera aspis* in the plantar skin.

This coating of the internal vessel walls with colloidal material is identical with the phenomenon which I described years ago under the name "endothelial activation" (Jancsó, 1941, 1947, 1955). At the time I reported that after local or systemic administration of histamine the

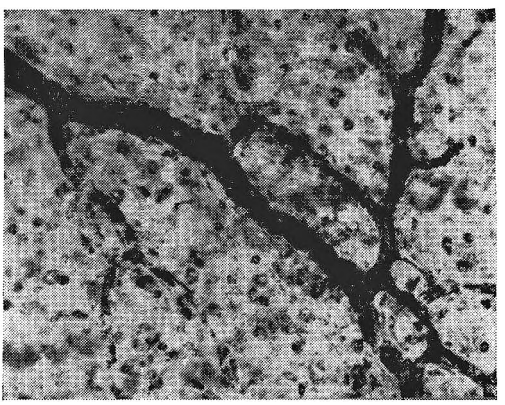


FIG. 3. Deposition of intravenous injected colloidal silver in the plantar skin of a rat after subplantar injection of 6 μg. of *Vipera aspis* venom.

vessels undergo a characteristic change which manifests itself by the deposition of the circulating Indian ink on the internal surface of the small veins. I also established (Jancsó, 1947) that this phenomenon can be inhibited with antihistamines, furthermore, that it also appears after the application of different histamine liberators. In addition, I also established that the endothelial cells soon engulf the carbon particles adsorbed on their surface and store them in granular form, that is to say, in this instance the endothelial cells fulfil a function which otherwise is a privilege of cells belonging to the reticulo-endothelial system. Some hours or days later the engulfed material appears in the surrounding tissue stored in phagocytic cells; by the intervention of some mechanism as yet incompletely understood the carbon particles emerge from the blood-vessels.

After my original report numerous authors interested themselves in this phenomenon (Benacerraf, McCluskey and Patras, 1959; Biozzi, Menè and Óváry, 1948; Gözsy and Kátó, 1957; Maltoltsy and Maltoltsy, 1951; Selye, Lemire and Cantin, 1959; Törö, 1942; Zemplényi, Fodor and Lojda, 1960). Besides the term "endothelial activation" the designations "endothelial colloidopexis" and "angiotaxis" were suggested.

This latter name proposed by Selye and others (1959) is perhaps the most adequate term. Till now attention has been focused chiefly on the phagocytic activity of the endothelium and on its possible rôle in the defence against infections (Gözsy and Kátó, 1957) or in the localisation of disease in the body (Benacerraf and others, 1959), respectively. In other papers the inhibitory action of antihistamines, phenothiazine derivatives and cortisone on this phenomenon was studied. Alksne (1959) confirmed with the electron-microscope that histamine prompts endothelial cells to take up colloidal particles from the blood stream.

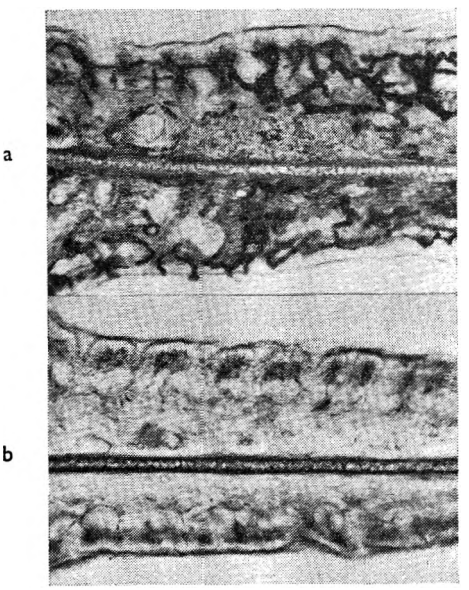


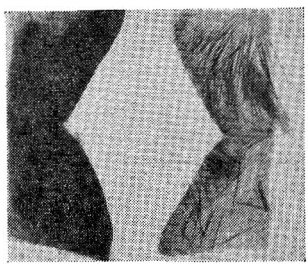
FIG. 4. a. Fixation of colloidal silver in the ear of a normal rat after painting with xylol; b. ear of a rat pretreated with Thrombodym 250 mg./kg. intravenously. No silver deposition after the application of the inflammatory stimulus.

At the moment, however, almost nothing is known about the mechanism of the angiotaxis phenomenon. Why do colloidal particles attach themselves to the vessel wall? What is the real cause of this conspicuous change in the properties of the vascular wall? In my book *Speicherung* published in 1955 (Jancsó, 1955) I put forward the hypothesis that angiotaxis is caused by a clotting process taking place in the vascular wall. The monomeric or low polymeric soluble fibrin produced during this coagulation process reacts immediately with the circulating colloidal particles forming a coloured precipitate on the internal surface of the vessels. Thus, in terms of this hypothesis, the coating on the inflamed vessel walls consists of silver and fibrin. (See also Jancsó and Jancsó-Gábor, 1960; Jancsó, 1960.)

It was tempting to consider such an explanation because an outstanding property of fibrinogen is that it adsorbs colloidal particles intensively in

the course of its coagulation (Jancsó, 1955). It is easy to show that coloured clots are formed if, after the injection of colloidal silver or carbon, an intravascular coagulation is induced by thrombin or any other coagulant. The microscopic picture of angiotaxis is also consistent with the coagulation-hypothesis since, besides typical parietal coatings, coloured precipitates and plugs can be detected in the inflamed vessels. Such findings suggest that some kind of precipitation takes place in the inflamed vessels.

To test our conception we made experiments with various anticoagulants. Surprisingly, heparin proved to be ineffective, but with several other anticoagulants important positive results were obtained (Jancsó and Jancsó-Gábor, 1960; Jancsó, 1960). Thus the expected effect



a b

FIG. 5. Silver deposition in the conjunctiva after instillation of a 0·5 per cent capsaicin solution. Transparent eyelid preparations; (a) from a control rat, (b) from a rat pretreated with Thrombodym, 220 mg./kg. intravenously.

could be achieved with compounds containing rare earth metals, as well as with sodium polyanetholesulphonate (Liquoid) and also suramin. These compounds were found in our experiments to exert a primary and specific inhibitory effect upon the process of angiotaxis. Moreover, these anticoagulants are able to inhibit the gross increase of vascular permeability caused by inflammatory agents. In this way, they prevent the escape of the colloidal silver particles from the terminal vascular bed and also much diminish the extent of oedema formation. Rare earth metals such as lanthanum, cerium, neodymium, praseodymium and samarium proved to be effective even in the form of their inorganic salts. In most experiments, however, we used the commercial preparations Helodym 88 (the didymium salt of β-acetylpropionic acid) or Thrombodym (the neodymium salt of sulpho-isonicotinic acid). Fig. 4 shows transverse sections of xylol painted ears of rats illustrating the characteristic effect of rare earths. Colloidal silver, 100 mg./kg., was injected into a rat and its ear was painted immediately afterwards for 10 sec. with xylol. Another animal was treated before this procedure with 250 mg./kg. of Thrombodym. The animals were killed 3 hr. later. Whereas in the ear of the control an intense accumulation of silver can be seen in the vessels and in histocytes, in the ear of the pretreated animal, silver deposits are absent. Likewise, the silver deposition in the conjunctiva which follows

the instillation of a strong, 0·5 per cent, capsaicin solution into the eye could be also totally prevented by a high dose of Thrombodym or Helodym 88 (Fig. 5).

A similar preventive effect could be established in inflammations induced by subplantar injection of various other agents, for example, compound 48/80, 5-HT, dextran, kallikrein, staphylococcus toxin, bee venom and diverse snake venoms. In these experiments, some plugs could often be detected in the minute vessels, but no typical angiotaxis or extravascular storage occurred.

In further experiments it could be shown that these anticoagulants are also able to inhibit the development of oedematous swelling to a great extent. Table I shows the effect of seven different oedema-producing agents on the rat paw and their inhibition by rare earths and Liquoid.

TABLE I

INHIBITION BY ANTICOAGULANTS OF OEDEMA FORMATION IN THE HINDPAW OF THE RAT INDUCED BY VARIOUS INFLAMMATORY AGENTS. AMPUTATION AND WEIGHING OF THE PAWS 1 HR. AFTER APPLICATION OF THE INFLAMMATORY AGENT. ALL FIGURES ARE MEANS FOR 10 ANIMALS WEIGHING 120–150 G.

Inflammatory agent	Dose subplantar in 0·1 ml.	Wt. increase of oedema-leg controls per cent.	Inhibition of oedema formation (per cent.)		
			Helodym 88* 9 ml./kg. i.v.	Thrombodym † 2 × 6 ml./kg. i.v.	Liquoid 50 mg./kg. i.v.
Comp. 48/80	10 μg.	51·6	69·0	90·7	80·7
5-Hydroxytryptamine ..	5 μg.	71·6	61·8	—	—
Peptone (Witte)	5 mg.	60·0	79·2	—	80·4
Dextran	0·5 mg.	56·8	—	70·0	—
Kallikrein (Padutin) ..	2 U	57·7	72·5	—	64·1
Saliva (human)	0·1 ml.	67·2	78·2	93·2	68·5
Staphylococcus culture filtrate	0·1 ml.	51·8	76·5	—	69·0

* 1 ml. = 9 mg. didymium metal. † 2·2 per cent solution.

In these experiments, in addition to compound 48/40, dextran, 5-HT and kallikrein, other substances were also used, the oedema-producing effect of which was detected in the course of our experiments. These substances were human saliva, staphylococcus toxin and peptone (Witte). The effect of these agents was much inhibited, even to an extent of 90 per cent, by anticoagulant substances.

According to our investigations the venom of different snakes in a dose of 4–20 μg. induces in the rat paw huge oedemas lasting for hours. Bee venom has a similar effect. Table II shows that these oedemas may be also strongly inhibited by anticoagulants.

These results are in complete agreement with the experiments made with colloidal silver which proved that anticoagulants prevent the escape of silver particles from the vessels. All these experiments provide evidence that anticoagulant agents are capable of preventing the extreme increase of vascular permeability which is the most characteristic symptom of inflammation.

It is important to note that, in contrast to the symptoms just discussed, the hyperaemic response is not influenced by anticoagulants. The ear

of rats pretreated with anticoagulants and painted with xylol becomes vivid red and warm although no oedematous swelling occurs. In the same way, after instillation of a strong, 0·1–0·5 per cent, capsaicin solution into the eye the usual conjunctival oedema does not develop, but the dilatation of the vessels is well observed. Probably, inflammatory hyperaemia is produced by vasoactive substances liberated from the tissues and neither the liberation nor the action of these substances is affected by anticoagulant agents.

This persistence of hyperaemia may account for the fact that the oedematous swelling of the rat paw cannot be prevented completely by anticoagulants. The dilatation of the terminal vessels may lead, through raising the intravascular hydrostatic pressure, to increased filtration and moderate accumulation of fluid in the tissue.

TABLE II

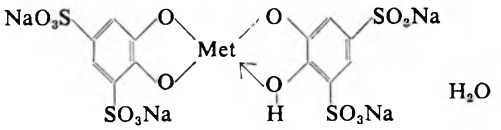
OEDEMA-PRODUCING EFFECT OF ANIMAL VENOMS AND ITS INHIBITION BY ANTICOAGULANT AGENTS. AMPUTATION AND WEIGHING OF THE HIND PAWS 1 HR. AFTER SUBPLANTAR APPLICATION OF THE TOXIN. ALL FIGURES MEANS FOR 8 ANIMALS WEIGHING 110–140 G.

Venom	Dose subplantar in 0·1 ml.	Wt. increase of oedema-leg controls per cent	Inhibition of oedema formation per cent	
			Helodym 88 9 ml./kg. i.v.	Liquoid 50 mg./kg. i.v.
Apis mellifera	Venom content of 1 bee	63·4	74·2	83·0
Naja naja	1 mouse U	64·1	66·0	79·5
"	0·5 "	46·3	71·0	—
Ancistrodon piscivorus ..	10 µg	56·7	64·1	73·9
Crotalus durissus	20 µg.	60·0	55·9	—
Sistrurus miliaris	10 µg.	58·0	78·5	84·4
Vipera ammodytes	12 µg.	58·8	64·8	68·6
Vipera aspis	5 µg.	62·4	79·0	75·2
Vipera berus	5 µg.	59·7	61·0	—

From all these observations we drew the conclusion that some clotting process must be involved in the mechanism of acute inflammation taking place in the wall and internal surface of the terminal vessels. Since the formation of fibrin must, in consequence of its pronounced adsorptive properties, result in a fixation of the circulating colloidal particles to the internal vascular wall, this conception provides a plausible explanation for the phenomenon of angiotaxis. At the same time it explains in an acceptable way the prevention of angiotaxis by anticoagulant agents. Moreover, our findings suggest that the gross increase of vascular permeability is also in some way connected with this coagulation process in the vascular structure.

Our experimental work was made difficult by the fact that intravenous injection of both Thrombodym and Helodym is not well tolerated by rats. Unless the injection is administered extremely slowly respiratory, arrest, convulsions and death occur. Recently I have succeeded in synthetising new compounds of rare earth metals having anticoagulant properties and which are free from such acute toxic effects. These compounds are rare earth complexes of pyrocatechol sodium disulphonate

in which the metal atom is attached to the oxygen atoms of two phenolic residues (I)



(I)

Such complexes of lanthanum, neodymium, praseodymium and samarium do not cause shock-like symptoms in rats and they are exceptionally well tolerated by rabbits too. These new compounds proved to be also very effective in counteracting inflammatory reactions in rats induced by subplantar injection of bee venom, cobra venom,

TABLE III

OEDEMA-PRODUCING EFFECT OF VARIOUS COAGULANTS ON THE HIND PAW OF THE RAT AND ITS INHIBITION BY ANTICOAGULANTS. WEIGHING OF THE PAWS 1 HR. AFTER SUBPLANTAR INJECTION OF THE CLOTTING AGENT. ALL FIGURES MEANS FOR 8 ANIMALS, WEIGHING 110–140 G.

Coagulant	Dose subplantar in 0·1 ml.	Wt. increase of oedema-leg controls per cent	Inhibition of oedema formation per cent		
			Helodym 88 9 ml./kg. i.v.	Thrombodym 2 × 6 ml./kg. i.v.	Liquoid 50 mg./kg. i.v.
Thrombin	20 U	51·3	56·0	87·4	79·6
Thromboplastin (rat brain)	0·1 ml.	67·3	76·7	86·5	87·0
Cephalin (pig brain)	5 mg.	52·8	72·5	—	69·1
Russell's viper venom	4 μg.	53·0	—	60·0	69·0
Ninhydrin	0·8 mg.	62·3	82·9	—	66·7
Sodium 1,2-naphthoquinone-4-sulphonate	2 mg.	46·3	—	—	62·2

compound 48/80 or dextran. With high doses of 250–350 mg./kg. the inhibition of the oedematous swelling may even exceed 80 per cent.

Since in the light of our results the development of inflammatory symptoms is intimately connected with a local coagulation phenomenon it was logical to assume that agents possessing clotting activity will exert a definite inflammatory effect if injected into the tissues of rats. To test this assumption we injected various coagulants into the foot pad of rats (Table III). As expected, we found that bovine thrombin, thromboplastin from rat brain, and unpurified cephalin from pig or rabbit brain are very effective in inducing oedematous swelling. In the same way, Russell's viper venom which is a recognised powerful thromboplastic substance caused much oedema even in a dose of 4 μg. Furthermore, it could be established that anticoagulants also exert in these experiments a considerable inhibitory action. The oedema induced by Russell's viper venom proved to be relatively resistant, but with high doses of Liquoid it could be almost completely prevented. Similar results could be obtained with ninhydrin and sodium 1,2-naphthoquinone-4-sulphonate which, according to Chargaff and Ziff (1941), are direct coagulants of fibrinogen. All these oedemas are associated with pronounced angiotaxis. Fig. 6 shows the action of thromboplastin in the plantar skin,

and Fig. 7 the action of 25 units of thrombin in the subcutis of the dorsal region. This effect of thrombin suggests very impressively that thrombin gains access to the blood thus causing parietal clotting and that the coatings are formed by a fibrinous precipitate with adsorbed silver.

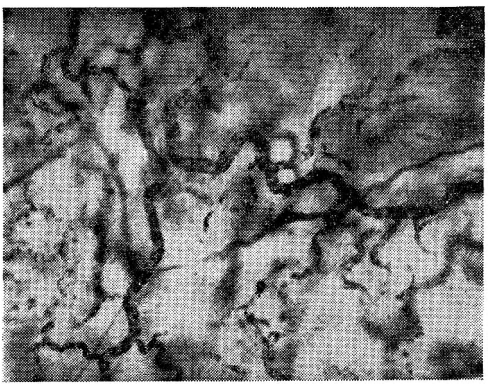


FIG. 6. Silver deposition in the plantar skin of a rat after subplantar injection of rat brain thromboplastin. Intense angiotaxis. The distended lympthatic network also contains colloidal silver.

Pretreatment with rare earths or Liquoid inhibits angiotaxis in the usual manner.

In the course of further experiments we attempted to prove the correctness of our conception in a direct way. Our experimental plan was to induce an afibrinogenaemia in rats and then to observe how these animals

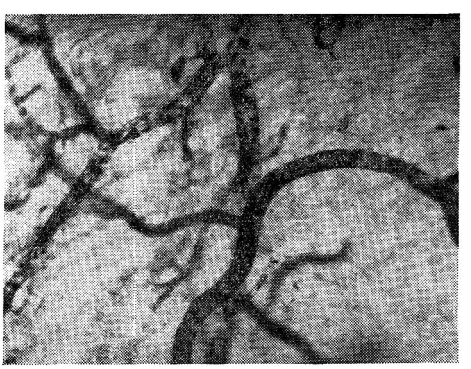


FIG. 7. Angiotaxis in the blood vessels of the dorsal skin region of a rat evoked by the subcutaneous injection of 25 units of bovine thrombin.

reacted to inflammatory stimuli. If our working hypothesis is really correct then the main symptoms of inflammation will fail to occur in such animals because the possibility of coagulation is precluded.

It could be foreseen that an *in vivo* defibrination of rats would not be an easy task. There are numerous data in the literature proving that

cautious intravenous infusion of coagulants in dogs or rabbits may cause a transitory total disappearance of fibrinogen. Such an effect could be for instance achieved with snake venoms (Mellanby, 1909; Rocha e Silva, 1955), thrombin (Basinger and Allen, 1951; Brayton and Zucker, 1957; de Nicola and Rosti, 1949; Jürgens and Studer, 1948; Quick, Hussey, Harris and Peters, 1959; Warner, Brinkhous, Seegers and Smith, 1939) or thromboplastin (Basinger and Allen, 1951). In our work we used thrombin. We succeeded only after long experimentation to elaborate a procedure with which fairly permanent defibrination could be achieved in rats. First 7–8 ml./kg. of carbon tetrachloride was injected subcutaneously into rats weighing 200–250 g. The defibrination was effected 40–48 hr. later by administering intravenously 120, 240 and finally 300 units of thrombin at intervals of 15 min. The inflammatory

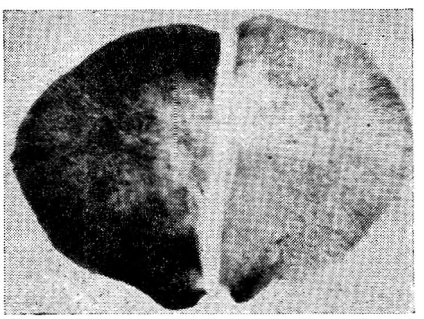


FIG. 8. Silver deposition in the xylol-painted ear; (a) ear of the control rat, (b) ear of the "defibrinated" rat.

agents were applied 5–15 min. after the last thrombin injection. Colloid silver was given, also, immediately afterwards in most of the experiments.

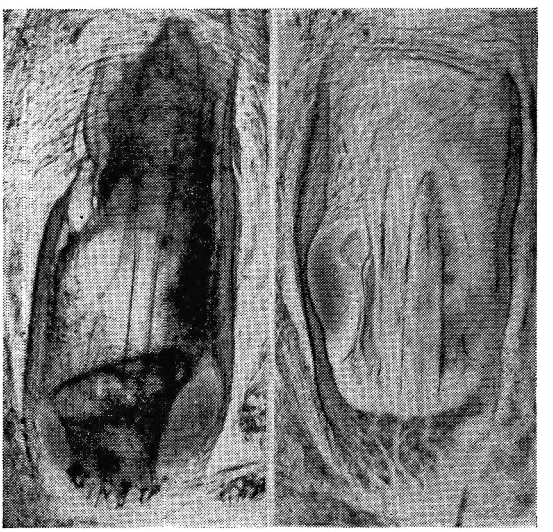
In recent experiments, before the thrombin injections, 300–400 units of heparin were first injected intravenously, to be followed at intervals of 15 min. by three or four injections each of 600 units of thrombin. After heparin-pretreatment the animals tolerate defibrination better; this may be because the fibrin is formed in the circulation in less coarse aggregates. Pretreatment with carbon tetrachloride is probably advantageous because it inhibits the replacement of fibrinogen through impairment of the liver. Others have shown that fibrinogen restituition fails to occur after elimination of the liver (Meek, 1912; Drury 1919).

The blood of rats treated in these ways becomes totally incoagulable; even 60–100 min. after the last thrombin injection the blood does not clot if thrombin is added, or only a small fragile clot appears. Normal blood clots in a coherent column even at a dilution of 200 times.

The inflammatory experiments made on the defibrinated animals led to results which fulfilled our expectations. It became evident that absence of fibrinogen favourably influences the inflammatory reactions in the same way as do anticoagulant agents. But the hyperaemic reaction is not modified; for instance, painting the ear with xylol produces a

vivid redness. Oedema, however, fails to develop. Likewise, instillation of a capsaicin solution into the eye causes only vasodilatation without conjunctival oedema. If colloidal silver is injected into the blood stream, the fixation of the metal in the affected area fails to occur, or only minute deposits are observed. Pictures of transparent preparations clearly show the diametrically opposed behaviour of normal and defibrinated animals (Fig. 8).

Similarly no oedema and no conspicuous silver deposition could be detected after subplantar or subcutaneous injection of compound 48/80, kallikrein, staphyococcus toxin, various snake venoms or thrombin. Microscopic examinations revealed that both angiotaxis and storage in the extravascular histiocytes is either totally absent or quite insignificant.



a b

FIG. 9. Silver deposition in the blood sinus of sinus hairs after the intravenous administration of dextran, 0·4 ml. of a 6 per cent solution of "Macrodex" before the silver injection; (a) sinus hair of a *normal* rat; silver stained fibrin net in the blood sinus; (b) sinus hair of a *defibrinated* rat. Total absence of silver in the blood sinus.

A particularly interesting picture appears in the lacunae of the sinus hairs after an intravenous injection of dextran which is followed by much oedema of the nasal region (Fig. 9). In the non-defibrinated rat every blood sinus contains a brown coloured typical fibrin net evidencing clearly that because of the inflammation in the cavity fibrin is formed which adsorbs much colloidal silver. The snout of the defibrinated rat, on the other hand, was not swollen and in the sinuses no silver fixation can be observed.

These findings suggest that fibrinogen indeed plays a central rôle in the mechanism of the acute inflammatory reaction. They render very probable the conception that the cause of the angiotaxis-phenomenon

arises in the conversion of fibrinogen to fibrin on the inflamed vessel walls. This fibrin in turn forms a precipitate with the circulating colloidal particles. Similarly, the findings are consistent with the assumption that a gross increase of vascular permeability is also in close causal connection with this coagulation process. Apparently fibrin formation is able to increase greatly the number of large pores in the vessel wall.

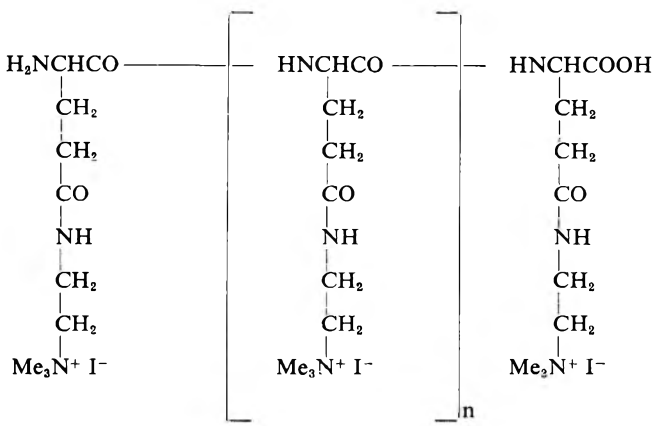
I must, however, confess that owing to haemodynamic reasons the experiments should be interpreted cautiously. Although the animals showing conspicuous prostration after the defibrination were discarded, in such experiments one must always take into account that the blood pressure falls and the blood supply of the peripery becomes deficient. We also established this deficiency in our defibrinated rats by a method once recommended by Rous and Gilding (1929) for control of the blood supply. We injected Patent Blue V into the blood stream of the defibrinated rats and observed the speed and depth of staining of the tissues by the highly diffusible dye. The experiments showed that the blood supply to the hind paws is strongly diminished; but, the circulatory supply to the fore legs and nasal region is fairly well maintained. In the staining of the ears there was no marked delay and the blood supply was appreciably enhanced if the ears were painted with xylol. Therefore, it is important that the effect of defibrination on the inflammatory reaction can be well demonstrated in the parts of the body just mentioned, the blood supply of which is not much impaired. It is even more important that the characteristic effect could also be clearly demonstrated in the diaphragm which may be considered as a permanently active muscle. Even after a severe haemorrhage Rous and Gilding (1929) found the diaphragm to be well supplied with blood. If 50 μg. of compound 48/80 was injected i.p. into rats pronounced angiotaxis could be provoked in the vessels of the diaphragm and the surrounding histiocytes were filled with colloidal silver. In defibrinated animals all this could be detected only in traces.

From all these findings it may be inferred that these defibrination experiments may after all be accepted and strongly support the view that fibrinogen is needed for the development of inflammatory reactions.

Yet we must admit that the assumed rôle of fibrinogen could be proved in a truly convincing manner only if these experimental results could be reproduced by a more adequate method of defibrination, and one which does not impair the circulation. Whilst we were searching for such a method, we discovered the interesting action of polyanthinium compounds on blood coagulation. It was soon revealed that with the aid of these compounds the desired aim can be achieved.

The compounds in question were prepared by Kovács and Kótai (1959) in the Institute of Organic Chemistry of the University of Budapest. Not long ago they synthethised from α-poly-L-glutaminic acid and ethylenediamine a soluble basic polypeptide derivative which was named "polyanthin". Kovács and Kótai recently synthethised some new derivatives in which one of the amino groups of the ethylenediamine is linked to the polypeptide chain, while the other one is quaternised with

alkyl groups. Most of our experiments were carried out with trimethyl-polyanthinium iodide. (II).



(II)

Trimethyl-polyanthinium iodide

The following compounds were also used: dimethylethyl-polyanthinium iodide and -methylsulphate and dimethyloxyethyl-polyanthinium chloride. The molecular weight of these compounds is approximately 15,000.

Theoretical considerations prompted me to investigate the effect of these interesting new compounds on blood coagulation. It was tempting to consider the possibility that not only polymers carrying electronegative groups such as heparin or heparinoids, but also macromolecular

TABLE IV

DEFIBRINATION OF THE CIRCULATING BLOOD BY POLYANTHINIUM COMPOUNDS

Species	Compound dose i.v.	Fibrin titre observed after hr.											
		0	1	2	3	4	5	6	10	20	24	30	48
Rabbit	A 70 mg./kg.	200		o	sm	sm	2	2		20			200
Rabbit	B 25 mg./kg.	250	o	o				2	20				250
Cat	A 65 mg./kg.	200			o			sm		20	50	100	200
Dog	A 65 mg./kg.	200		o		o		o		100			200
Rat	A 100 mg./kg.	200	o	o				sm			100		200
Rat	C 75 mg./kg.	200	o	o	o			sm			100		200

o = blood totally incoagulable. sm = small clot in undiluted blood.

A = trimethyl-polyanthinium-iodide; B = dimethylethyl-polyanthinium-iodide; C = dimethyloxyethyl-polyanthinium-chloride.

compounds of polycation character may interact with clotting factors and exert an anticoagulant activity. Animal experiments revealed that these compounds really possess a considerable anticoagulant and anti-thrombic activity. Their mode of action is quite different to that of recognised anticoagulant substances. Injected intravenously they induce a rapid fall of the fibrinogen content of the blood and in higher doses an

afibrinogenaemia lasting for several hours. After 24–48 hr. the fibrinogen level again becomes normal.

As Table IV shows, this peculiar effect could be equally well demonstrated on rats, rabbits, cats and dogs. In these experiments the fibrin titre was determined by the method of Schneider (1952) with minor modifications. Citrated blood is diluted and the highest dilution in which visible coagulation occurs when thrombin is added is determined. As can be seen, before the experiment is made, clot formation is detectable in a dilution of 200 or 250, whereas, at the peak of the effect, even the undiluted blood does not clot or only a small clot is formed. Thrombin was added as a powder to the undiluted blood.

TABLE V

INHIBITION BY POLYANTHINIUM COMPOUNDS OF THE INFLAMMATORY OEDEMA OF THE HIND PAW OF RATS. ALL FIGURES MEANS FOR 6 ANIMALS WEIGHING 160–200 G.

Compound mg./kg. dose i.v.	Interval min.	Inflammatory agent subplantar	Killed after min.	Wt. increase of oedema-paw		Inhibition per cent
				pretreated per cent	control per cent	
A 100	60	comp. 48/80 10 μg.	60	14·3	45·4	68·6
A 125	60	dextran 600 μg.	80	14·8	58·0	74·5
A 100	60	kaolin 0·1 ml. 10 per cent	140	13·0	60·0	78·0
A 100	60	cephalin 5 mg.	60	13·0	46·6	71·8
C 65	60	cobra venom 1 mouse U	60	11·7	45·7	74·4

The animals tolerated large, fibrinogen-depleting doses well. In 3 cats, after administration of 65 mg./kg. of the trimethyl derivative, the blood pressure showed only minimal changes during the experiment. The blood pressure was measured without using anaesthesia by means of a polythene tube inserted into the aorta.

In view of the fibrinogen-depleting effect it could be expected that the polyanthinium compound will exert an antithrombotic action too. We investigated, in rats whether pretreatment with polyanthinium exhibits a protective action against lethal thrombosis caused by an intravenous injection of thrombin. The animals were given 100 mg./kg. of trimethylpolyanthinium iodide intravenously (LD50 = 160 mg./kg.). It could be established that even tremendous doses of thrombin, such as 2000 units, did not evoke thromboembolic death. In controls the injection of 400 units is nearly always fatal. For these experiments the standardised thrombin preparation Thrombofort-"Richter" was used.

As these findings show, with the aid of polyanthinium compounds, the aim which was always in our mind in the course of our work is easy to achieve. A single injection of polyanthinium brings about a lasting disappearance of fibrinogen, whilst the animal maintains its fit condition. Thus we possess a simple and reliable method with which it could be

unequivocally decided how inflammatory reactions develop in the absence of fibrinogen.

The inflammatory experiments on polyanthinium treated animals revealed important results which confirmed our previous conclusions in every respect. We found that on such animals oedematous inflammation is so reduced that it can be hardly detected by inspection and the local fixation of injected colloidal silver is totally absent.

Table V shows the far reaching inhibitory effect of polyanthinium compounds on the oedematous swelling of the paw of the rat due to

a b

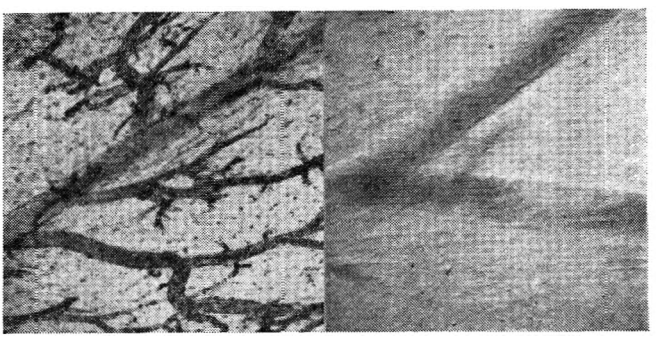
FIG. 10. Prevention of the inflammatory fixation of intravenous injected colloidal silver by trimethyl-polyanthinium iodide, 100 mg./kg. intravenously 1 hr. before application of the inflammatory stimulus. Plantar skin of a normal (a) and of a polyanthinium-treated rat (b) after subplantar injection of 10 μg. of compound 48/80.

compound 48/80, dextran, kaolin and cobra toxin, respectively. The inflammatory agents were applied in these experiments 1 hr. after the polyanthinium compounds, at which time the blood is already free of fibrinogen.

The local hyperaemic reaction was not inhibited by the polyanthinium compounds; this is in accordance with our previous experiments which all showed that this symptom is independent of clotting factors. The xylol-painted ear of the pretreated animals becomes vividly red just like that of the normal animals, but, the oedematous swelling fails to occur. In the same way, if a capsaicin solution of 0·1 per cent is instilled into the eye vasodilatation is obvious, but the usual conjunctival oedema does not develop.

To study the silver fixation phenomenon rats were defibrinated with trimethyl-polyanthinium iodide and dimethylethyl-polyanthinium methyl-sulphate respectively. Of the former 100 mg./kg. of the latter 80 mg./kg.

was administered intravenously and then we waited for 1 hr. for the blood to become free of fibrinogen. Subsequently, different inflammatory substances were applied locally and ultimately 120 mg./kg. of colloidal silver was injected into the tail vein. The experiments demonstrated that the usual silver deposition does not take place. Neither angiotaxis nor granular accumulation in the histiocytes of connective tissue could be observed. Thus, no visible silver deposition was found in the xylol-painted ear and capsaicin-treated conjunctiva. The same negative result was obtained in the plantar skin and musculature after subplantar injection of compound 48/80, 10 and 20 μg., dextran, 600 μg., toxin of the rattle snake *Crotalus durissus*, 10 μg., and thrombin, 40 U., respectively. In the controls, as usual, ample deposits of silver could be



a b

FIG. 11. Silver fixation in the omentum of the rat after the intraperitoneal injection of a suspension of fine glass particles; (a) normal rat; ample deposition of the intravenous injected colloidal silver on the internal surface of the vessels and in extravascular histiocytes, (b) "defibrinated" rat: no visible silver deposition. Defibrination was effected by the intravenous injection of 80 mg./kg. of dimethylethyl-polyanthinium methylsulphate 1 hr. before the application of the inflammatory stimulus.

observed in all inflammatory sites. Figs. 10 and 11 show in an impressive way, the difference in response of the depleted animals and of the controls. It should be noted that in the Kupffer-cells of the liver a fine and even storage of silver could be observed in the polyanthinium-treated animals too.

All these findings strongly support our view that in rats fibrinogen plays indeed a fundamental rôle in the mechanism of acute oedematous inflammation.

According to current views the increase in vascular permeability is an outcome of the direct action of the so-called mediators of the inflammatory reaction such as histamine or 5-HT. It is generally believed that it is an intrinsic property of these substances to induce increased vascular permeability. I do not want to deny the important rôle played by these mediators in the elicitation of inflammatory responses. For instance, the experiments of Rowley and Benditt (1956), Parratt and West (1957, 1958), Doepfner and Cerletti (1957) and Stenger (1958) suggest very strongly that dextran- or eggwhite-oedema in rats is mainly caused by

the liberation of 5-HT or 5-HT plus histamine. There is, however, no proof that these mediators exert their effect by a simple direct action on the vascular wall. The whole affair seems to be far more complicated since our results suggest that the intervention of the coagulation system is a pre-requisite to typical inflammatory vascular reaction.

The assumed coagulation process in the vascular wall may be initiated by the inflammatory agent itself or by endogenous thromboplastic factors produced under the influence of these agents. Unfortunately, we are at present completely ignorant of the nature of such endogenous thromboplastic factors.

In retrospect it may be stated that a wealth of observations suggest that the blood clotting system plays an important rôle in the mechanism of inflammation in addition to its function in haemostasis and thrombus-formation. Apparently, a coagulation process is involved in the inflammatory tissue reaction taking place in the walls of the terminal vessels. In all probability it is this coagulation process which induces the most characteristic vascular inflammatory reactions, that is, the angiotaxis phenomenon and the excessive increase in permeability. Such a conception would then be in good agreement with the observation that neither angiotaxis nor gross oedema formation can be achieved in the absence of fibrinogen or if the coagulation is inhibited by an appropriate anticoagulant.

REFERENCES

Alksne, I. F. (1959). *Quart. J. exp. Physiol.*, **44**, 51–66.
Basinger, C. and Allen, J. G. (1951). *J. Lab. clin. Med.*, **38**, 784.
Benacerraf, B., McCluskey, R. T. and Patras, D. (1959). *Amer. J. Path.*, **35**, 75–91.
Biozzi, G., Menè, G. and Óváry, Z. (1948). *Clinica Nuova*, **6**, 393–402.
Biozzi, G., Menè, G. and Óváry, Z. (1948a). *Rev. d'Immunol.*, **12**, 320–334.
Biozzi, G., Menè, G. and Óváry, Z. (1949). *Lo Sperimentale*, **99**, 341–351; 352–362.
Brayton, R. G. and Zucker, M. B. (1957). *Proc. Soc. exp. Biol. N.Y.*, **96**, 418–421.
Chargaff, E. and Ziff, M. (1941). *J. biol. Chem.*, **138**, 787–788.
de Nicola, P. and Rosti, P. (1949). *Boll. Soc. ital. biol. sper.*, **25**, 1184.
Doepfner, W. and Cerletti, A. (1957). *Int. Arch. Allergy*, **10**, 348–354.
Feldberg, W. (1966). *Ibid.*, **8**, 15–31.
Gözsy, B. and Kátó, L. (1957). Studies on Phagocytic Stimulation. Institute of Microbiol. and Hyg., University of Montreal.
Jancsó, N. (1941). *Ber. Physiol.*, **126**, 475.
Jancsó, N. (1942). *Magyar Orvosi Archivum*, **43**, 367–368.
Jancsó, N. (1947). *Nature, Lond.*, **160**, 227–228.
Jancsó, N. (1955). Speicherung, Stoffanreicherung im Reticuloendothel und in der Niere, Akadémiai Kiadó, Publishing House of the Hung. Acad. Sci., Budapest.
Jancsó, N. (1960). *Klin. Wschr.*, **11**, 1067–1068.
Jancsó, N. (1960). *Bull. Millard Filmore Hosp.*, 1960, **7**, 53–77.
Jancsó, N. and Jancsó-Gábor, A. (1960). *Arch. exp. Path. Pharmak*, **238**, 83–84.
Jürgens, R. and Studer, A. (1948). *Helv. Physiol. Acta*, **6**, 130–149.
Kovács, K. and Kótai, A. (1959). *Acta Chim. Hung.*, **21**, 453–460.
Matoltsy, G. and Matoltsy, M. (1951). *J. Pharmacol.*, **102**, 237–249.
McMaster, 0. and Drury, 0. (1919). *Proc. Soc. exp. Biol., N.Y.*, **26**, 490. cited by Heinlein, H. (1943). *Z. exp. Med.*, **112**, 535–558.
Meek, 0. (1912). *Amer. J. Physiol.*, **30**, 161, cited by Heinlein, H. (1943). *Z. exp. Med.*, **112**, 535–558.
Mellanby, J. (1909). *J. Physiol.*, **38**, 28–40.
Parratt, J. R. and West, G. B. (1957). *J. Physiol.*, **139**, 27–41.
Parratt, J. R. and West, G. B. (1958). *Brit. J. Pharmacol.*, **13**, 65–70.
Paton, W. D. M. (1957). *Pharmacol. Rev.*, **9**, 269–328.
Quick, A. J., Hussey, C. V., Harris, J. and Peters, K. (1959). *Amer. J. Physiol.*, **197**, 791–794.

Roche e Silva, M. (1955). *Histamine*, Springfield: Thomas.
Rous, P. and Gilding, H. P. (1929). *J. exp. Med.*, **50**, 189–211.
Rowley, D. A. and Benditt, E. P. (1956). *Ibid.*, **103**, 399–411.
Selye, H., Lemire, Y. and Cantin, M. (1959). *Med. exp.*, **1**, 11–16.
Schneider, C. L. (1952). *Amer. J. Obstet. Gynec.*, **64**, 141–147.
Spector, W. G. (1958). *Pharmacol. Rev.*, **10**, 475–505.
Stenger, E. G. (1958). *Arzneimitt.-Forsch.*, **8**, 693–694.
Törö, I. (1942). *Z. mikrosk.-anat. Forsch.*, **52**, 552.
Warner, E. D., Brinkhous, K. M., Seegers, W. H. and Smith, H. P. (1939). *Proc. Soc. exp. Biol., N.Y.*, **41**, 655–657.
Zemplényi, T., Fodor, J. and Lojda, Z. (1960). *Quart. J. exp. Physiol.*, **45**, 50–54.

RESEARCH PAPERS

EFFECTS OF SALICYLATE ADMINISTRATION ON REPUTED INDICES OF ADRENAL CORTICAL ACTIVITY IN THE RAT

BY G. F. BLANE* AND K. A. MUNDAY

From the Department of Physiology and Biochemistry, Southampton University

Received May 15, 1961

Changes in the urinary sodium and potassium concentration and 17-ketosteroid excretion patterns of rats treated with large doses of salicylate have been compared with those seen in animals given a standard pituitary-adrenal stimulus (exposure to cold). The significance of the general lack of correlation of responses to these two forms of treatment is discussed in the light of the frequently accepted theory that salicylates stimulate the adrenal cortex.

THE claim of Hetzel and Hine (1951) that salicylates stimulate the adrenal gland by way of the pituitary to increased release of cortical hormones has been supported by many observations in experimental animals and man. In a recent review Smith (1959) emphasises the general acceptability of the evidence, disputing only that the antirheumatic effects of salicylate are mediated via the adrenal cortex.

The conclusions of the early workers in this field were based on adrenal ascorbic acid or cholesterol depletion in rats after heavy dosage with salicylate (Robinson, 1951; van Cauwenberge, 1951) and the absence of these responses in the hypophysectomised animal (van Cauwenberge, 1951). More recently, conclusive evidence of increased levels of circulating plasma 17-hydroxycorticosteroids after treatment with salicylate has been obtained for rats (Done, Ely and Kelley, 1958; Roskam, 1957; van Cauwenberge, 1954); dogs (Done, Ely and Kelley, 1958); guinea-pigs (Good, Done, Ely and Kelley, 1957); and man (Done, Ely and Kelley, 1955; Roskam, 1956).

The increased plasma corticosteroid response to massive salicylate dosage is not paralleled in the urinary steroid metabolites where there is considerable disparity in reports of levels after salicylate administration. While increase in urinary excretion of reducing corticoids is reported by van Cauwenberge and Huesghem (1952), they found no consistent response in 17-ketosteroid output of salicylate-treated adults with rheumatic diseases. Pellegrini and Sala (1952) and Roskam (1956) report similar findings. Other workers confirm the absence of a consistent urinary increase after salicylate treatment in either 17-ketosteroids (Böe and Stöa, 1953; Bonati, Bertolani and Lorenzini, 1951; Henly, 1952; van Cauwenberge and Huesghem, 1951); the 17-hydroxycorticosteroids (Smith, Gray and Lunnor, 1954), or reducing steroids (Böe and Stöa, 1953). In fact Done and co-workers (1955, 1958) report consistent depression of steroid excretion during salicylate treatment.

* Present address: Sub-Department of Pharmacology, The University College of the West Indies, Jamaica, W.I.

Until recently there appear to have been no detailed studies of urinary sodium and potassium levels after salicylate administration, and yet the sodium : potassium ratio of the urine is known as a sensitive index of adrenal mineralocorticoid activity (Hetzel, McSwiney, Mills and Prunty, 1956; Simpson and Tait, 1955). However, in 1959, Hetzel, Charnock and Lander reported low sodium and high potassium levels in the urine of human subjects within a few hr. after administration of 5 g. of sodium salicylate, a finding which is in close accord with our own preliminary observations in man (Blane, 1957).

The plasma and urinary electrolyte changes resulting from acute cold exposure have been shown in rats to be compatible with a theory of increased adrenal activity in both normal (Munday and Blane, 1960, 1961) and hypothermic animals (Munday, Blane, Chin and Machell, 1958). In the normal rat so stressed there is also a marked increase in the short-term (12–36 hr.) output of 17-ketosteroids (Munday and Blane, 1960). It was decided therefore to test the value of the same criteria in the assessment of salicylate as a pituitary-adrenal stimulant. The response to salicylate of the urinary sodium and potassium levels, and the 17-ketosteroid excretion was studied in rats.

Experimental Methods

Male rats of the Hooded strain were used and during the 12-hr. experimental periods were housed in metabolism cages constructed to avoid dilution or contamination of urine by drinking fluid.

Normally rats were supplied with a standard cube diet and water. Solid food however was withheld during the test periods to avoid urine contamination and instead a 5 per cent glucose in 0·85 per cent saline solution was available *ad libitum*. This short-term change of diet has been shown to be without effect on plasma electrolyte and 17-ketosteroid levels (Munday and Blane, 1960).

Techniques used in the measurement of urinary electrolytes and 17-ketosteroids were the same as have been described previously (Munday and Blane, 1960).

Both adrenal glands were removed from rats of 100 to 150 g. under pentobarbitone anaesthesia. Until required they were maintained in draught-free cages and supplied with 1 per cent saline and rat cake. On this régime their plasma electrolyte levels remained within the normal range. Careful macroscopic examination at autopsy confirmed the completeness of adrenalectomy in all animals.

Acetylsalicylic acid was given to rats by stomach-tube as 1·0 ml. of 6 per cent solution in 2 per cent sodium carbonate. Since the intact animals used in most experiments weighed between 300 and 350 g. the dose of 60 mg. per rat corresponded to something less than 20 mg. per 100 g. weight. Proportionately reduced doses were used for the smaller adrenalectomised rats. Other groups of rats were given sodium salicylate instead of acetylsalicylic acid at an increased dose (30 g./100 g.) corresponding to the reported lessened therapeutic activity of the sodium salt. The "control" animals received a 1·0 ml. placebo of sodium bicarbonate

solution containing an amount of sodium equivalent to that given to the "experimental" rats in their salicylate solution.

RESULTS

Rats supplied with glucose in saline produced large volumes of clean urine in the 12-hr. test period and the urinary sodium and potassium

TABLE I

EFFECTS OF ACETYLSALICYLIC ACID, SODIUM SALICYLATE AND EXPOSURE TO COLD ON THE 12-HR. VALUES OF URINARY SODIUM, POTASSIUM AND 17-KETOSTEROID EXCRETION IN MALE RATS

	Controls	Acetylsalicylic acid	Sodium salicylate	Cold exposure (0° C.)
Na	150·7 ± 0·56 (12)	141·1 ± 0·63 (12)	143·9 ± 1·42 (12)	137·6 ± 0·89 (10)
K	13·73 ± 0·62 (12)	34·25 ± 2·78 (12)	38·30 ± 2·19 (12)	15·89 ± 0·27 (10)
Na:K	11·21 ± 0·52 (12)	4·37 ± 0·33 (12)	3·86 ± 0·19 (12)	8·68 ± 0·14 (10)
17-KS	53·9 ± 1·45 (18)	26·2 ± 1·02 (8)	23·6 ± 1·11 (8)	62·5 ± 2·14 (8)

Na and K concentrations as m-equiv., 17-KS as μg./12 hr./rat, numbers of animals in parenthesis means ± standard errors.

concentrations as well as the 17-ketosteroid excretion were found to vary within very narrow limits under these conditions (Table I).

Urine collected in the immediate 12-hr. period after administration of either salicylate showed a much depressed Na:K ratio by virtue of a fall in sodium level and rise in potassium (Table I). The directional similarity of these electrolyte changes to those obtained on cold exposure

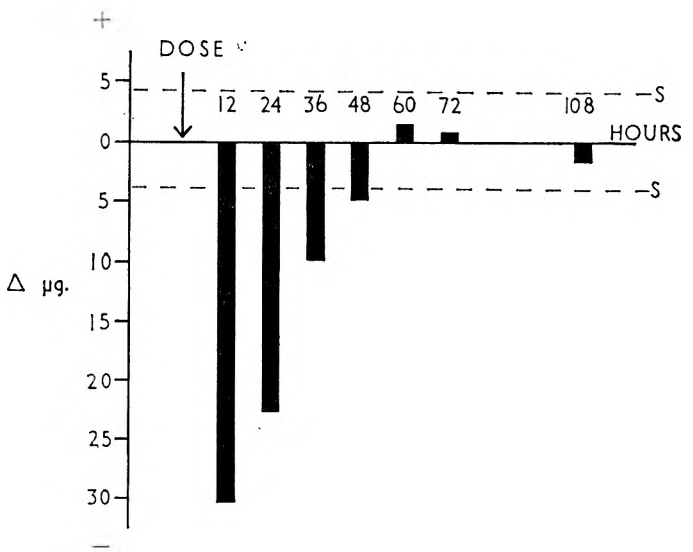


FIG. 1. Typical effect of a single dose of sodium salicylate on the urinary 17-ketosteroid excretion of a group of 8 male rats. Base line = mean value of 52·9 μg./12 hr./rat from 16 rats with S the standard deviation. Columns represent the mean change in 17-ketosteroid excretion from the control level (Δ μg.) measured for the whole group of salicylate-treated animals at 12 hr. intervals. These mean differences at 12, 24 and 36 hr. are highly significant with $P < 0{\cdot}005$. Dose at zero time = 30 mg./100 g. rat.

is also illustrated in Table I and supports the contention of earlier authors that salicylate stimulates the pituitary-adrenal axis.

In direct contrast to the increased 17-ketosteroid excretion on short-term cold exposure, the 17-ketosteroid excretion of rats treated with acetylsalicylic acid or sodium salicylate was significantly diminished (Table I). Longer term experiments provided conclusive evidence that the urine 17-ketosteroid excretion of rats is greatly reduced during the first 12 hr. after dosing with salicylate and that there is a gradual return to normal over the following 36 hr. (Fig. 1).

Bilaterally adrenalectomised rats were given sodium salicylate and their urinary sodium and potassium levels determined after 12 hr. (Table II). In the adrenalectomised control animals the régime on which they

TABLE II

EFFECTS OF SODIUM SALICYLATE ON URINARY SODIUM AND POTASSIUM CONCENTRATION OF ADRENALECTOMISED RATS

	Na	K	Na/K
Adrenalectomised untreated ..	160·2 ± 2·62 (14)	14·1 ± 1·01 (14)	11·68 ± 0·72 (14)
Adrenalectomised treated with sodium salicylate	190·1 ± 6·25 (14)	49·7 ± 4·00 (14)	3·94 ± 0·23 (14)

Na and K concentrations as m-equiv., numbers of animals in parenthesis, means ± standard errors.

were maintained caused only a slight elevation of both sodium and potassium urine concentrations and the Na : K ratio was within the normal range. On treatment with salicylate there was, by contrast with intact animals, a marked rise in the urinary sodium level. However, the potassium concentrations rose at the same time to such high levels that the Na : K ratio was as low in these adrenalectomised as in the intact animals.

DISCUSSION

It has been established earlier that the urinary sodium and potassium changes in rats caused by exposure to cold are dependent on the integrity of the adrenal gland (Munday and Blane, 1960). That an essentially similar change in electrolyte pattern should have been observed in man receiving heavy doses of salicylate (Hetzel, Charnock and Lander, 1959) and is now shown in intact rats appears to further support the long-established hypothesis that salicylates have an effect in stimulating the pituitary-adrenal axis. There are difficulties in the way of accepting this interpretation.

In all salicylate-treated intact animals, loss of potassium was massive compared to that seen in cold-stressed rats and furthermore potassium excretion was also greatly increased in adrenalectomised rats receiving salicylate. It appeared therefore that potassium excretion may be affected by salicylate without the intervention of adrenal corticosteroids. Guest, Rapoport and Roscoe (1945) found the potassium loss in men treated with acetylsalicylic acid or sodium salicylate to be secondary to an alkalosis caused by hypernoea, and it is noteworthy that the rats in our experiments were frequently observed to be hyperventilating after dosage

with salicylate. The high urinary potassium in salicylate treated rats may represent—in part at least—a loss of fixed base consequent on respiratory alkalosis. A further possible alternative explanation is suggested by the *in vitro* work of Hicklin (1959) and Manchester, Randle and Smith (1958) which has yielded some evidence that salicylate may act directly to bring about potassium loss from tissues through interference with the energy supplies for active transport of this ion. Hence, for the present, potassium loss in salicylate-treated subjects and the Na : K ratio must be rejected as reliable indices of adrenal activity.

Sodium excretion on the other hand was reduced in intact salicylate-treated rats and raised in adrenalectomised animals receiving the same treatment. This could be interpreted to suggest that a sodium-retaining factor is released from the adrenal cortex in increased amounts by salicylate and that urinary sodium concentration may provide an adequate indication of the level of adrenal activity in these circumstances, at least for mineralocorticoid output.

The reduced urinary steroid excretion in subjects receiving salicylate presents another problem and should not immediately be taken as an indication that adrenal activity is depressed. Done, Ely and Kelley (1958) have found consistently low urinary 17-hydroxycorticosteroid and 17-ketosteroid levels in human subjects and animals treated with salicylate at a time when plasma corticosteroid levels were consistently high. Similar plasma changes did not occur in hypophysectomised or adrenalectomised experimental animals. In considering mechanisms to account for the low levels of urinary steroid metabolites while plasma corticosteroid levels are high it is of interest that salicylate may be secreted in a significant proportion as the glucuronide (Kapp and Coburn, 1944). Steroids too are mainly excreted as conjugates, and combination with glucuronic acid in the liver is known as a major pathway (Dorfman and Ungar, 1954). Salicylate might therefore act as a competitive inhibitor of corticosteroid excretion in a manner comparable to that already demonstrated for *N*-acetyl-*p*-aminophenol (Corte and Johnson, 1958). In this instance there would be a concentration of circulating corticosteroid while the steroid-glucuronide levels in the urine, including the 17-ketosteroid fraction, would fall.

The demonstration of Na : K ratios as low in adrenalectomised as in intact rats treated with salicylate, and the dramatic fall in 17-ketosteroid excretion of salicylate-treated rats shows clearly the inadequancy of these indices in the assessment of salicylate as a pituitary-adrenal stimulant. Sodium alone followed an excretion pattern that paralleled that seen in animals given a standard stress. Further detailed studies are required in this field with particular attention paid to the effects of salicylate on plasma corticosteroid levels, having regard to the possibility that these may be raised only because renal clearance of steroids is inhibited.

References

Blane, G. F. (1957). Ph.D. Thesis, University of Southampton.
Böe, J. and Stöa, K. F. (1953). *Acta endocr., Copenhagen*, **12**, 201–206.

Bonati, B., Bertolani, F. and Lorenzini, R. (1951). *Farmaco*, **6**, 719–725.
Cauwenberge, H. van (1951). *Lancet*, **2**, 374–375.
Cauwenberge, H. van (1954). *C.R. Soc. Biol., Paris*, **148**, 1297–1300.
Cauwenberge, H. van and Heusghem, C. (1951). *Lancet*, **1**, 771–773.
Cauwenberge, H. van and Heusghem, C. (1952). *Acta med. scand.*, **141**, 265–283.
Corte, G. and Johnson, W. (1958). *Proc. Soc. exp. Biol., N.Y.*, **97**, 751–755.
Done, A. K., Ely, R. S. and Kelley, V. C. (1955). *Metabolism*, **4**, 129–142.
Done, A. K., Ely, R. S. and Kelley, V. C. (1958). *Ibid.*, **7**, 52–69.
Dorfman, I. J. and Ungar, F. (1954). *Metabolism of Steroid Hormones*, Minneapolis: Burgess Publishing Co.
Good, A., Done, A. K., Ely, R. S. and Kelley, V. C. (1957). *Metabolism*, **6**, 346–349.
Guest, G. M., Rapoport, S. and Roscoe, C. (1945). *J. clin. Invest.*, **24**, 770–774.
Henly, A. A. (1952). *Ann. Rep. West London Hosp.*, 24–26.
Hetzel, B. S., Charnock, J. S. and Lander, H. (1959). *Metabolism*, **8**, 205–213.
Hetzel, B. S. and Hine, D. C. (1951). *Lancet*, **2**, 94–97.
Hetzel, B. S., McSwiney, R. R., Mills, I. H. and Prunty, F. T. G. (1956). *J. Endocrin.*, **13**, 112–124.
Hicklin, J. A. (1959). *Nature, Lond.*, **184**, 2029.
Kapp, E. M. and Coburn, A. F. (1942). *J. biol. Chem.*, **145**, 549–565.
Manchester, K. L., Randle, P. J. and Smith, G. H. (1958). *Brit. med. J.*, **1**, 1028–1030.
Munday, K. A. and Blane, G. F. (1960). *J. Endocrin.*, **20**, 266–275.
Munday, K. A. and Blane, G. F. (1961). *Comp. Biochem. Physiol.*, **2**, 8–21.
Munday, K. A., Blane, G. F., Chin, E. F. and Machell, E. S. (1958). *Thorax*, **13**, 334–342.
Pellegrini, U. and Sala, I. (1952). *Lattante*, **23**, 869–871.
Robinson, F. B. (1951). *Brit. med. J.*, **1**, 300.
Roskam, J. (1956). *Schweiz. med. Wschr.*, **86**, 1269–1273.
Roskam, J. (1957). *Chem. Abstr.*, **51**, 7576–7577.
Simpson, S. A. and Tait, J. F. (1955). *Recent Progr. Hormone Res.*, **11**, 183–219.
Smith, M. J. H. (1959). *Ann. rheum. Dis.*, **18**, 298–300.
Smith, M. J. H., Gray, C. H. and Lunnon, J. B. (1954). *Lancet*, **1**, 1008–1009.

ANTITRICHOMONAL AGENTS 5-NITROTHIAZOLES, 5-NITROPYRIDINES AND 5-NITROPYRIMIDINES

By R. M. Michaels and R. E. Strube

From the Research Division, The Upjohn Company, Kalamazoo, Michigan

Received April 21, 1961

Some 5-nitrothiazoles, 5-nitropyridines and 5-nitropyrimidines have been synthesised and evaluated against *Trichomonas vaginalis* and *Trichomonas foetus in vitro* and *in vivo* in the mouse, hamster and monkey. Although 2-amino-5-nitropyrimidine, 2-acetamido-5-nitropyrimidine and 2-trifluoroacetamido-5-nitropyrimidine were highly active in mice infected with *T. vaginalis* the activity was not superior to the standard 2-acetamido-5-nitrothiazole. The nitropyrimidines were highly species specific, being much more active *in vivo* against *T. vaginalis* than against *T. foetus*. One compound, 2-amino-5-nitropyrimidine was selected as the best of this series because of the high blood levels obtained after a single oral dose in a monkey, and its presence in a biologically active form.

Trichomonas vaginalis (Donné, 1836) and *Trichomonas foetus* are flagellate parasites of the genito-urinary tract of man and bovine, respectively. In the female, *Trichomonas vaginalis* causes an overt disease of the genito-urinary tract (Kean, 1955; Kean and Wolinska, 1956; Perl, Guttmacher and Raggazoni (1956), while the male serves primarily as an asymptomatic carrier (Perl and others, 1956). Evidence is accumulating that the parasite localises in Skene's glands, Bartholin's glands and the urinary tract (Kean, 1955; Kean and Wolinska, 1956; Perl and others, 1956), thus making it extremely difficult to eliminate the infection by local treatment.

2-Amino-5-nitrothiazole and a few of its acyl derivatives, in particular 2-acetamido-5-nitrothiazole (Cuckler, Kupferberg and Millman, 1955; Prince, 1960) are active in animals infected with *T. vaginalis* and *T. foetus*. The antibiotics Trichomycin and Fervenulin are also active (Hosaya, Soeda, Komatsu, Okada and Watanabe, 1953; Deboer, Dietz, Evans and Michaels, 1959–1960). Early clinical evaluation of these compounds was promising (Perl and others, 1956; Plentyl, Grey, Nelson and Da Lali, 1956; Magara, Nittono and Senda, 1955), but later they were shown to be without effect (Gardner and Dukes, 1956; Barnes, Bontwood, Haines, Lewington, Lister and Haram, 1957; Catterall and Nicol, 1957). Because of its toxicity, Fervenulin has not been tested in man. The only compound which has been found effective in laboratory animals (Cosar and Julou, 1959) and man is the recently discovered 1-(2'hydroxyethyl)-2-methyl-5-nitroimidazole. Cure rates above 85 per cent in females have been reported when the drug was used locally (Durel, Coutre, Collart and Girot, 1960) or systemically (Durel and others, 1960; Nicol, Barrow and Redmond, 1960; Rodin, King, Nicol and Barrow, 1960; Wilcox, 1960) and in males treated systemically (Nicol and

others, 1960; Rodin and others, 1960; Sylvestre, Gallai and Ethier, 1959; Sylvestre, Belanger and Gallai, 1960).

The need remains for other effective systemic treatments for this disease.

Chemical

The biological action of isosteric compounds often differs only in degree, therefore, we investigated isosteric compounds of 2-amino-5-nitrothiazole and 2-acetamido-5-nitrothiazole. The replacement of the sulphur atom of these thiazoles by the CH=CH and CH=N groups gave the isosteric compounds 2-amino-5-nitropyridine, 2-amino-5-nitropyrimidine and their corresponding acetyl derivatives. The *in vitro* antitrichomonal activities of 2-amino-5-nitrothiazole and its acetyl derivative are similar but in mice, the acetyl compound is much more active (Tables I and II). Whether this greater activity is due to more favourable distribution of the acetyl derivative in the animal body or whether the compound is actually more active in the body is not yet known. As a working hypothesis it was postulated that there might be a relation between the rate of deacylation and activity. For this reason the synthesis of the more rapidly hydrolysable trifluoroacetyl derivatives of 2-amino-5-nitrothiazole, 2-amino-5-nitropyridine and 2-amino-5-nitropyrimidine was undertaken.

In the evaluation of a series of 5-nitropyridine derivatives, 2-hydroxy-5-nitropyridine was found to be active in mice. This prompted the synthesis and evaluation of the isosteric compounds 2-hydroxy-5-nitrothiazole and 2-hydroxy-5-nitropyrimidine (Roblin, Winnech and English, 1942; Hale and Brill, 1912).

Experimental

2-*Trifluoroacetamido*-5-*nitrothiazole.* To a stirred suspension of 2-amino-5-nitrothiazole (14·5 g.; 0·10 mole) in 100 ml. of ether was added over 5 min. 50 g. (0·24 mole) of trifluoroacetic anhydride. After 15 min. the solution was treated with charcoal, filtered and distilled. The residual, slightly yellow product was washed with light petroleum and dried. Yield, 18·5 g.; m.p. 150–151°. Found: C, 25·21; H, 1·31; N, 17·40. $C_5H_2F_3N_3O_3S$ requires C, 24·98; H, 0·84; N, 17·43 per cent.

2-*Hydroxy*-5-*nitrothiazole.* This compound was synthesised as described by Babo and Prijs (1950), the product melted at 143–144° decomp. (Babo and Prijs gave m.p. 136° with decomp. at 142°). Found: C, 24·87; H, 1·77; N, 19·25; S, 21·57. $C_3H_2N_2O_3$ requires C, 24·66; H, 1·39; N, 19·17; S, 21·94 per cent.

2-*Acetamido*-5-*nitropyridine.* Acetylchloride (7·9 g.; 0·10 mole) was added dropwise over 30 min. to a cooled solution (10–15°) of 2-amino-5-nitropyridine (13·9 g.; 0·10 mole) in a mixture of dry acetone (100 ml.) and dry pyridine (10 ml.). Stirring was continued for another 30 min. at room temperature and for 15 min. at reflux temperature. The reaction mixture was poured into water (200 ml.) and the solid formed was removed by filtration. After recrystallisation from ethanol a slightly tan coloured

product was obtained melting at 198–199°. Tschitschibabin and Posdnjakow (1926) gave m.p. of 196° using acetic anhydride.

2-Trifluoroacetamido-5-nitropyrimidine. 2-Amino-5-nitropyrimidine (Roblin and others, 1942; Hale and Brill, 1912) (14·0 g.; 0·10 mole) was heated under reflux with trifluoroacetic anhydride (100 ml.) for 3 hr. with stirring. After cooling to room temperature the product was removed by filtration and recrystallised from toluene. The almost colourless product (12·9 g.) melted at 131–133·5°. Found: C, 30·45; H, 1·28; N, 23·50. $C_6H_3F_3N_4O_3$ requires C, 30·52; H, 1·28; N, 23·50 per cent.

Biological

The culture of *Trichomonas vaginalis* (Trussell and Plass, 1940) and *Trichomonas foetus* (Morgan and Campbell, 1946) made possible the *in vitro* testing of compounds for direct trichomonacidal properties. Effective contact agents have been found against *T. vaginalis* (Johnson and Trussell, 1943; Lynch, Holley and Salmirs, 1955; MacDonald and Tatum, 1948; Seneca and Ides, 1953; Trussell, 1947) and *T. foetus* (Morgan and Campbell, 1946; MacDonald and Tatum, 1948) *in vitro* and against *T. foetus in ovo* (Pierce and Morgan, 1950).

Further evaluation of compounds requires an *in vivo* system. The Rhesus monkey (*Macacus mulatta*) is the only species other than man which harbours *T. vaginalis* naturally and is susceptible to experimental vaginal infection. But few chemotherapeutic studies have been made with this animal.

Recently, Schnitzer, Kelly and Leiwant (1950) reported that white mice can be infected intraperitoneally, subcutaneously or intramuscularly with *T. vaginalis*, *T. foetus* and *T. gallinae*. Mice infected subcutaneously with either *T. foetus* or *T. vaginalis* have been used to study the effect of local treatment with either chemicals or antibiotics (Lynch and Stephens, 1955–56; Lynch, English, Morrison and Maven, 1954; Lynch, Holley and Margison, 1955). The systemic activity of compounds has been investigated in mice infected intraperitoneally with either *T. foetus* (Cuckler and others, 1955) or *T. vaginalis* (Bushby and Copp, 1955; Hosaya and others, 1953).

To ascertain whether drugs given systemically reach the vaginal secretions in an active form, a vaginal infection is required; this has been obtained by Kradolpher (1954) in golden hamsters with *T. foetus*.

Experimental

Materials

Cultures of *T. vaginalis** and *T. foetus* strain MSC† and strain L‡ were maintained at 37° in fluid thioglycollate medium§ and 5 per cent

* Obtained through the courtesy of Dr. A. C. Cuckler of the Merck Institute for Therapeutic Research, Rahway, New Jersey, U.S.A.

† Obtained through the courtesy of Dr. W. D. Lindquist of Michigan State University, East Lansing, Michigan, U.S.A.

‡ Obtained through the courtesy of Dr. D. T. Clark of Michigan State University, East Lansing, Michigan, U.S.A.

§ Fluid thioglycollate medium obtained from Baltimore Biological Laboratories Baltimore, Maryland, U.S.A. Medium 01–140.

horse-serum. For the cultivation of *T. vaginalis*, the pH was adjusted to 6·0. No adjustment in pH was required for the growth of *T. foetus*. Unless otherwise indicated, experiments with *T. foetus* were with the MSC strain.

Determination of in vitro *End Points*

Aliquots of logarithmically growing (24 hr.) flask cultures of either species were diluted with fluid thioglycollate medium to contain 110,000 cells per ml. checked by direct counts. The "medium-inoculum" was prepared by combining 10 volumes of diluted cells with 5 volumes of horse-serum and 85 volumes of fluid thioglycollate medium. Solutions or finely divided suspensions containing 2 mg. of drug per ml. were prepared. Serial two-fold dilutions of these were made in 0·85 per cent saline. 0·5 ml. of each drug dilution was added to an experimental tube. Control tubes received an equal volume of saline. The medium-inoculum was added to each tube in 4·5 ml. amounts using a Cornwall semi-automatic syringe. The cells were agitated during this process by a magnetic stirrer. The highest concentration of any chemical tested was 200 μg./ml. against an initial concentration of 10,000 cells/ml.

After 22 hr. incubation at 37°, while the cells were still growing logarithmically, determinations of cell populations were made by direct counts. The 90 per cent inhibitory level was calculated arithmetically using the expression per cent inhibition equals $(C - E)C \times 100$, where C = number of cells per ml. in a control tube and E = number of cells per ml. in an experimental tube. When the end point could not be calculated in this manner, the results were plotted on semi-logarithmic paper, and the end point read graphically.

Plasma Level Determinations

Plasma levels of 2-acetamido-5-nitropyrimidine were determined microbiologically by titration, in the way just described, against a standard. Controls included plasma obtained before the administration of drug, together with an estimate of the 90 per cent inhibitory level of this compound.

Evaluation of Compounds in Animals Infected by the Subcutaneous Route

Groups of 5 adult male albino mice (CF 1 strain) or female golden hamsters, were infected subcutaneously with 200,000, 24 hr. cells, contained in 0·2 ml. Compounds for treatment of these infections were homogenised in 50 per cent Plazmoid*. Beginning with the Tolerated Dose (in this study the T.D. is defined as the maximum amount of drug in mg./kg./day which can be administered for six consecutive days and still allow weight gains comparable to those of the control animals), drugs were given in serial two-fold dilutions either orally or intraperitoneally. The treatment was first given immediately after infection, and continued once daily for five days. Controls were dosed with 50 per cent Plazmoid. Since the pathogenicity of the cultures varied, an

* Sterile solution of 5 per cent gelatin.

estimate of the 50 per cent infective-dose (ID50), the number of organisms successfully infecting 50 per cent of the animals, was made for each individual experiment.

Animals were killed and examined seven days after infection. The presence of trichomonads in the lesions at the site of injection was verified by microscopic examination of an eosin stained wet smear.

The 50 per cent curative dose (CD50) and the ID50 were calculated according to the method of Reed and Muench (1938), while the ED50 was obtained graphically by the method of Litchfield and Wilcoxon (1949).

The relative activity of the compounds was obtained by comparing their Therapeutic Quotients. For our purpose we define the Therapeutic Quotient to be the Tolerated Dose of a compound divided by the CD50.

Evaluation of Compounds in Hamsters Infected Intravaginally

Four to six months before study, virgin female hamsters were infected intravaginally on three successive days with 100,000 to 200,000 (48 hr.) *T. foetus* cells contained in 0·05 ml. Vaginal smears for microscopic examination were made daily for 6 days before beginning treatment and only animals showing positive smears were used.

Groups of 5 animals were treated orally once daily for six consecutive days with suspensions of the drug in 50 per cent Plazmoid. The drug suspensions were so prepared that the animals in any one group received the same amount of a drug on a mg./kg. basis.

During the treatment and for two additional weeks, vaginal smears were examined daily. After a rest period of 2 weeks, this procedure was repeated for an additional week. Animals which became negative during the course of treatment and remained so during the entire follow-up period were considered to be cured.

Results and Discussion

A series of 2-substituted-5-nitropyridines, pyrimidines and thiazoles have been examined for their *in vitro* activity against *T. vaginalis* and *T. foetus*. *In vitro* end points are listed in Table I both in μg./ml. and in μMoles/l., since it is only on the latter basis that a direct comparison of activities can be made. With two exceptions (2-acetamido-5-nitropyridine and 2-hydroxy-5-nitropyrimidine), all of these compounds were more active *in vitro* against *T. vaginalis* than *T. foetus*. The 2-acetamido-5-nitropyridine was inactive, while in the pyrimidine and thiazole series the 2-hydroxy compounds were nearly so. Of the remaining compounds the activities are close together, so that there is only a ten-fold difference between the least and most active. Within the limitation of the few substituent groupings at C (2) studied no radical was found to enhance activity. Except in the pyridines, the difference in activity is not more than threefold. The activity of 2-amino-5-nitropyridine was 5 times as great as 2-hydroxy-5-nitropyridine and 6 times that of the trifluoroacetamido compound (Table I).

The most active compounds were 2-amino-5-nitropyridine, 2-amino-5-nitrothiazole and 2-acetamido-5-nitrothiazole.

There was almost a direct correlation of *in vitro* and *in vivo* results (Table II). All compounds inactive *in vitro* were inactive *in vivo*. In the pyridine and pyrimidine series activity followed the same pattern

TABLE I

THE EFFECT OF 5-NITROPYRIDINES, PYRIMIDINES AND THIAZOLES AGAINST *T. vaginalis* AND *T. foetus* IN VITRO

Compound	*T. vaginalis* End point		*T. foetus* End point	
	µg./ml.	µM/l.	µg./ml.	µM/l.
Pyridines				
2-Amino-5-nitropyridine	0·8	3·6	0·9	4·0
2-Acetamido-5-nitropyridine	+200	—	+200	—
2-Trifluoroacetamido-5-nitropyridine	5·0	21·2	6·2	26·4
2-Hydroxy-5-nitropyridine	2·5	17·8	3·1	22·0
Pyrimidines				
2-Amino-5-nitropyrimidine	2·1	15·0	2·7	19·2
2-Acetamido-5-nitropyrimidine	6·2	34·0	3·1	17·0
2-Trifluoroacetamido-5-nitropyrimidine	3·1	13·1	5·2	22·0
2-Hydroxy-5-nitropyrimidine	+200	—	150·0	1060·0
Thiazoles				
2-Amino-5-nitrothiazole	0·62	4·3	0·8	5·5
2-Acetamido-5-nitrothiazole	0·62	3·3	0·8	4·3
2-Hydroxy-5-nitrothiazole	62·0	424·0	140·0	960·0

TABLE II

THE ACTIVITY OF 5-NITROPYRIDINES, PYRIMIDINES AND THIAZOLES IN MICE INFECTED SUBCUTANEOUSLY WITH *T. vaginalis*

Compound	CD50*		TD†		TQ‡	
	Oral	I.P.	Oral	I.P.	Oral	I.P.
Pyridines						
2-Amino-5-nitropyridine	78·8§	77	200	250	2·5	3·2
2-Acetamido-5-nitropyridine	Inactive					
2-Trifluoroacetamido-5-nitropyridine	188§	194§	600	400	3·2	2·1
2-Hydroxy-5-nitropyridine	144§	134§	1000	400	6·9	3·0
Pyrimidines						
2-Amino-5-nitropyrimidine	35·3§	28·6§	250	200	7·1	7·0
2-Acetamido-5-nitropyrimidine	77·0	71·0	500	200	6·5	2·8
2-Trifluoroacetamido-5-nitropyrimidine	58·8§	50·0	500	200	8·5	4·0
2-Hydroxy-5-nitropyrimidine	Inactive					
Thiazoles						
2-Amino-5-nitrothiazole	—	70·6	—	100	—	1·4
2-Acetamido-5-nitrothiazole	11·6§	11·1§	100	100	8·6	9·0
2-Trifluoracetamido-5-nitrothiazole	Inactive					
2-Hydroxy-5-nitrothiazole	Inactive					

* CD50 Median curative dose mg./kg.
† TD = Tolerated dose.
‡ TQ = Therapeutic Quotient.
§ Geometric mean of more than one trial.

in vitro and *in vivo*. A comparison of the *in vivo* activity on a molar basis shows that the same number of moles of 2-amino-5-nitropyrimidine and 2-trifluoroacetamido-5-nitropyrimidine are required for cure. This was to be expected since experimentally it was found that the half-life of 2-trifluoro-acetamido-5-nitropyrimidine in solution is 4 min. Trifluoroacetic acid is not active systemically, nor does it influence the

CD50 of 2-amino-5-nitropyrimidine when the two are administered simultaneously at the same molar ratio as is found in 2-trifluoroacetamido-5-nitropyrimidine. While 2-amino and 2-acetamido-5-nitrothiazole were equally effective *in vitro*, the 2-acetamido compound was more than six times as active *in vivo* intraperitoneally.

However, *in vivo* chemotherapeutic efficacy is a resultant of the effect of the drug on the parasite and the host. There was a host variation in toxicity of compounds in the pyridine and pyrimidine series. When the Therapeutic Quotient of the compounds is calculated for the oral route, it is evident that 2-hydroxy-5-nitropyridine is twice as effective as other compounds in the pyridine series and that all the compounds in the pyrimidine series are of about the same efficacy. These, together with

TABLE III

THE EFFECT OF 5-NITROPYRIDINES, PYRIMIDINES AND THIAZOLES AGAINST *T. foetus* AND *T. vaginalis* IN LOCAL SUBCUTANEOUS LESIONS IN MICE

Compound	I.P.		Oral	
	T. vaginalis	*T. foetus*	*T. vaginalis*	*T. foetus*
Pyridines				
2-Amino-5-nitropyridine	77·0*	89·2	—	—
2-Acetamido-5-nitropyridine	Inactive	—	—	—
2-Trifluoroacetamido-5-nitropyridine	194	—	—	—
2-Hydroxy-5-nitropyridine	134	—	144·0	260·0
Pyrimidines				
2-Amino-5-nitropyridimidine	28·6	—	35·3	77·5
2-Acetamido-5-nitropyrimidine	71·0	—	71·0	177·0
2-Trifluoracetamido-5-nitropyrimidine	50·0	100·0	58·8	142·0
2-Hydroxy-5-nitropyrimidine	Inactive			
Thiazoles				
2-Amino-5-nitrothiazole	70·6	56·6	—	—
2-Acetamido-5-nitrothiazole	11·1	14·1	—	—
2-Trifluoroacetamido-5-nitrothiazole	Inactive	—	—	—
2-Hydroxy-5-nitrothiazole	Inactive	—	—	—

* CD 50 mg./kg.

2-acetamido-5-nitrothiazole, had Therapeutic Quotients of 6·5 or more against *T. vaginalis*. Compounds in the pyrimidine series exhibited species specificity, since they were almost three times as active against *T. vaginalis* as against *T. foetus*. (Table III.)

2-Hydroxy-5-nitropyridine and 2-acetamido-5-nitrothiazole were given by mouth to treat hamsters infected intravaginally with *T. foetus*. When hamsters were treated with 500 mg./kg. of the pyridine derivative it was consistently found that 100 per cent of the animals were cured, but none were cured by half this dose (Table IV). Both compounds were less active against the vaginal infection than against the local infection since it took almost 1·5 times as much of the pyridine derivative orally and almost four times as much of the thiazole compound given intraperitoneally to achieve the same level of cure (CD50) in the hamsters as in mice (Tables III and IV).

Even at an infecting dose of 1×10^6 organisms, hamsters were refractory to subcutaneous infection with the MSC strain of *T. foetus*. Three additional strains of recent isolation were obtained*. One of these,

* Through the courtesy of Dr. D. T. Clark of Michigan State University, East Lansing, Michigan, U.S.A.

strain L, infected hamsters when injected subcutaneously. When the infectivity of strain MSC and L was compared subcutaneously in mice it was found that the ID50 for the former was 66,600 and for strain L, 2,140.

In a single experiment mice and hamsters were infected subcutaneously with *T. foetus* (L) and treated orally with a 2-hydroxy-5-nitropyridine,

TABLE IV

A VAGINAL INFECTION IN HAMSTERS TREATED WITH 2-HYDROXY-5-NITROPYRIDINE AND 2-ACETAMIDO-5-NITROTHIAZOLE BY MOUTH

Compound	Dose mg./kg./day	No. cured / No. treated	Per cent cured	CD50
2-Hydroxy-5-nitropyridine ..	500	5/5	100	350
	250	0/5	0	—
2-Acetamido-5-nitrothiazole ..	100	5/5	100	50
	50	2/5	40	—
	25	1/4	25	—
Plazmoid	—	0/5	0	—

2-amino-5-nitropyrimidine or 2-acetamido-5-nitrothiazole. The pyridine and pyrimidine were found to be significantly less active in hamsters than in mice (Table V). The apparent differences in response to the subcutaneous and vaginal infections were entirely due to the host.

The specificity of 2-amino-5-nitropyrimidine against *T. vaginalis* and its stability in acid and basic solutions led us to study this compound in

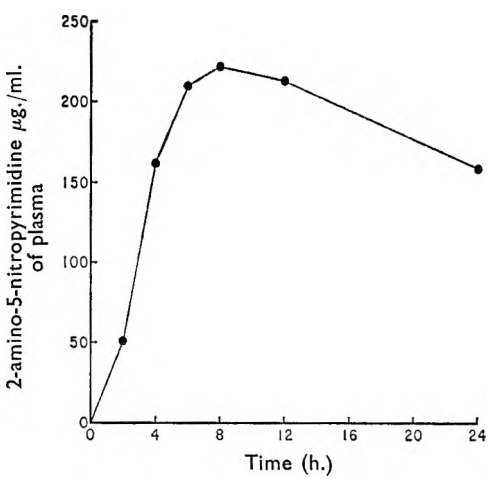


FIG. 1. The concentration of 2-amino-5-nitropyrimidine in μg./ml. of plasma after oral administration to a monkey.

more detail. Plasma concentrations were estimated microbiologically in one nonfasted monkey after oral administration of an aqueous suspension of the drug at 500 mg./kg. The data shown in Fig. 1 shows

TABLE V

THE EFFICACY OF THREE COMPOUNDS BY MOUTH IN MICE AND HAMSTERS INFECTED SUBCUTANEOUSLY WITH *T. foetus* (L)

Compound	ED50 and range mg./kg.		Significant (+) or not Significant (−)*
	Mice	Hamsters	
2-Amino-5-nitropyrimidine ..	108 (76–145)	196 (145–265)	+*
2-Hydroxy-5-nitropyridine ..	165 (126–214)	312 (268–350)	+
2-Amino-5-nitrothiazole	30 (20–45)	16 (7·6–33·5)	−

* Significance at the 5 per cent level of confidence.

that the drug, present in a microbiologically active form is slowly absorbed; probably from the intestine and is even more slowly excreted. The peak drug levels were obtained in 8 hr. The plasma level 24 hr. after drug administration is 50 times the amount of drug needed to kill the organism *in vitro*.

The stability of 2-amino-5-nitropyrimidine in acid, base and body fluids, its ability to produce good blood levels in monkeys, absorption in a biologically active form and ability to cure experimental infections in mice indicate that it should be an effective systemic treatment for the clinical disease evoked by *Trichomonas vaginalis*.

Qualitative differences in the carbohydrate metabolism of *T. vaginalis* and *T. foetus* exist but despite these, various investigators have used these species indiscriminately in the search for compounds which will inhibit *T. vaginalis*. Results from these experiments indicate that the two species probably utilise similar metabolic pathways *in vitro* but that *T. foetus* may change its metabolic pathway *in vivo*. If *T. foetus* only had been used to evaluate the compounds in the pyridine and pyrimidine series, their therapeutic potentialities would have been missed.

REFERENCES

Babo, H. V. and Prijs, B. (1950). *Helv. Chim. Acta*, **33**, 306–313.
Barnes, J., Boutwood, A., Haines, E., Lewington, W., Lister, E. and Haram, B. J. (1957). *Brit. med. J.*, **1**, 1160–1162.
Bushby, S. R. M. and Copp, F. C. (1955). *J. Pharm. Pharmacol.*, **7**, 112–117.
Catterall, R. D. and Nicol, C. S. (1957). *Brit. med. J.*, **2**, 29–31.
Cosar, C. and Julou, L. (1959). *Ann. Inst. Pasteur*, **96**, 238–241.
Cuckler, A. C., Kupferberg, A. B. and Millman, N. (1955). *Antibiotics & Chemotherapy.*, **5**, 540–550.
Deboer, C., Dietz, A., Evans, J. S. and Michaels, R. M. (1959–50). *Antibiotics Ann.*, p. 220–226, New York: Medical Encylopedia, Inc.
Durel, P., Coutre, J., Collart, P. and Girot, C. (1960). *Brit. J. vener. Dis.*, **36**, 152–153.
Gardner, H. L. and Dukes, C. D. (1956). *Obstet. Gynecol.*, **8**, 591–596.
Hale, W. J. and Brill, H. C. (1912). *J. Amer. chem. Soc.*, **64**, 567–570.
Hosaya, S., Soeda, M., Komatsu, N., Okada, K., Watanabe, S. (1953). *Antibiotics (Japan)*, **6**, 92–97.
Johnson, G. and Trussell, R. E. (1943). *Proc. Soc. exp. Biol. N.Y.*, **54**, 245–249.
Kean, B. H. (1955). *Amer. J. Obstet. Gynecol.*, **70**, 397–402.
Kean, B. H. and Wolinska, W. H. (1956). *Amer. J. clin. Pathol.*, **26**, 1142–1145.
Kradolpher, F. (1954). *Exp. Parasitol.*, **3**, 1–8.
Litchfield, J. T., Jr. and Wilcoxon, F. (1949). *J. Pharmacol.*, **96**, 99–113.
Lynch, J. E., English, A. R., Morrison, J. and Maven, I. (1954). *Antibiotics Chemother.*, **4**, 899–904.
Lynch, J. E., Holley, E. C. and Margison, J. E. (1955). *Ibid.*, **5**, 508–514.
Lynch, J. E., Holley, E. C. and Salmirs, A. M. (1955). *Ibid.*, **5**, 300–304.

Lynch, J. E. and Stephens, C. R. (1955–56). *Antibiotics Ann.*, p. 466–472, New York: Medical Encyclopedia, Inc.
MacDonald, E. M. and Tatum, A. L. (1948). *J. Immunol.*, **59**, 301–308.
Magara, M., Nittono, H. and Senda, T. (1955). *Antibiotic Med.*, **1**, 394–397.
Morgan, B. B. and Campbell, H. M. (1946). *Amer. J. vet. Research*, 7, 45–51.
Nicol, C. S., Barrow, J. and Redmond, A. (1960). *Brit. J. vener. Dis.*, **36**, 151–153.
Perl, G., Guttmacher, A. F. and Raggazoni, H. (1956). *Obstet. Gynecol.*, **7**, 128–136.
Pierce, A. E. and Morgan, J. (1950). *J. infect. Dis.*, **87**, 96–98.
Plentyl, A. A., Gray, M. J., Neslen, E. D. and DaLali, S. J. (1956). *Amer. J. Obstet. Gynecol.*, **71**, 116–120.
Prince, H. N. (1960). *Nature, Lond.*, **186**, 816–818.
Reed, L. J. and Muench, H. (1938). *Amer. J. Hyg.*, **27**, 493–497.
Roblin, R. O. Jr., Winnek, P. S. and English, J. P. (1942). *J. Amer. chem. Soc.*, **64**, 567–570.
Rodin, P., King, A. J., Nicol, C. S. and Barrow, J. (1960). *Brit. J. vener. Dis.*, **36**, 147–151.
Schnitzer, R. J., Kelly, D. R. and Leiwant, B. (1950). *J. Parasitol.*, **36**, 343–349.
Seneca, H. and Ides, D. (1953). *Amer. J. Trop. Med. Hyg.*, **2**, 1045–1049.
Sylvestre, L., Belanger, M. and Gallai, Z. (1960). *Can. med. Assoc. J.*, **83**, 1195–1199.
Sylvestre, L., Gallai, Z. and Ethier, J. (1959). *First Canadian Symposium on N.G.U., Montreal*, 230–238.
Tshitshibabin, A. E. and Posdnjakow, A. W. (1926). *Beilstein*, **22**, II, 337.
Trussell, R. E. (1947). *Trichomonas vaginalis and Trichomoniasis*, pp. 51–64, Springfield, Illinois: Charles C. Thomas.
Trussell, R. E. and Plass, M. D. (1940). *Amer. J. Obstet. Gynecol.*, **40**, 883–890.
Wilcox, R. R. (1960). *Brit. J. vener. Dis.*, **36**, 175–177.

ANTITRICHOMONAL AGENTS
TRIAZEN DERIVATIVES

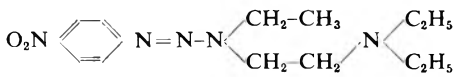
By R. E. Strube and R. M. Michaels

From the Research Division, The Upjohn Company, Kalamazoo, Michigan

Received April 21, 1961

Six new triazens have been synthesised. One of these compounds, 3-(2′-diethylaminoethyl)-3-ethyl-1-(*p*-nitrophenyl)-triazen was active in mice against *Trichomonas vaginalis* but inactive against *Trichomonas foetus*.

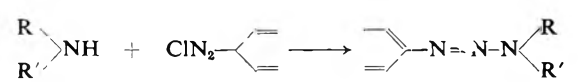
As outlined previously (Michaels and Strube, 1961), there is a need for a suitable systemically active agent against *Trichomonas vaginalis*. While screening many compounds the *in vitro* and *in vivo* activity of 3-(2′-diethylaminoethyl)-3-ethyl-1-(*p*-nitrophenyl)triazen against *T. vaginalis* was uncovered. Therefore, the syntheses and evaluation of a number of triazen derivatives related to I was undertaken.



I

Chemical

Triazens are derivatives of the unknown base triazen, $HN=N-NH_2$, a compound consisting of a chain of three nitrogen atoms, two being united by a double bond. A number of triazen derivatives are important intermediates for the dyestuff industry (Saunders, 1949). These triazens are prepared by treating aromatic diazonium salts with secondary amines carrying solubilising groups such as sulphonic acid, carboxyl or hydroxyl groups. Attempts to solubilise certain insoluble sulpha drugs by



transforming the aromatic amino group to a solubilising triazen group have been made with the supposition that the active sulpa drug would be liberated again *in vivo*.

The triazens reported in this communication were prepared by treating aromatic diazoniumchlorides with 1-diethylamino-2-monoethylamino-ethane $(C_2H_5)_2NCH_2CH_2NHC_2H_5$.

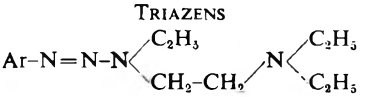
Experimental

General procedure for preparing triazens of type I. A filtered aqueous solution of the benzenediazonium salt, prepared in the usual way from the aromatic amine (0·1 mole), was added dropwise in about 30–45 min. from a cooled dropping funnel to a cooled (0–35°), stirred solution of 1-diethylamino-2-monoethylaminoethane (Damiens, 1951) (0·1 mole) in water (150 ml.) containing an excess of 30 per cent sodium carbonate

over that required for the neutralisation of the acid. After adding the diazonium salt solution, stirring was continued for 30 min. The triazen, which separated as a dark coloured oil, was extracted with ether and

TABLE I

TRIAZENS



Comp. No.	Triazens	Ref.	B.P.°/ mm.	Yield per cent	Analysis					
					Calculated			Found		
					C	H	N	C	H	N
1	3-(2-Diethylaminoethyl)-3-ethyl-1-(*p*-nitrophenyl)-triazen	Saunders (1949a)	195°/0·08	60	57·32	7·90	23·81	57·41	7·77	23·62
2	1-(*p*-Chlorophenyl)-3-(2-diethylaminoethyl)-3-ethyltriazen	Shirley (1951)	127–134°/ 0·02–0·1	68	59·45	8·14	12·54 Cl = 19·81	59·87	8·58	12·50 Cl = 19·11
3	3-(2-Diethylaminoethyl)-3-ethyl-1-(*p*-methoxyphenyl)-triazen	Shirley (1951a)	145°/0·07	46	64·71	9·42	20·13	64·41	9·26	19·56
4	3-(2-Diethylaminoethyl)-3-ethyl-1-(*p*-methylsulphonylphenyl)-triazen	Waldron and Reid (1923)	214–217°/ 0·08–0·09	61	55·18	8·03	17·16 S = 9·82	55·17	7·85	16·92 S = 10·03
5	3-(2-Diethylaminoethyl)-3-ethyl-1-(*p*-cyanophenyl)-triazen	Waldron and Reid (1923)	167–171°/ 0·03–0·04	67	66·88	8·51	25·62	66·28	8·38	25·26
6	3-(2-Diethylaminoethyl)-3-ethyl-1-(*o*-nitrophenyl)-triazen	Jacobs, Heidelberger and Rolf (1918) Shirley (1951b)	162–166°/ 0·04–0·06	54	57·32	7·90	23·81	57·14	8·07	23·2

dried over anhydrous magnesium sulphate. After removing the solvent the residual triazen was distilled under reduced pressure. For this distillation the distilling flask was immersed as far as possible in the oil bath. Table I summarises the results.

Biological

The methods for evaluation of compounds against *Trichomonas vaginalis* and *Trichomonas foetus* have been outlined by Michaels and

TABLE II

In vitro ACTIVITIES AGAINST *T. vaginalis* AND *T. foetus*

Compound No.	*T. vaginalis* Endpoint		*T. foetus* Endpoint	
	μg./ml.	μM/l.	μg./ml.	μM/l.
1	500	1700	200	682
2	500	1770	100	355
3	200	1720	200	1720
4	Inactive	Inactive		
5		Inactive *in vivo*		
6		Inactive *in vivo*		

Strube (1961). All the triazens mentioned were inactive *in vivo* against *T. foetus*. Besides 3-(2'-diethylaminoethyl)-3-ethyl-1-(*p*-nitrophenyl)-triazen, none of the other triazens were active in mice against *T. vaginalis*. The median curative doses (CD50) of this compound were 25 mg./kg./day and 20·6 mg./kg./day by the oral and intraperitoneal route of administration, respectively. The tolerated dose was 100 mg./kg. by either route. Table II summarises the results obtained *in vitro*.

REFERENCES

Damiens, R. (1951). *Ann. Chim.*, **6**, 835–879.
Jacobs, W. A., Heidelberger, M. and Rolf, I. P. (1918). *J. Amer. chem. Soc.*, **40**, 1582.
Michaels, R. M. and Strube, R. E. (1961). *J. Pharm. Pharmacol.*, **13**, 601–610.
Saunders, K. H. (1949). *The Aromatic Diazo-Compounds and Their Technical Application*, 2nd ed., pp. 170–175, London: Edward Arnold and Co.
Saunders, K. H. (1949a). *Ibid.*, p. 6.
Shirley, D. A. (1951). *Preparation of Organic Intermediates*, p. 81. New York: John Wiley and Sons, Inc.
Shirley, D. A. (1951a). *Ibid.*, p. 194.
Shirley, D. A. (1951b). *Ibid.*, p. 216.
Waldron, W. R. and Reid, E. E. (1923). *J. Amer. chem. Soc.*, **45**, 2399–2417.

THE BREAKDOWN OF ATROPINE BY BACTERIA*

By W. Kedzia, J. Lewon and T. Wiśniewski

From the Department of Medical Microbiology of the Medical Academy, Gdansk, and the Hospital Pharmacy Division of the State Clinic Hospital No. 1, Gdansk, Poland

Received April 26, 1961

744 strains of micro-organisms have been examined for their ability to break down atropine. Many strains of the genera *Corynebacterium* and *Pseudomonas* may occur as contaminants of atropine and eye preparations containing atropine.

Sterility tests on atropine eye drops (Polish Pharmacopoeia III) often demonstrated the presence of Gram-negative motile rods which could survive and even multiply in the eye drops. There are many reports of soil bacteria which are able to break down tropine alkaloids (Lypacewicz, 1930; Küster, 1952; Mozejko, 1955). We have now examined the organisms isolated from contaminated atropine preparations and from other related environments for their ability to break down atropine.

Experimental

Materials and methods. Altogether 744 strains of micro-organisms belonging to 15 genera were examined. The strains were isolated from atropine eye drops, from solid atropine, from the air of the Pharmacy Division rooms at the Clinic Hospital No. 1 in Gdansk, and the Department of Microbiology, from clinical material, from soil and from a departmental stock culture collection. The micro-organisms were isolated from air by the plate sedimentation method, from soil by Lypacewicz's 1930 method, and from drugs and preparations by Kedzia and Barteczko's method (1959). Media used were Lypacewicz's (1930) and a modification of this, and also 1 per cent atropine eye drops.

The number of viable cells was determined by Chabbert's method 1955) and a surface plate method, the quantity of alkaloids in the media and the drugs by *p*-dimethylamidobenzaldehyde (Wasicky, 1915), or a colorimetric method (Vitali, 1881) and a modification of this method (Allport and Wilson, 1939; Allport and Jones, 1942).

Results

Table I shows the occurrence of atropine breakdown by micro-organisms of 15 genera; 54 strains out of the 744 were able to break down the alkaloid; 38 of these belonged to the genus *Pseudomonas*, 10 to the genus *Corynebacterium*, 3 strains to *Bacillus*, one to *Aspergillacae* and one to *Saccharomyces*.

The ability to break down atropine was not a stable property. The storage of strains in meat broth agar led to a loss of this ability in less than 45 days. Frequent subculturing on meat broth agar shortened

* Research work subsidised by the Committee for Pharmaceutical Sciences at the Polish Academy of Sciences.

this time. Strains which had lost their ability to break down atropine could again acquire this character after making 7–15 subcultures on meat broth agar containing atropine. Fig. 1 shows the time course of atropine breakdown in a modified Lypacewicz's medium caused by two

TABLE I

ABILITY OF THE ISOLATED STRAINS TO BREAKDOWN ATROPINE

	Total	Number of positive strains
1. *Micrococcus*	88	1
2. *Sarcina*	291	—
3. *Gaffkya*	11	—
4. *Neisseria*	18	—
5. *Staphylococcus*	4	—
6. *Escherichia*	48	—
7. *Serratia*	19	—
8. *Proteus*	17	—
9. *Achromobacter*	12	—
10. *Pseudomonas*	41	38
11. *Corynebacterium*	11	10
12. *Erwinia*	2	—
13. *Bacillus*	66	3
14. *Aspergillaceae*	70	1
15. *Saccharomyces*	1	1
16. *Non-identified*	45	—
	744	54

of the most active strains belonging to the genera *Corynebacterium* and *Pseudomonas*, and by filtrates of 48-hr. cultures of these strains. Active strains of *Corynebacterium* and *Pseudomonas* immediately after isolation destroyed almost 100 per cent of the alkaloids present in the medium in 36–48 hr. of incubation at 30°. Active strains belonging to other genera destroyed atropine at a slower rate and to a lesser extent.

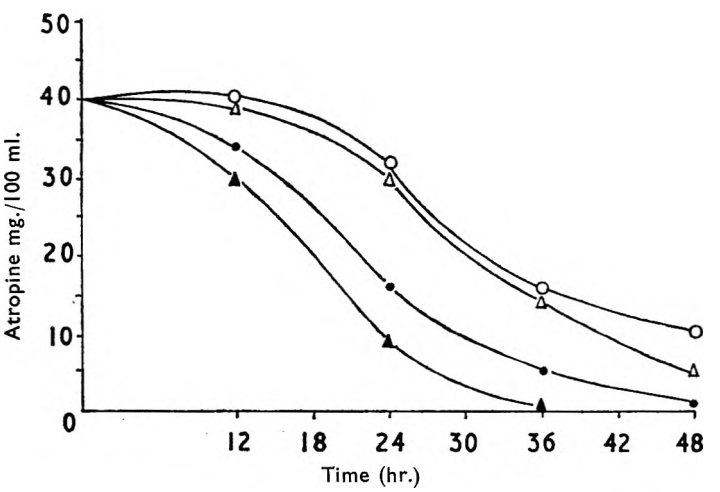


FIG. 1. The time: concentration course of atropine breakdown caused by cultures and filtrates of the *Corynebacterium* (No. 31) and *Pseudomonas* (No. 32) strains on the modified Lypacewicz's medium.

—●—●— No. 32, culture. —○—○— No. 31, culture. —▲—▲— No. 32, filtrate of 48-hr. culture. —△—△— No. 31, filtrate of 48-hr. culture.

Sterile filtrates of 48-hr. cultures of *Pseudomonas* and *Corynebacterium* strains caused atropine breakdown indicating the presence of alkaloid inactivating enzymes in the filtrates. Atropine-destroying strains were also able to break down hyoscine (scopolamine). The maximum atropine concentration was 0·5 per cent of strains belonging to the genus *Corynebacterium*, and about 3·4 per cent for strains belonging to genus *Pseudomonas*.

Table II shows the ability to cause atropine breakdown by strains isolated from the air of the hospital dispensary and from atropine eye drops contaminated while in use, and from solid atropine. Of the 555

TABLE II

ABILITY TO BREAKDOWN ATROPINE OF STRAINS ISOLATED FROM AIR OF THE HOSPITAL PHARMACY, FROM ATROPINE EYE DROPS CONTAMINATED IN THE COURSE OF THEIR USE AND FROM SOLID ATROPINE

Strains isolated from	Number of isolated strains	Number of isolated strains breaking-down atropine	
		Coryne-bacterium	*Pseudo-monas*
Air	303	1	–
Eye drops	202	–	8
Solid atropine	50	–	2
Total	555	1	10

strains 11 caused atropine breakdown. Eight of these were isolated from atropine eye drops, 2 from solid atropine and 1 from the dispensary air. All the atropine-destroying strains isolated from eye drops and solid atropine belonged to the genus *Pseudomonas*. The strain isolated from air belonged to *Corynebacterium*. A loss of about 20 per cent was found in 2 of the 8 samples of atropine eye preparation from which alkaloid-destroying strains had been isolated.

DISCUSSION

The ability of bacteria to break down atropine can be frequently observed in micro-organisms of the genera *Corynebacterium* and *Pseudomonas*. Micro-organisms breaking down atropine are common contaminants of eye preparations, and the air of the rooms where drugs are prepared may be the source of contamination. Species of *Corynebacterium* are of little importance as atropine inactivators, since the amount of atropine present is bacteriostatic for these organisms, but *Pseudomonas* species are able to degrade atropine in the concentration commonly used.

REFERENCES

Allport, N. L. and Wilson, E. S. (1939). *Quart. J. Pharm. Pharmacol.*, **12**, 399–408.
Allport, N. L. and Jones, N. R. (1942). *Ibid.*, **15**, 238–250.
Chabbert, Y. (1955). *Ann. Biol. Clin.* **13**, 37–40.
Kedzia, W. and Barteczko, I. (1959). *Farmacja Polska*, **15**, 85–89.
Küster, E. (1952). *Zbl. Bakter. Parasit.*, **158**, 350–357.
Lypacewicz, J. (1930). *Acta Soc. Bot. Polon.*, **7**, 553–581.
Mozejko, M. (1955). Ref. XIII. Zjazdu Midrob. Pol. Poznan.
Vitali, D. (1881). *Zbl. Anal. Chem.*, **20**, 563–574.
Wasicky, R. (1915). *Ibid.*, **54**, 393–397.

THE MECHANISM OF THE LOCAL INFLAMMATORY REACTION INDUCED BY COMPOUND 48/80 AND DEXTRAN IN RATS

BY A. JORI, A. P. BENTIVOGLIO AND S. GARATTINI

From Department of Pharmacology, Medical School, University of Milan, Italy

Received May 23, 1961

The butanolamide of 1-methyl-lysergic acid (UML) and cyproheptadine are strong inhibitors of the local inflammatory reaction induced by 5-hydroxytryptamine (5-HT) in rats, but only cyproheptadine is active against histamine. As cyproheptadine is more active than UML in reducing the local oedema reaction induced by dextran and compound 48/80 in intact and adrenalectomised rats, it is suggested that both histamine and 5-HT are involved in the reaction.

THE local inflammatory reaction induced by compound 48/80 or dextran in rats is thought to be mediated by a release of 5-hydroxytryptamine (5-HT) and histamine (Engelhardt and Schwabe, 1960; Halpern, Liacopoulis and Liacoupoulos-Briot, 1959; Parratt and West, 1957; von Mörsdorf and Fehres, 1959; West, 1961). Exogenous 5-HT is many times more active than histamine in producing this reaction (Sparrow and Wilhelm, 1957; Spector and Willoughby, 1957; Ungar, Kobrin and Sezesny, 1959) and the problem needed further investigation.

Two recently-introduced drugs have been used: (i) UML, the butanolamide of 1-methyl-lysergic acid, a specific powerful anti-5-HT agent with few central actions (Berde, Doepfner and Cerletti, 1960; Doepfner and Cerletti, 1958; Hillebrecht, 1959) and (ii) cyproheptadine (1-methyl-4, 5-dibenzo[a,e]-cycloheptatrienylidene piperidine), a powerful anti-5-HT and antihistamine agent (Stone, Wenger, Ludden, Stavorski and Ross, 1961). Gelfand and West (1961) have recently shown that UML reduces the oedema reaction produced in rats by dextran and by compound 48/80 but is ineffective against histamine. The effect of these drugs on the local inflammatory reactions induced by 5-HT, histamine, dextran and compound 48/80 have therefore been studied in both intact and adrenalectomised rats.

EXPERIMENTAL

Methods

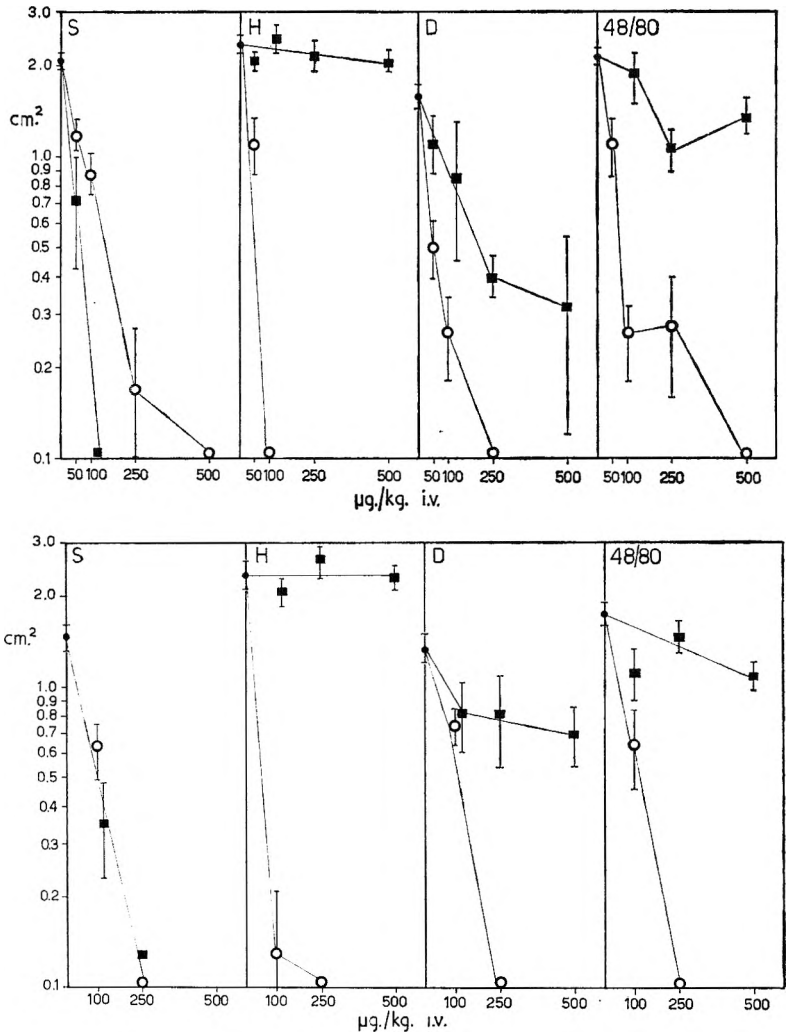
The abdominal skin of groups of female Sprague-Dawley rats (weighing about 150 g.) was depilated 24 hr. before the test. UML bimaleate (kindly supplied by Sandoz, Ltd., Milan) and cyproheptadine hydrochloride (kindly supplied by Merck, Sharp and Dohme, West Point, Pa.) were injected intravenously 30 min. before 2 mg. Evans Blue dye (0·4 per cent in water) and then four intradermal injections were made on each rat with 0·1 ml. each of 5-HT (2·5 μg./ml.), histamine (1 mg./ml.), dextran (1·2 mg./ml.) and compound 48/80 (10 μg./ml.). These doses produced similar degrees of blueing. The responses were evaluated by multiplying together the two maximum diameters of blueing 30 min. after the intradermal injections.

Control experiments were made to test the reaction arising from saline and that from the different sensitivities of the various areas of the abdominal skin.

Similar experiments were also made in rats which had been adrenalectomised 72 hr. previously and maintained on food and salt water. The doses of the oedema-producing agents were half those used in intact animals.

Results and Discussion

The results using intact rats are shown in Fig. 1. Small doses of UML prevent the oedema and blueing induced by 5-HT but have no anti-



FIGS. 1 and 2. Ordinates—product of the 2 maximum diameters of blueing (log scale). Abscissae—intravenous doses of cyproheptadine (○) or UML (■) given 30 min. before Evan's Blue dye.
The vertical bars represent the standard error of the mean.
Upper figure shows results obtained in unoperated rats;
lower figure in adrenalectomised rats.
S = Serotonin (5-HT) H = Histamine D = Dextran.

histaminic effect and only slightly reduce the effects after dextran or compound 48/80. Cyproheptadine is very active on both histamine and on 5-HT, although less active on 5-HT than is UML. Dextran and 48/80 induced oedema are more markedly decreased by cyproheptadine than by UML.

In Table I are shown the percentages of rats responding to the treatment. The results using adrenalectomised rats are plotted in Fig. 2 and are similar to those with intact animals, except that UML is slightly less active

TABLE I

THE EFFECT OF CYPROHEPTADINE AND UML ON THE BLUEING RESPONSE IN RATS INDUCED BY 5-HT, HISTAMINE, DEXTRAN AND COMPOUND 48/80

Treatment (μg./kg. i.v.)		Experimental condition	No. rats showing blueing/No. rats treated (and per cent) (controls = 100)			
			5-HT	Histamine	Dextran	48/80
Cyproheptadine	50	Intact rats	7/7 (100)	7/7 (100)	6/7 (85)	7/7 (100)
	100	"	9/10 (90)	3/10 (30)	5/10 (50)	8/10 (80)
	250	"	5/9 (55)	1/9 (11)	0/9 (0)	6/9 (66)
	500	"	0/5 (0)	0/5 (0)	0/5 (0)	0/5 (0)
UML	50	"	3/4 (75)	4/4 (100)	4/4 (100)	4/4 (100)
	125	"	0/4 (0)	4/4 1(00)	3/4 (75)	4/4 (100)
	250	"	0/7 (0)	7/7 1(00)	4/7 (57)	7/7 1(00)
	500	"	0/7 (0)	7/7 1(00)	2/7 (28)	7/7 1(00)
	1000	"	0/4 (0)	4/4 (100)	0/4 (0)	4/4 (100)
Cyproheptadine	100	adrenalectomised	6/7 (85)	4/7 (57)	7/7 (100)	7/7 (100)
	250	"	3/4 (75)	0/4 (0)	3/4 (75)	2/4 (50)
	500	"	1/4 (25)	0/4 (0)	1/4 (25)	1/4 (25)
UML	125	"	3/4 (75)	4/4 (100)	4/4 (100)	4/4 (100)
	250	"	2/6 (33)	6/6 (100)	4/6 (66)	6/6 (100)
	500	"	2/9 (22)	9/9 (100)	9/9 (100)	9/9 (100)

against dextran and 5-HT. This suggests that the two drugs are chiefly acting directly on the oedema producing agents without interfering with adrenal function to any great extent. Since drugs with specific anti-histaminic effects only slightly reduce the dextran and 48/80 induced local oedema in rats (Halpern, Liacopoulos and Liacopoulos-Briot, 1959; Parratt and West, 1957) it is concluded that these are best explained on the basis of a participation of, at least, both mediators 5-HT and histamine.

REFERENCES

Berde, B., Doepfner, W. and Cerletti, A. (1960). *Helv. physiol. Acta*, **18**, 537–544.
Doepfner, W. and Cerletti, A. (1958). *Int. Arch. Allergy*, **12**, 89–97.
Engelhardt, G. and Schwabe, U. (1960). *Klin. Wschr.*, **38**, 145–152.
Gelfand, M. and West, G. B. (1961). *Int. Arch. Allergy*, **18**, 286–291.
Halpern, B. N., Liacoupoulis, P. and Liacopoulos-Briot, M. (1959). *Arch. Int. Pharmacodyn.*, **119**, 56–101.
Hillebrecht, J. (1959). *Arzneimitt.-Forsch.*, **9**, 625–628.
Mörsdorf, K. von and Fehres, L. (1959). *Med. Exp.*, **1**, 58–63.
Parratt, J. R. and West, G. B. (1957). *J. Physiol.*, **139**, 27–41.
Sparrow, E. M. and Wilhelm, D. L. (1957). *J. Physiol.*, **137**, 51–65.
Spector, W. G. and Willoughby, D. A. (1957). *J. Path. Bact.*, **74**, 57–65.
Stone, C. A., Wenger, H. C., Ludden, C. T., Stavorski, J. M. and Ross, C. A. (1961). *J. Pharmacol.*, **131**, 73–84.
Ungar, G., Kobrin, S. and Sezesny, B. R. (1959). *Arch. int. Pharmacodyn.*, **123**, 71–77.
West, G. B. (1961). *Int. Arch. Allergy*, **18**, 56–61.

THE STABILITY OF ANTIBACTERIALS IN POLYETHYLENE GLYCOL MIXTURES

By L. V. Coates, Melanie M. Pashley and K. Tattersall

From Smith & Nephew Research Ltd., Hunsdon Laboratories, Ware, Herts.

Received June 14, 1961

The antibacterial activity of penicillin and bacitracin is rapidly destroyed in polyethylene glycol bases. Aminoacridine, neomycin, oxytetracycline and streptomycin are affected less. Attempts to improve the stability of incorporated penicillin by modifications to the base, such as reformulation, adjustment of pH to neutrality and heat treatment, were unsuccessful. Commercially available polyethylene glycols, particularly the lower molecular weight polymers, showed oxidising activity thought to be due to peroxides. Catalase and horse blood destroyed the oxidising activity of polyethylene glycol bases and prevented the destruction of added penicillin during a test period of 10 days. The addition of reducing agents, although apparently removing the oxidising activity when tested chemically, did not improve the stability of penicillin mixed with the treated bases. Since catalase did not completely protect aminoacridine, neomycin and oxytetracycline from inactivation by polyethylene glycol bases, it was concluded that other inactivating factors were operative.

Consideration of the physical properties of polyethylene glycols, especially their water miscibility and excellent solvent qualities for many organic medicaments, suggests their usefulness as ointment bases for topical therapy. There is, however, conflicting evidence regarding their compatibility with antibiotics, in particular, penicillin.

Aburaya and Shirahiga (1952) reported that a polyethylene glycol base was the best of four tested and the penicillin level was still effective after storage for 35 days at room temperature. Meleney (1946) reported losses of 50 per cent with penicillin in these bases after storage for 6–12 weeks at 5°. On the other hand, Ferlauto and Clymer (1947) and Sherwood and Mattocks (1951) record rapid inactivation in polyethylene glycol mixtures. Simone and Popino (1955), in their investigations of neomycin, observed that penicillin was unstable in polyethylene glycol mixtures. Bacitracin has been noted by a number of authors to be unstable in polyethylene glycol 400 or mixtures (Bond, Himelick, and MacDonald, 1947; Plaxco and Husa, 1956; Simone and Popino, 1955).

As most of this work was carried out on polyethylene glycols manufactured in the United States, an investigation of the compatibility of presently available material from British sources was thought necessary. The following report describes the compatibility of these ointment bases with a range of antibacterials and a more detailed study of the mechanism responsible for the destruction of added penicillin.

Materials and Methods

The method of assay was a modified agar plate diffusion technique. The following medium with a very low content of sulphonamide antagonists was chosen. 10 g. Difco Certified Casamino acids; 10 g. Oxoid

Lab Lemco; 3 g. sodium chloride; 12 g. of appropriate agar and water to 1,000 ml. The final pH was 7·2–7·4 and the medium was sterilised by autoclaving at 10 lb./sq. in. for 20 min.

The choice of agar was dictated by the following considerations. Oxoid New Zealand Agar had been found to give the greatest diffusion with quaternary ammonium compounds and this agar was used for all assays except the large molecular weight antibiotics, bacitracin, neomycin and polymyxin B, where, following a recommendation by Bechtle and Scherr (1958), Ionagar No. 2 (Oxoid) was used.

The molten agar was poured into plates to a depth of at least 3 mm. and when solidified the surface was flooded with a suspension of the test organism standardized to approximately 100×10^6 organisms per ml.

TABLE I

CONCENTRATION OF ANTIBACTERIAL SUBSTANCES IN OINTMENT BASES AND ASSAY ORGANISMS

Antibacterial substance	Concentration in base	Assay organism
Aminoacridine	10 mg./g.	*Staph. pyogenes* Oxford H strain
Bacitracin	200 i.u./g.	" " " "
Benzalkonium chloride	10 mg./g.	" " " "
Chloramphenicol	2·5 mg./g.	" " " "
Chlorhexidine diacetate	2·5 mg./g.	" " " "
Chlortetracycline	5 mg./g.	" " " "
Neomycin sulphate	100 i.u./g.	" " " "
Oxytetracycline	1000 µg./g.	" " " "
Penicillin G	100 i.u./g.	" " " "
Penicillin V	100 i.u./g.	" " " "
Phenoxetol	200 mg./g.	*Escherichia coli*
Polymyxin B	100 µg./g.	" "
Streptomycin (sulphate)	1000 µg /g.	*Staph. pyogenes* Oxford H strain
Sulphathiazole	20 mg./g.	*Escherichia coli*

After drying the plates, holes of 6·25 mm. diameter were cut through the medium and the bottom of the wells sealed with a drop of molten medium. The polyethylene glycols used in the experimental formulations were obtained from Union Carbide Co. Ltd. and from Shell Chemical Co. Ltd. A 20 g. sample of each experimental base was used and the antibacterial was added either directly to the base at 45–48° or from a concentrated aqueous solution to the base at 45–48°. The final water concentration never exceeded 1–2 per cent. Two drops of the molten base, incorporating the antibacterial, were added to each test well, and standards were similarly added. Four test samples and four samples of each of five levels used to produce a standard curve, were assayed on every occasion. Test and standard samples were randomly distributed and the plates incubated at 37° for 18 hr. The resulting zones of inhibition were measured with callipers. To obviate temperature effects the standard solutions were held at 45–48° for the same period as the experimental bases.

Antibacterial mixtures were assayed immediately after mixing and after storing for 24 hr. at 28°, a temperature which it was thought could be encountered under some storage conditions and during topical therapy. Standard solutions were stored in the same conditions and results were calculated as percentage potency remaining and are comparative with

aqueous solutions maintained in the same environment. Concentrations of antibacterials and assay organisms used are in Table I.

Results

Results of initial testing using base A (83 per cent polyethylene glycol 400; 17 per cent polyethylene glycol 4000) and the repeat tests where there was inactivation of an antibacterial substance, are found in Table II.

TABLE II

Potency remaining in ointment base A

Antibacterial substance	Potency remaining per cent	
	Immediate	24 hr. at 28°
Aminoacridine	12 (51)	14 (20)
Bacitracin	19	0
Benzalkonium chloride	100	100
Chloramphenicol	100	100
Chlorhexidine diacetate	100	100
Chlortetracycline	100	78
Neomycin	12 (38)	0 (37*)
Oxytetracycline	100 (42)	10 (45)
Penicillin G	0 (0)	0 (0)
Penicillin V	70	0
Phenoxetol	100	100
Polymyxin B	100	100
Streptomycin sulphate	47 (80)	47 (27)
Sulphathiazole	100	100

* 24 per cent after 3 days at 28°. ( ) repeated test.

With the confirmation that polyethylene glycols had a rapid inactivating effect on penicillin, attempts were made to improve the stability of penicillin by modifications to the ointment formulation. Tests on the individual constituents had shown that the higher molecular weight polyethylene glycols and glycerol were not so incompatible with penicillin.

TABLE III

Effect of ointment base formulation on penicillin stability

Ointment base	Ingredients per cent							Penicillin potency per cent	
	Polyethylene glycol			Carbitol	Hexylene glycol	Glycerol	Water	Immediate	24 hr. at 28°
	400	1500	4000						
A	83	—	17	—	—	—	—	0	0
B	—	20	20	60	—	—	—	0	0
C	—	—	25	—	75	—	—	100	0
D	—	—	45	—	—	35	20	100	0

The results are recorded in Table III, and show that penicillin stability is increased in formulations in which polyethylene glycols 400 and 1500 are omitted.

In view of the limited increase in stability achieved with this approach, analytical investigations of polyethylene glycol mixtures were undertaken, which showed that heavy metals were absent. It was found, however, that the mixtures had oxidising activity when tested with an acid potassium iodide solution. This was thought to be due to peroxides as detailed tests for other common oxidising agents proved negative. Chemical

methods of improving penicillin stability, such as adjustment of the pH to 7·3 with alkali, and incorporation of antoxidants were tried, but the lack of success achieved can be seen in Table IV.

TABLE IV

EFFECT OF CHEMICAL AND PHYSICAL TREATMENTS OF OINTMENT BASE ON PENICILLIN STABILITY

Treatment of ointment base A	Penicillin potency per cent	
	Immediate mixing	24 hr. at 28°
Control base	0	0
Base autoclaved at 20 lb./sq. in. for 30 min.	77	0
Base adjusted to pH 7·3	67	0
Base adjusted to pH 7·3 and autoclaved	77	0
Base with Na metabisulphite 0·02 per cent	100	0
Base with ascorbic acid 0·02 per cent	100	0
Base with ferrous sulphate 0·02 per cent	100	0
Base at pH 7·3 ferrous sulphate 0·02 per cent	72	0
Base with Na dithicnite 0·02 per cent	48	0
Base with Na dithicnite 0·1 per cent	11	0
Base with Na dithicnite 0·2 per cent	23	0
Base with Na thioglycollate 0·01 per cent	28	0
Base with Na thioglycollate 0·22 per cent	80	0
Base with isopropanol 5–10 per cent	0	0

TABLE V

THE EFFECT OF CATALASE AND OTHER BIOLOGICAL MATERIALS ON PENICILLIN STABILITY IN OINTMENT BASE A

Additions to ointment base A (100 g.)	Penicillin per cent		
	Immediate	24 hr. at 28°	3 days at 28°
Base alone	38 (0)	0 (0)	— (—)
Base with 5 mg. per cent catalase	100	0	—
Base with 10 mg. per cent catalase	100	59	—
Base with 50 mg. per cent catalase	100 (100)	100 (64)	100 (52)
Base with 100 mg. per cent catalase	100	100	100*
Base with 10 per cent horse blood	100 (100)	100 (75)	100 (—)*
Base with 10 per cent horse serum	100 (70)	0 (41)	— (—)

* No loss after 10 days at 28°. ( ) repeated test.

TABLE VI

THE EFFECT OF CATALASE ON THE STABILITY OF OTHER ANTIBACTERIALS IN OINTMENT BASE A

Antibacterial substance	Potency per cent			
	Immediate mixing		24 hr. at 28°	
	Without catalase	With 50 mg. catalase/100 g.	Without catalase	With 50 mg. catalase/100 g.
Aminoacridine	51	68	20	59
Neomycin	38	74	37 (24)*	78 (54)*
Oxytetracycline	22	42	33	45

( )* At 3 days.

Bases were then prepared in which the oxidising activity, if peroxide, would be removed by the addition of beef liver catalase (Oxoid). The tests showed this addition to protect the penicillin (Table V). The protective action of catalase was then investigated with other antibacterial substances affected by polyethylene glycol mixtures. The results are given in Table VI.

Discussion

Penicillin is rapidly inactivated by ointment bases containing polyethylene glycols from British sources and other antibacterial substances are sensitive but less so. Taking penicillin as the most sensitive to inactivation, we have investigated some of the possible causes. That acid pH was not solely responsible was shown by the fact that adjustment of the mixtures to pH 7·3 had only a slight stabilising effect and that Penicillin V, which is acid stable, was also destroyed within 24 hr. of addition to an unneutralised polyethylene glycol mixture. The failure in our own analytical studies to detect the presence of heavy metals and the work of Sherwood and Mattocks (1951) who showed that dimercaprol did not improve the stability of penicillin in polyethylene glycol mixtures, exclude heavy metal contamination as the cause of the inactivation. Chemical tests showed that the mixtures had oxidising activity thought to be due to peroxides. Since penicillin is known to be readily inactivated by oxidising agents, penicillin stability is likely to be improved by removal of such influences. The result of experiments in which catalase or horse blood were added to the ointment base, before the addition of penicillin, support the hypothesis that the major factor for penicillin destruction is peroxide. However, it is difficult to explain why the antoxidants, although themselves compatible with penicillin at the concentrations tested, failed to protect penicillin to the same extent as catalase.

Our results further show that antibacterials other than penicillin are inactivated by the mixtures. It is particularly interesting that chlortetracycline was not inactivated, whereas oxytetracycline is markedly affected.

The loss of activity by other antibacterials in the presence of polyethylene glycols does not appear to be due to the same causes that inactivate penicillin, since catalase has little protective action. One possible exception to this is bacitracin which according to Anker, Johnson, Goldberg and Meleney (1948) is sensitive to the presence of H_2O_2.

References

Aburaya, H. and Shirahige, H. (1952). *Japan J. Pharm. Chem.*, **21**, 45–51.
Anker, H. S., Johnson, B. A., Goldberg, J. and Meleney, F. L. (1948). *J. Bact.* **55**, 249–255.
Bechtle, R. M. and Scherr, G. H. (1958). *Antibiot. Chemother.*, **8**, 599–606.
Bond, G. C., Himelick, R. E. and MacDonald, L. H. (1949). *J. Amer. pharm. Ass. Sci. Ed.*, **38**, 30–34.
Ferlauto, R. J. and Clymer, H. A. (1947). *Science*, **105**, 130–131.
Meleney, F. L., Johnson, B. A., Pulaski, E. J. and Colonna, F. (1946). *J. Amer. med. Ass.*, **130**, 121–124.
Plaxco, J. M. and Husa, W. J. (1956). *J. Amer. pharm. Ass., Sci. Ed.*, **45**, 141–145.
Sherwood, R. R. and Mattocks, A. M. (1951). *Ibid.*, **40**, 90–92.
Simone, R. M. and Popino, R. P. (1955). *Ibid.*, **44**, 275–280.

A COMPARISON OF ACTIVITY OF QUININE AND QUINIDINE ON THE ISOLATED ELECTRICALLY STIMULATED RAT VENTRICLE STRIP

BY L. MOLINENGO AND G. SEGRE

From the Department of Pharmacology and Experimental Therapeutics, University of Turin Medical School, Turin, Italy

Received June 19, 1961

In isolated and electrically stimulated rat ventricle strips, the activity of quinine and quinidine sulphate on excitability, rheobase and maximal rate of stimulation has been measured. By testing the two drugs on the same preparation at the same concentrations, from 5×10^{-6} to 3×10^{-5}, no statistically significant difference was detected in the relative potency of the two drugs. These findings are discussed in relation to the observed clinical inactivity of quinine.

THE first observations on the antiarrhythmia activity of cinchona alkaloids in man are attributed to Wenckebach (1923). Frey (1918) showed later the higher activity of quinidine. The superiority of quinidine over quinine in auricular fibrillation was thereafter generally accepted, but the experimental evidence for this superiority is scanty. Alexander, Gold, Katz, Levy, Scott and White (1947) reported a clinical trial on patients with cardiac arrhythmias in which quinine was clearly inferior to quinidine. More recently Benthe (1956) reported quinidine to be a little more active than quinine on conduction velocity and on absolute refractory time in the ventricle strip of the frog.

In the present work we have studied the relative activity of quinine and quinidine on excitability, rheobase and on the maximal rate of stimulation (Dawes, 1946) using the rat right ventricle strip.

METHOD

Strips of the right ventricle of adult albino rats (Feigen, Masuoka, Thienes, Saunders and Sutherland, 1952) were placed on the electrode unit of Alles and Ellis (1948) in a bath at 32° with Krebs-Henseleit solution, and stimulated by square pulses from a Grass stimulator. The displacement of an optical level was projected on a screen and used as an index of adequate stimulus. Recordings were made before and after 30 min. contact with the drug.

A complete curve of excitability was obtained by plotting the voltages of stimulation against duration (from 100 to 0·01 msec.). The equation of Weiss (1901) and Hoorveg (1892)

$$V = a/t + R \quad .. \quad .. \quad .. \quad .. \quad .. \quad .. \quad (1)$$

where V = voltage; t = duration (in msec.); a = constant; R = rheobase, was transformed in a linear equation as follows, to permit an easier calculation.

$$2 + \log (V - R) = A + B (2 + \log 1/t) \quad .. \quad .. \quad (2)$$

The slope B of the straight line (2) is not affected by the presence of an antifibrillatory drug; it is therefore possible to express the action of the

drug by measuring the displacement of the straight line (Libonati and Segre, 1960). The straight line (2) was calculated from the experimental values by the method of least squares (Figs. 1 and 2).

The per cent effect of a drug on the excitability is given by

$$100\ \frac{(V - R)' - (V - R)}{(V - R)}$$

where $(V - R)$ = the difference between voltage and rheobase, the voltage being calculated from equation (2) at 1 msec.; and $(V - R)'$ = the same difference in presence of the drug.

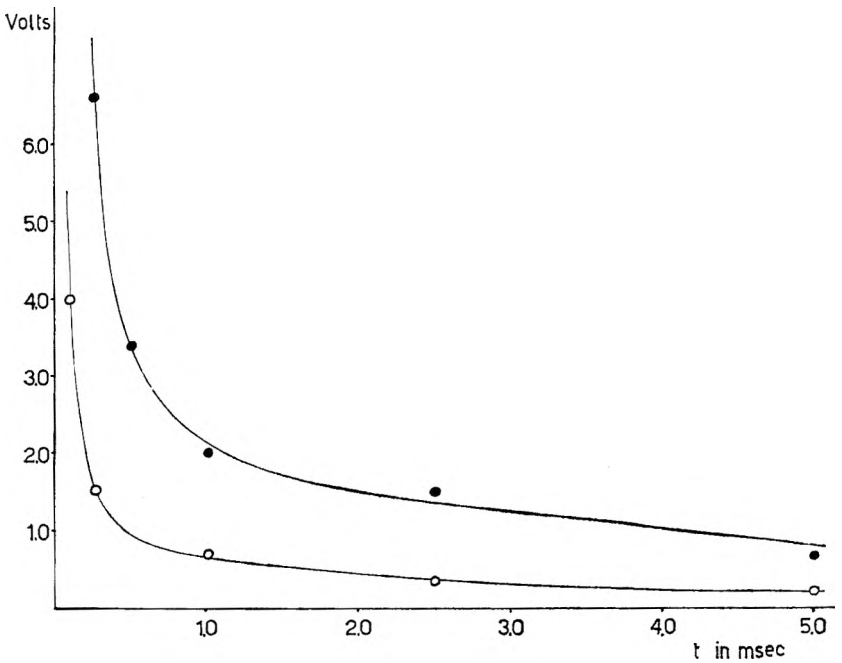


FIG. 1. Effect of quinidine sulphate (3×10^{-5}) ●—● on excitability curve ○—○.

The value of the rheobase was obtained by the same excitability curve and corresponds to the voltage applied for 100 msec.

The per cent effect of a drug on the rheobase was

$$100\ \frac{R' - R}{R}$$

where R = rheobase and R′ = rheobase in presence of the drug.

The maximal rate of stimulation (MRS) was determined at 5 V and 1 msec.; MRS can be accurately estimated because of an abrupt change in the rhythm of the contractions by overcoming the maximal rate.

The per cent effect of a drug on MRS was calculated as

$$100\ \frac{MRS - MRS'}{MRS}$$

where MRS′ = MRS in presence of the drug.

COMPARISON OF ACTIVITY OF QUININE AND QUINIDINE

The drugs used were commercial samples of quinidine sulphate and quinine sulphate. No difference in relative potency was detected whether quinine or quinidine was first introduced into the bath.

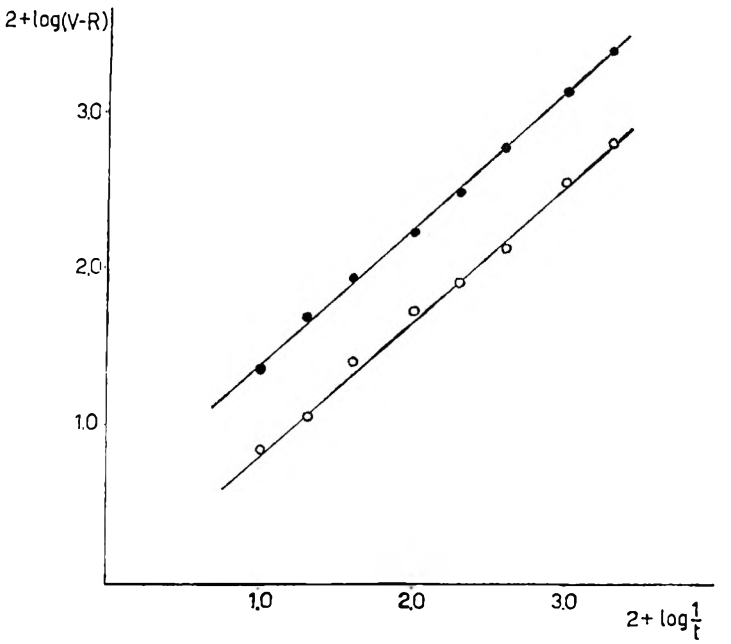


FIG. 2. Linear transformation of the values of the experiment of Fig. 1.

TABLE I

EFFECTS OF QUINIDINE AND OF QUININE ON THE EXCITABILITY CURVE

Concentration	Effect of quinidine (per cent) (A)	Effect of quinine (per cent) (B)	A − B	t	P
5×10^{-6}	0·00	0·00	0·00		
	15·93	12·23	+3·70		
	33·13	64·25	−31·12		
	10·17	0·00	+10·17		
	42·41	62·60	−20·19		
	31·60	4·47	+57·13	0·26	>0·8
1×10^{-5}	18·16	17·90	+0·26		
	25·30	43·48	−18·18		
	7·20	8·94	−1·74		
	12·83	0·00	+12·83		
	96·25	34·57	+61·68	0·80	>0·4
2×10^{-6}	15·82	17·13	−1·31		
	95·07	279·83	−184·76		
	124·56	163·56	−39·00		
	36·65	132·44	−95·79		
	58·68	97·15	−38·47		
	47·36	13·47	+33·89		
	500·66	26·03	+474·63	0·27	>0·7
3×10^{-5}	289·50	106·25	+183·25		
	656·00	57·81	+598·19		
	671·70	370·47	+301·23	2·32	>0·1
		On the total		1·41	>0·1

Results

Tables I, II and II show the effects produced in 21 experiments on each parameter by the two drugs. (A — B) indicates the difference (per cent) of the effect between the two drugs on the same preparation. These differences were statistically analysed by the Student's "*t*" test at each concentration and for all the values of the tables. The levels of significance do not show difference ($P > 0.05$) between quinine and quinidine as far as the three parameters are concerned.

By using the Lineweaver and Burk (1934) double reciprocal transformation, the values obtained for excitability and MRS enable the best

TABLE II

Effects of quinidine and of quinine on rheobase

Concentration	Effect of quinidine (per cent) (A)	Effect of quinine (per cent) (B)	A − B	*t*	P
5×10^{-6}	8·69	0·00	+8·69		
	17·07	0·00	+17·07		
	23·81	25·00	−1·19		
	100·00	95·83	+4·17		
	26·47	35·29	−8·82		
	17·39	13·64	+3·75	1·10	>0·3
1×10^{-6}	4·35	0·00	+4·35		
	46·70	0·00	+64·70		
	12·19	6·00	+6·19		
	118·51	139·28	−20·77		
	88·57	76·47	+12·10	0·95	>0·3
2×10^{-6}	7·14	20·00	−12·86		
	11·54	0·00	+11·54		
	72·73	68·00	−4·73		
	500·00	28·00	+472·00		
	9·37	21·05	−11·68		
	65·91	44·44	+21·47		
	5·26	0·00	+5·26	1·02	>0·3
3×10^{-6}	247·83	190·91	+56·92		
	400·00	58·82	+341·18		
	284·61	233·33	+51·28	1·56	>0·2
		On the total		1·81	<0·05 >0·1

straight line to be calculated between 1 ÷ per cent effect (y) and 1 ÷ concentration $\times 10^5$ (x) for the two drugs; one obtains:

	Quinidine	Quinine
Excitability ..	$y = 0.01374 + 0.02303x$	$y = 0.006271 + 0.04072x$
MRS	$y = 0.00633 + 0.02439x$	$y = 0.00163 + 0.03757x$

Putting now $y = 0.04$, the concentrations giving 25 per cent effect may be derived.

	Quinidine	Quinine
Excitability ..	$x = 0.8770 \times 10^{-5}$	$x = 1.2074 \times 10^{-5}$
MRS	$x = 0.7244 \times 10^{-5}$	$x = 0.9025 \times 10^{-5}$

It is seen that these values are very similar, agreeing with the estimate non-significance yielded by the *t* test.

At high dosage levels it was noticed that quinidine displayed a more marked negative inotropic effect.

Discussion

The effects of quinidine on excitability, rheobase and MRS correspond to those found in this laboratory with the same method (Libonati and Segre (1960).

The findings of Benthe (1956) on the ventricular strips of the frog show that quinidine is a little more active than quinine at two concentrations, 10^{-5} and 10^{-4}, on the absolute refractory period; however, the experimental

TABLE III

Effects of quinidine and of quinine on maximal rate of stimulation

Concentration	Effect of quinidine (per cent) (A)	Effect of quinine (per cent) (B)	A − B	t	P
5×10^{-6}	25·00	13·95	+11·05		
	11·82	16·00	−4·18		
	17·39	34·78	−17·39		
	30·00	35·94	−5·94		
	38·23	7·69	+30·54		
	12·76	8·00	+4·76	0·75	>0·4
1×10^{-5}	37·50	30·23	+7·27		
	34·40	40·00	−5·60		
	29·09	40·95	−11·86		
	34·00	35·13	−1·13		
	16·00	17·64	−1·64	1·31	>0·2
2×10^{-5}	54·00	100·00	−46·00		
	32·29	60·00	−27·71		
	52·17	100·00	−47·83		
	100·00	40·42	+59·58		
	44·17	30·00	+14·17		
	92·64	100·00	−7·36		
	82·76	55·00	+27·76	0·48	>0·7
3×10^{-5}	89·23	58·26	+30·97		
	100·00	100·00	0·00		
	100·00	100·00	0·00	0·81	>0·5
		On the total		0·08	>0·9

conditions and the test used were different from ours and he did not investigate the relative activities of the two drugs on the parameters investigated by us.

Only one clinical trial has been reported, in which the activity of the two drugs has been compared in man (Alexander and others, 1947); this trial showed no appreciable therapeutic activity of quinine. On the other hand it must be remembered that in the standard books of pharmacology and therapy no mention is made to the use of quinine in cardiac arrhythmias which is also true of a recent review on the clinical treatment of atrial fibrillation (Migheli, 1958).

The experimental results of the present work do not show differences in potency between quinine and quinidine on the excitability parameters in isolated rat ventricle. To explain the discrepancy between the experimental and the clinical findings it might be worth while to make a controlled clinical trial in which the absorption rate, the distribution in blood and myocardium, the excretion and the inactivation rate of quinine and quinidine were compared.

References

Alexander F., Gold H., Katz, L. N., Levy, R. L., Scott, R. and White, P. D. (1947). *J. Pharmacol.*, **90**, 191–201.
Alles, C. A. and Ellis, C. H. (1948). *Ibid.*, **94**, 416–425.
Benthe, H. F. (1956). *Arch. exp. Path. Pharm.*, **299**, 82–91.
Dawes, G. S. (1946). *Brit. J. Pharmacol.*, **1**, 90–112.
Feigen, G. A., Masuoka, D. T., Thienes, C. H., Saunders, P. R. and Sutherland, G. B. (1952). *Stanford Med. Bull.*, **10**, 27–31.
Frey, W. (1918). *Klin. W.*, **55**, 849–853.
Hoorveg, J. L. (1892). *Pflüger's Arch.*, **52**, 87–108.
Libonati, M. and Segre, G. (1960). *Arch. Ital. Sci. Farmacol.*, **25**, 166–180.
Lineweaver, H. and Burk, D. (1934). *J. Amer. chem. Soc.*, **56**, 658–666.
Migheli, B. (1958). *Omnia Therapeutica*, **9**, 225–267.
Weiss, M. G. (1901). *C.R. Soc. biol., Paris*, **53**, 466–468.
Wenckebach, K. F. (1923). *J. Amer. med. Ass.*, **81**, 472–474.

A NOTE ON THE INFLUENCE OF A METABOLITE OF ADRENALINE ON WATER DIURESIS IN RATS

By D. Roberts and Mary F. Lockett

From the Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.3

Received May 23, 1961

Solutions of an isoprenaline-like metabolite of adrenaline, and of isoprenaline itself which had been equated in potency both by depressor action on the mean arterial pressure of rats and by their inhibitory action on the rat uterus, equated also in effect on water diuresis in rats. The metabolite, like isoprenaline, reduced the rates of excretion of water, sodium and potassium and raised the pH of the urine.

A METABOLITE of (−)-adrenaline, indistinguishable from isoprenaline both in R_F value and in pharmacological action (Lockett, 1954, 1957, 1959) has been found in the plasma of blood withdrawn from the lower aorta (Eakins and Lockett, 1961). It is therefore expected to be present also in the plasma of renal arterial blood. Earlier work from this laboratory has shown that in unanaesthetised rats the subcutaneous injection of very small quantities of isoprenaline (0·75 μg. per 100 g.) diminishes the rates of excretion of sodium, potassium, ammonium and chloride and raises the pH of the urine (Botting, Farmer and Lockett, 1961; Farmer and Lockett, 1961, Lockett, 1959). We were interested, for these reasons, to compare the actions of isoprenaline and this metabolite of adrenaline on renal function in rats during water diuresis.

METHODS

Preparation of the metabolite for injection. Rabbits, 1·5–2·7 kg., were anaesthetised by slow injection of 5 ml. of 20 per cent urethane per kg. into a marginal ear vein. A tracheal cannula was inserted and 5 mg./kg. harmeline in 10 ml. of 0·9 per cent aqueous NaCl was similarly injected; then a polythene cannula was introduced through a femoral artery so that its tip lay just above the bifurcation of the aorta. Next heparin, 1,000 u/kg., was injected into an ear vein and the needle fixed in position for an infusion of (−)-adrenaline bitartrate 10 μg./ml. in 0·9 per cent NaCl, begun 30 min. after the injection of harmeline and 5 min. before bleeding, and maintained at a rate of 4 μg./min. Blood was collected from the femoral arterial cannula into heparinised ice-cold tubes; plasma was separated without delay. The metabolite was isolated from protein free extracts of plasma by ascending paper chromatography, using phenol-hydrochloric acid as solvent in an atmosphere of carbon dioxide, and eluates were prepared for biological use as previously described (Lockett, 1954, 1957). These eluates were assayed in terms of (±)-isoprenaline activity by their depressor action on the mean arterial pressure of rats anaesthetised with pentobarbitone sodium and by inhibition of the response of a quiescent dioestrus rat uterus to a fixed dose of acetylcholine, submaximal in effect (Eakins and Lockett, 1961).

Renal function tests. Male wistar rats, weighing 225–275 g., accustomed to stomach-tubes and handling, were fasted for 12 hr. before experiments. Each received an hydrating dose of tap water, 2 ml./100 g. weight, by stomach-tube, at zero time. At 1 hr., 5 ml. of tap water/100 g. weight was similarly administered and the bladder was emptied by gentle suprapubic pressure. A subcutaneous injection of isoprenaline, adrenaline metabolite or of normal saline was now given and the rat was placed in an individual cage for the collection of all urine excreted in the subsequent 90 min., when the bladder was again emptied. Two groups of 12

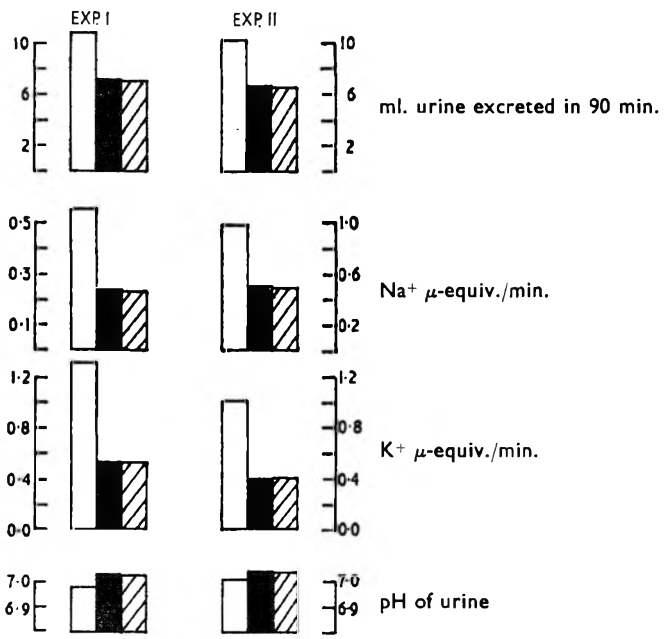


FIG. 1. A comparison of the effects of isoprenaline and a metabolite of adrenaline (assayed as isoprenaline) on the excretion of water, sodium and potassium in the 90 min. after administration of a water-load equivalent to 5 per cent weight. The mean results of two cross-over tests, each on 12 rats, are shown. Open rectangles, control values. Black rectangles, subcutaneous (±)-isoprenaline 0·75 μg./100 g. (Exp. I) and 1·0 μg./100 g. (Exp. II) weight. Hatched rectangles, Exp. I, 0·7 μg. and Exp. II, 1·0 μg. (±)-isoprenaline-like activity per 100 g. in eluate of metabolite injected subcutaneously (see also Table I).

rats were subjected to a 3 part cross-over test, made every third day, in which each animal received the three different subcutaneous injections in an order determined by randomisation within and between groups. Concentrations of Na^+ and K^+ in urine were measured in a flame photometer, and the pH of urines was determined by a glass electrode.

Drugs. (±)-Isoprenaline hydrochloride (Winthrop Sterling Inc.), Harmeline (L. Light & Co. Ltd.), (−)-adrenaline bitartrate (Burroughs Wellcome Ltd.), and heparin, Liquemin (Roche Products Ltd.) were obtained commercially.

RESULTS

Two three part cross-over experiments were used to compare the submaximal effects of subcutaneous injections of (±)-isoprenaline and of the isoprenaline-like metabolite of adrenaline on the elimination of a water-load by rats. The concentrations of metabolite in the eluates were assayed biologically for isoprenaline-like activity; these answers enabled a calculated dose to be used in the cross-over tests. The mean results of these two experiments are shown in Fig. 1, and the significance of differences caused by the administration of either isoprenaline or the metabolite are examined in Table I. In the first experiment the effects of 0·75 μg.

TABLE I

COMPARISON OF THE EFFECTS OF (±)-ISOPRENALINE AND A METABOLITE OF (−)-ADRENALINE ON THE EXCRETION OF WATER, SODIUM AND POTASSIUM BY RATS, IN THE FIRST 90 MIN. AFTER ADMINISTRATION OF A STANDARD WATER LOAD. THE VALUES SHOWN ARE MEANS ± STANDARD ERRORS OF THE MEAN. SIGNIFICANCE OF DIFFERENCES WAS DETERMINED BY *t* TEST, ONE ASTERISK, P = <0·05; TWO, P = <0·001.

Expt. I	Control	Isoprenaline 0·75 μg./100 g.	Metabolite as Isoprenaline 0·7 μg./100 g.
Water load excreted in 90 min., per cent ..	81·5 ± 3·55	52·6 ± 2·76**	52·0 ± 3·42**
Urine. m-equiv. Na^+/litre	8·5 ± 0·93	6·8 ± 0·63*	7·3 ± 0·88
m-equiv. K^+/litre..	9·1 ± 0·75	5·4 ± 0·71**	6·0 ± 0·53**
μ-equiv. Na^+/min.	0·9 ± 0·14	0·5 ± 0·06**	0·5 ± 0·04**
μ-equiv. K^+/min..	1·0 ± 0·09	0·4 ± 0·05**	0·4 ± 0·04**
pH	7·02 ± 0·03	7·28 ± 0·05*	7·24 ± 0·03**
Expt. II		1·0 μg./100 g.	1·0 μg./100 g.
Water load excreted in 90 min., per cent. ..	83·8 ± 2·78	51·8 ± 5·80**	51·6 ± 5·3**
Urine. m-equiv. Na^+/litre	3·9 ± 0·50	2·4 ± 0·32**	2·4 ± 0·31**
m-equiv. K^+/litre..	10·9 ± 1·05	6·7 ± 0·95**	6·3 ± 0·73**
μ-equiv. Na^+/min.	0·46 ± 0·06	0·20 ± 0·02**	0·19 ± 0·02**
μ-equiv. K^+/min..	1·31 ± 0·14	0·52 ± 0·06**	0·52 ± 0·07**
pH	6·94 ± 0·04	7·13 ± 0·01**	7·10 ± 0·03**

isoprenaline per 100 g. weight are compared with those of 0·7 ± 0·05 μg. isoprenaline-like activity of the metabolite per 100 g. weight in 12 rats weighing 250 ± 1·0 g. The second experiment differed in that the dose of metabolite was increased to 1·0 ± 0·1 μg./100 g. and compared with 1·0 μg. isoprenaline in rats weighing 254 ± 5·3 g. Fig. 1 demonstrates that isoprenaline and the metabolite caused equivalent reduction in the standard water load per cent excreted in 90 min. and similarly reduced not only the rates of excretion of Na^+ and K^+ (Fig. 1), but also their concentration in the urine (Table I). Both substances raised the pH of the urine, similarly. All these induced changes were of high significance (Table I). No significant differences were demonstrable by *t* tests between the effects of isoprenaline and the metabolite of adrenaline.

REFERENCES

Botting, R. M., Farmer, J. and Lockett, M. F. (1961). *Arch. Internat. de Physiol.*
Eakins, K. E. and Lockett, M. F. (1961). *Brit. J. Pharmacol.*, **16**, 108.
Farmer, J. and Lockett, M. F. (1961). *J. Pharm. Pharmacol.*, **13**, 412–415.
Lockett, M. F. (1954). *Brit. J. Pharmacol.*, **9**, 498–505.
Lockett, M. F. (1957). *Ibid.*, **12**, 86–96.
Lockett, M. F. (1959). *J. Physiol.* (*Lond.*), 146, 15P.

PHARMACOPOEIAS AND FORMULARIES

THE EXTRA PHARMACOPOEIA (MARTINDALE) SUPPLEMENT 1961*

REVIEWED BY EDWARD G. FELDMANN, PH.D.
Director of Revision, U.S.A. National Formulary

THE Extra Pharmacopoeia Supplement represents a departure from the established practice of publication of new editions of each of the two volumes of the Extra Pharmacopoeia every four to five years. The present book, which appears at a time when the revised version of Volume II would normally be issued, is intended to serve a dual purpose. In addition to bringing up to date the material carried in the latest edition of Volume II (23rd edition, 1955) it also provides new and revised information for the more recent Volume I (24th edition, 1958). This explanation is necessary to understand the scope of the Supplement and the reason why a new Volume II has not been issued. The preface states that this change in publication policy was prompted by the most rapid change in "the composition, general properties, actions and uses, dosage, and toxic effects of the drug and medicinal preparations in current use, and it is this information with which Volume I almost entirely concerned".

The Supplement in itself is deceivingly small in physical appearance. Actually, however, it contains over 300 pages which are filled with a vast variety of useful information. Moreover, because of the fact that most of the material has not appeared in previous editions of the Extra Pharmacopoeia, it may well be that this single book presents more new information than any two-volume set of the previous editions. Those using "Martindale", however, must now exercise normal caution in checking the Supplement first before referring to Volumes I and II, in order to assure that the information they seek is the latest provided. The slight inconvenience this causes is more than compensated by the economies in size and cost which have been afforded.

The Supplement is divided into seven main sections: (1) analytical addenda, (2) bacteriological and clinical notes, (3) sterilisation, (4) disinfectants, (5) blood transfusion, (6) formulae of proprietary medicines, and (7) new drugs and proprietary medicines. In addition, a list of abbreviations, a directory of manufacturers, and a well-prepared index are also provided.

The analytical addenda section gives informative summaries of scientific literature reports which deal with substances added to the latest editions of the British Pharmacopoeia, the British Pharmaceutical Codex, and the British Veterinary Codex. Also included are revisions of the subsections on barbiturates and sulphonamides, along with a number of abstracts of papers on miscellaneous analytical procedures of pharmaceutical interest. The bacteriological and clinical notes section brings up to date the corresponding discussions which appeared in Volume II. The sections on

* Pp. xi + 315 (including Index). The Pharmaceutical Press, London, 1961 32s. 6d. (Postage U.K. 1s.)

disinfectants and on sterilisation, which are treated separately, likewise are intended to revise the corresponding chapters in Volume II to a significant extent. In this connection the recent scientific literature has been thoroughly reviewed. As a consequence, those responsible for compiling these sections have wisely chosen to cite liberally the original references, which further enhances the value of the summaries.

The new chapter on Blood Transfusion represents a very comprehensive review on the subject which was not treated in either Volume I or Volume II. This extensive chapter describes the clinical uses of blood transfusion; the various blood-group systems; the determination of blood groups; compatibility tests; blood collection, preservation, storage, and handling; transfusion practice; and transfusion reactions. Unquestionably then, this represents a near-classic work on the subject. Unfortunately, however, this chapter is not documented with original reference citations as in the case of the sections which have been discussed above. Such documentation is not perhaps as important here, and furthermore, the omission is somewhat offset by the list of general references given at the end of the chapter.

The last two sections of the Supplement then provide, respectively, a complete revision of the list of proprietary medicine formulae given previously in Volume II, and supplemental information on new drugs and proprietary medicines to that described in Volume I. The proximity of these two separate tabulations causes this reviewer to speculate that a single integrated list might well be considered for future editions. In such a tabulation suitable symbols, prominently placed to precede each item, might indicate whether that item is (1) a "counter" proprietary, (2) an "ethical" proprietary, or (3) a substance subject to restrictions.

A most remarkable and striking feature of the Supplement is the inclusion of very recent information. *Addenda* have been inserted toward the end of the book, and through this mechanism information on products introduced as recently as April, 1961, has been included. When the publication date of July 7, 1961, for the Supplement is considered, those familiar with the problems of publication will recognise this as quite a noteworthy feat.

To those possessing the latest two volumes in the series, the Supplement represents an indispensable adjunct. For others, it will be a useful reference tool, particularly in the wealth of new information and tabulations which it provides. Those responsible for its compilation are to be congratulated once again on the splendid service they have rendered to the pharmaceutical and medicinal communities.

LETTERS TO THE EDITOR

On the Chemical Structure of Lysolecithins

SIR,—For some years the action of phospholipids, especially of lysolecithins, on the cardiac muscle has been the subject of pharmacological studies in our institute. Therefore we have found the excellent Review Article on lysolecithin by N. Robinson (1961) in this Journal of great use to us. It seems to be of general value to report on recent results concerning the chemical structure and composition of lysolecithins.

When lecithin is attacked by lecithinase A, one fatty acid ester linkage is cleaved with the formation of lysolecithin and a long-chain fatty acid. In the past several attempts have been made to determine whether the α'- or the β-ester linkage in lecithin is attacked by lecithinase A. The problem seemed finally having been solved by Hanahan (1954) and Long and Penny (1954), who showed fairly conclusively that lecithinase A attacks only the α'-ester linkage. Thus it has been assumed that the fatty acid ester linkage in lysolecithin is in the β-position of the glycerol nucleus only (β-acyl lysolecithin).

More recent investigations by Marinetti and his colleagues (1959a) resulted in the presumption that lecithinase A is not specific for cleaving the α'-ester linkage only, since their lysolecithin, obtained by incubation with cobra venom, had also an α'-acyl structure. Later Tattrie (1959) was able to show that lecithin from egg yolk had only saturated fatty acids on the α'-position and unsaturated ones on the β-ester linkage of lecithin. The lysolecithin resulting from the incubation of such a lecithin with phospholipase A (from the venom of *Crotalus adamanteus*) contained only saturated fatty acids, as has been shown by gas chromatographic analysis. From this Tattrie concluded that lecithinase A cleaves the unsaturated β-linked fatty acids specifically. With this opinion, De Haas and van Deenen (1960) have agreed. They demonstrated by means of synthesised "mixed-acid" lecithins that lecithinase A splits exclusively the fatty acids attached to the β-position irrespective of whether they were saturated or unsaturated. Meanwhile, Hanahan and his colleagues (1960) have corrected their former results. In the summary of their recent publication they state: "Thus it seems that lecithinase A attacks specifically the β-ester position of lecithins forming α'-acyl lysolecithin."

Another way to form lysolecithin is the acid hydrolysis of plasmalogens. Prepared thus, lysolecithin is proabably not exclusively α'-acyl lysolecithin as described by Gray (1958), for Marinetti and his colleagues (1959b) reported, that in at least 68 per cent of all plasmalogens of beef heart muscle the fatty aldehyde residue was found to be attached to the α'-position. Therefore the acid hydrolysis of natural plasmalogens yields to a certain degree (up to 68 per cent for the beef heart muscle) β-acyl lysolecithin with a corresponding share of unsaturated fatty acids.

Institut für Pharmakologie,
Toxikologie und Pharmazie
der Tierärztlichen Fakultät
der Universität München,
Munich,
Germany.

A. PETTER.
W. SCHLEMMER.
K. ZIPF.

August 4, 1961.

REFERENCES

de Haas, G. H. and van Deenen, L. L. M., Lecture, 5th Intern. Conference on Biochemical Problems of Lipids, July, 1960 (Marseille).

Gray, G. M. (1958). *Biochem. J.*, **70**, 425–432.
Hanahan, D. J. (1954). *J. biol. Chem.*, **207**, 879; **211**, 313 and 321.
Hanahan, D. J., Brockerhoff, H. and Baron, E. J. (1960). *J. biol. Chem.*, **235**, 1917–1923.
Long, C. and Penny, I. F. (1954). *Biochem. J.*, **58**, XV.
Marinetti, G. V., Erbland, J. and Stotz, E. (1959a). *Biochim. biophys. Acta* (Amst.), **33**, 403–414.
Marinetti, G. V., Erbland, J. and Stotz, E. (1959b). *J. Amer. chem. Soc.*, **81**, 861–864.
Robinson, N. (1961). *J. Pharm. Pharmacol.*, **13**, 321–354.
Tattrie, N. H. (1959). *J. Lip. Res.*, **1**, 60–65.

Peroxide-Sulphuric Acid Test as an Indication of the Ripeness and Physiological Activity of Cannabis Resin

SIR,—Duquénois and Negm (1938) have reported a reaction with hydrogen peroxide and sulphuric acid as a very sensitive but not specific enough test for the identification of cannabis. This should not be mistaken for the "Duquénois-Negm" vanillin-acetaldehyde test for cannabis, described in the same paper, which has found a wide application for the identification of hemp resin. No further data on the use of the peroxide-sulphuric acid test for cannabis have been made available since.

To examine the reaction of various types of cannabis resin, 49 samples originating from 11 countries have been analysed by means of the following procedure. Cannabis was extracted by maceration in light petroleum (1:20)

TABLE I

Origin	Number of samples analysed	Ripeness	Colour obtained
Germany	5	Unripe	Pink
Switzerland	1	"	"
Yugoslavia	2	Intermediate	"
Morocco	1	"	"
Spain	4	"	Pink to reddish-brown
Cyprus	4	"	"
Greece	6	"	Reddish-brown to brown
Brazil	15	Ripe	Mostly brown
Costa Rica	1	"	Brown
Burma	1	Overripe	Greenish-brown
Cyprus	1	Spoiled	None
Canada	8	"	"

for 24 hr. 0·2 ml. of the extract was left to evaporate in a porcelain dish. To the residue 2 drops of a 20 per cent hydrogen peroxide and 0·5 ml. of concentrated sulphuric acid were added and the dish rotated gently for 1 min. The colour of the liquid was observed after 5 min.

Most samples gave colours which ranged from pink (temperate regions) to brown or greenish brown (mostly tropical samples). But some of the samples did not exhibit any colour at all. The results are summarised in Table I. For comparison, their ripeness has been recorded in the same Table. Full details of constants, based upon an indophenol reaction, are given elsewhere (Grlić, 1961).

To elucidate the differences obtained, the same test was made with pure cannabinolic compounds. Cannabidiol yields a pink product, which in higher concentration appears blood-red. Tetrahydrocannabidiol exhibits a violet colour. Six synthetic tetrahydrocannabinol homologues showed strong brown colours. Synthetic cannabiol showed at first a green colour going quickly

into greenish brown. Cannabidiolic acid acetate reacted to give a colour ranging in various concentrations from orange to pink.

The results have been compared with those obtained by the indophenol method and explained in accordance with the classification of cannabis resin described in previous papers (Grlić, 1961; Grlić and Andrec, 1961). Samples exhibiting a pink colour correspond to the unripe or intermediate type, containing mostly cannabidiol or cannabidiolic acid, which is readily converted into cannabidiol (Schultz and Haffner, 1960). Samples in which the phytochemical conversion process of the cannabinolic constituents was more advanced (ripe cannabis) yield a brown reaction product. This group contains predominantly tetrahydrocannabinols and consequently (Loewe, 1950) exhibits the highest physiological potency. The reaction was greenish brown for overripe samples, containing predominantly cannabinol, the final product of conversion. A negative reaction is exhibited by samples containing mostly disintegration products of cannabinolic constituents.

As it is seen, the peroxide-sulphuric test seems to be suitable as an indication of the progress of the ripening process in hemp resin. In addition, the test seems to be useful for a rapid and rough estimation of the potency of the drug. A highly potent cannabis will exhibit a strong brown colour owing to the presence of tetrahydrocannabinols. Cannabis yielding an orange, pink or red reaction contains precursors of physiologically active constituents. Such hemp may be considered as potentially active, being converted into an active form under favourable conditions. A greenish brown colour or a negative reaction indicates the loss of physiological activity.

Consequently, the proper use of the peroxide-sulphuric test does not appear to lie in its possible identification of the geographical source of drug, but to distinguish cannabis resin of various compositions and potency. This reaction may be of practical use as a simple and rapid substitute for the complicated chemical and biological methods where no quantitative analytical data are required.

Acknowledgements. I am indebted to the Division of Narcotic Drugs of the United Nations and to Professor Décio Parreiras, Faculty of Medicine, Rio de Janeiro, for the supply of cannabis samples; also to Dr. S. Loewe (College of Medicine, University of Utah, Salt Lake City), to Professor Dr. F. Korte and Dr. H. Sieper (Chemical Institute, Bonn University), and to Professor Dr. O. E. Schultz (Pharmaceutical Institute, University of Kiel) for the supply of cannabinolic compounds.

Institute for the
Control of Drugs,
Zagreb, Yugoslavia.

Lj. Grlić.

August 2, 1961.

References

Duquénois, P. and Negm, H. M. (1938). *Méd. Leg.*, **18**, 493–506.
Grlić, Lj. (1961). *Acta Pharm. Jug.*, **11** (in the press).
Grlić, Lj. and Andrec, A. (1961). *Experientia*, **17**, 325–326.
Loewe, S. (1950). *Arch. exp. Path. Pharmak.*, **211**, 175–193.
Schultz, O. E. and Haffner, G. (1960). *Arch. Pharm.*, **293**, 1–8.

New Glycosides from Senna

SIR,—The letter by Vickers in the August issue of this Journal (1961) has stimulated us to report the isolation of five anthracene glycosides from senna since our report of the separation of an active primary glycoside (Fairbairn, Friedmann and Ryan, 1958).

Rhein-8-glucoside. This has been isolated from Alexandrian senna pod and appears to be similar to that reported by Vickers. We have shown it to be a rhein-monoglucoside by chemical analysis. Ultra-violet absorption measurements indicate that the compound is identical with synthetic rhein-8-glucoside, prepared from authentic rhein anthrone-8-glucoside by aerial oxidation in sodium borate solution at pH 9·2, and differs from a sample of synthetic rhein-1-glucoside, kindly supplied by Dr. A. C. Bellaart. Our experience is that the glucoside isolated by us is not readily decomposed by sodium bicarbonate solution with the formation of rhein, which does not accord with the inference that can be drawn from Vickers' letter.

Rhein-8-diglucoside. A second glycoside isolated has been shown to be rhein-8-diglucoside. This glycoside, is much more soluble in water than the 1- or 8-monoglucosides. It can be converted into the 8-monoglucoside by treatment with 0·1N hydrochloric acid at room temperature.

Rhein anthrone-8-glucoside, which is virtually half a sennoside molecule, was also found by us to be present in senna pod. This has been identified by hydrolysis to rhein anthrone, which was characterised spectroscopically, and by the formation of a green compound (λ_{max} 640 mμ) with *p*-nitrosodimethylaniline in pyridine (Tsukida and Suzuki, 1954). The conversion of rhein anthrone-8-glucoside to rhein-8-glucoside has been shown to proceed slowly in sodium bicarbonate solution. Rhein anthrone-8-glucoside, in alkaline solution, can give end-products other than rhein-8-glucoside: for example, Stoll, Becker and Helfenstein (1950) were able to obtain sennosides, and under more vigorous conditions, rhein. We are investigating the reaction conditions and the mechanisms which produce these different oxidation products.

Primary glycoside. Fairbairn and others (1958) have already described an active primary glycoside (related to the sennosides) having a molecular weight of 1164. We now report the discovery of a further primary glycoside having a molecuar weight of about 2000. It is characterised by a greater solubility in water than any of the other substances we have isolated and can be degraded to the sennosides by mild hydrolysis.

Aloe-emodin glycoside. This has been isolated from the non-rhein fraction of senna-leaf glycosides. It occurs as a pale-orange substance, soluble in water and methanol. Hydrolysis produces aloe-emodin and glucose, and quantitative work suggests it is a monoglucoside. We have good evidence also for the presence in the leaf of an aloe-emodin anthrone glucoside.

The important question raised by the discovery of these new glycosides is what contribution they make to the total activity of senna. Experiments on mice show that the new primary glycoside (MW 2000) is more active than the primary glycoside described by Fairbairn and others (1958), and significantly more active than the sennosides. Rhein anthrone-8-glucoside is about as active as the sennosides whereas Vickers' rhein glucoside, according to his results, appears to be much less active. Our experience with Alexandrian senna pod indicates that the less active rhein glucoside it contains constitutes only about 4 to 8 per cent of the total acidic anthracene glycosides, and that the aloe-emodin glycosides are present only in insignificant quantities. As the result of routine assays on many samples, we confirm the earlier findings (Fairbairn, 1959) that the pharmacological activity as determined by bioassay (Lou, 1949) runs

parallel with the sennoside content determined by the chemical assay (Fairbairn and Michaels, 1950). Both assay procedures have been shown to reflect adequately the laxative activity in man (Browne, Edmunds, Fairbairn and Reid, 1957).

When dealing with other varieties of senna or with extracts this satisfactory relationship between chemical and biological assays may not hold owing to varying proportions of active glycosides and the presence of breakdown products.

School of Pharmacy,
University of London,
Brunswick Square,
London, W.C.1.

J. K. CRELLIN.
J. W. FAIRBAIRN.
C. A. FRIEDMANN.
H. A. RYAN.

Westminster Laboratories Ltd.,
Regents Park,
London, N.W.1.
September 7, 1961.

REFERENCES

Browne, J. C. McClure, Edmunds, V., Fairbairn, J. W. and Reid, D. D. (1957). *Brit. med. J.*, **1**, 436–439.
Fairbairn, J. W. and Michaels, I. (1950). *J. Pharm. Pharmacol.*, **2**, 807–812.
Fairbairn, J. W., Friedmann, C. A. and Ryan, H. A. (1958). *Ibid.*, **10**, *Suppl.* 186*T*–191*T*.
Fairbairn, J. W. (1959). *Planta Medica*, **7**, 408.
Lou, T. C. (1949). *J. Pharm. Pharmacol.*, **1**, 673–682.
Stoll, A., Becker, B. and Helfenstein, A. (1950). *Helv. Chim. Acta*, **33**, 313–336.
Tsukida, K. and Suzuki, N. (1954). *J. Pharm. Sci. Japan*, **74**, 1092–1096.
Vickers, C. (1961). *J. Pharm. Pharmacol.*, **13**, 509.