THE PHARMACY OF BLOOD, ITS PRODUCTS AND SUBSTITUTES*

THE BLOOD CLOTTING MECHANISM

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When blood clots one of the plasma proteins, fibrinogen, is transformed to a gel, fibrin. Although fibrinogen makes up only one thirtieth of the plasma protein, on clotting it gives the blood a solid consistency, sufficiently strong to prevent further bleeding in case of smaller lesions.

From William Hewson to H. P. Smith

The first description of fibrin is to be found in Marcello Malpighi's *Dissertatio de Polypo Cordis* in the year 1666[1]. When the coloured matter was washed away from the clotted blood a whitish fibrous substance remained. Emboli from the heart behaved in a similar way. About 100 years later Hewson[2,3] clearly differentiated "the coagulable lymph" which clotted spontaneously in the air at room temperature from "the clottable matter of serum" which like egg-white clotted at first on heating.

Hewson demonstrated that blood without contact with the air remains fluid for a long time in a suspended vein. He found that blood, to which sodium sulphate had been added, retained its clotting ability for a time on diluting afterwards with water; this was the first practical way of preserving blood for experimental purposes. Hewson was the first to point out that fluid blood deprived of red corpuscles, that is the plasma, contained all constituents necessary for the clot formation.

In Hewson's time the ideas about the fluidity of the blood were very simple. The most common view was that the motion of the blood separates the fibrin particles and the blood corpuscles, which were thought to participate in the clot formation. The escape of carbon dioxide, known since 1783 to occur in the blood, or of ammonia, when the blood leaves the vessels, was also thought to induce coagulation. In the middle of the previous century, Richardson, an English physician, was an ardent defender of the theory that ammonia kept the fibrin in solution in the circulating blood. Joseph Lister[4] disproved this view in his Croonian Lecture of 1863.

Andrew Buchanan[5] in Glasgow was the first to make observations indicating a catalytic reaction in the clotting process. In 1845 he showed that ascitic and hydrocele fluid clotted when an extract of clotted fibrin was added to them. The blood clot itself, muscle, and other tissues had a similar action when added to blood. According to Buchanan there is a resemblance between the clotting of blood and the clotting of the milk by rennin.

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The enzymatic theory of coagulation was put on a sound basis by Virchow[6]. The founder of cellular pathology took particular interest in blood coagulation. In 1845 he made one of his great contributions to medicine when he explained that pulmonary emboli are clots from disrupted thrombi of the peripheral veins. Our main conceptions of the enzymatic processes leading to coagulation originate from his institute. It was here that Alexander Schmidt was educated. In 1861 Schmidt's new theory about coagulation was presented to the Berlin Academy of Science. His theory postulated that the coagulation proceeds in two phases. In the first phase thromboplastic substances are liberated from blood cells and tissues, which are capable of transforming a plasma component, prothrombin, into the active clotting enzyme thrombin. In the second phase the thrombin produces insoluble fibrin from its soluble precursor, which Virchow in 1847 named fibrinogen. Thus the terms thrombin, prothrombin, thromboplastic substances and fibrinogen all have their origins in the school of Virchow.

A new component was introduced in the discussion, when Arthus and Pages[7] in 1890 found calcium salts to be necessary for coagulation. By adding decalcifying agents such as oxalate, fluoride or citrate to the blood a new way of keeping the blood fluid became available.

Olof Hammarsten[8] in Uppsala proved some years later that calcium is necessary only in the first phase of coagulation, but not for the action of thrombin.

The classical theory of Alexander Schmidt as demonstrated in Figure 1 was supplemented in the years around 1905 by the theory of Spiro, Fuld and Morawitz (see Morawitz[9]).

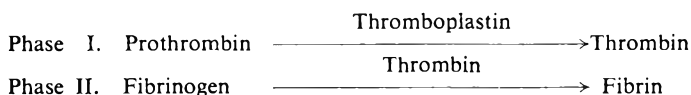

FIG. 1. The classical theory of blood coagulation.

Particular attention was paid to the study of the lipid component of thromboplastin by Bordet in Belgium and Howell in Baltimore. In this study Howell and MacLean discovered heparin in 1916 (see Jorpes[10]). Except for this, research on blood coagulation went into a 30-year hibernation period.

PROTHROMBIN ENTERS CLINICAL MEDICINE

In the nineteen-thirties interest in blood coagulation was given a new impetus. At the Iowa State University H. P. Smith introduced the principle of studying the different stages in the coagulation process on a quantitative basis with products as pure as possible. Thus the first reliable methods of determining prothrombin were elaborated. These are a two-stage method by Smith and others[11] and a one-stage method by Quick[12] in Milwaukee. A prothrombin deficiency was now found to be the cause of cholæmic bleeding[13], the hæmorrhages due to deficiency of vitamin K[14] and the bleeding tendency during the neonatal period[15].

The discovery of vitamin K in 1934 by Henrik Dam[16] in Denmark, and the work done by Charles and Scott[17] at the Connaught Laboratories of the University of Toronto on heparin in 1933 followed by the elucidation of its chemical nature in 1935[18] gave two new therapeutic possibilities of the utmost importance for medicine. In 1938 vitamin K was introduced as a prophylactic agent against the hypoprothrombinæmic bleeding tendency in obstructive jaundice and during the neonatal period. At about the same time heparin was found to be an efficient remedy for thrombo-embolic diseases. Nothing in the history of coagulation has exerted such a stimulating influence as these happenings particularly after Karl Paul Link's work. From 1940 to 1942 he studied the hæmorrhagic agent causing sweet-clover disease in cattle. The agent, named dicumarol, soon proved to be an excellent antithrombotic remedy (see Jorpes[19]). In his introduction to the number of the *British Medical Bulletin* (1955, **11**, No. 1), devoted to blood coagulation and thrombosis, Macfarlane points out that for every patient who dies as a result of deficient coagulation, there are thousands who die of thrombosis. Hence the importance of this new efficient specific therapy against thrombo-embolic disorders. Prothrombin now becomes a key substance in clinical analysis, almost as important as hæmoglobin.

New Coagulation Factors are Discovered

Thus, in the nineteen-thirties prothrombin was the central topic under discussion. During the next decade the main interest was focused on newly discovered coagulation factors concerned with prothrombin activation.

First consideration must be given to some early findings about the cause of hæmophilia. In 1911 Addis[20] found that a fraction of normal plasma accelerated the clotting of hæmophilic blood. He considered prothrombin to be the active component in it. During the period 1936 to 1937 Patek, Taylor *et al.*[21,22] at the Thorndike Memorial Laboratory in Boston were working on a plasma fraction found to be lacking in hæmophilia and hence called the antihæmophilic globulin. The globulin fraction, however, did not prove to be more useful than whole blood for the purpose of correcting the clotting deficiency in hæmophiliacs and no practical therapeutic results came from the work. These findings focused the interest upon the plasma proteins at a time when the cause of hæmophilia was considered to be exclusively a platelet deficiency.

Much more attention was paid to the discovery in 1943 by Paul Owren[23,24] in Oslo of a new factor, the absence of which causes a kind of hæmophilia, called by Owren parahæmophilia. He found a case with a congenital bleeding tendency in whose blood all known components of the coagulation system, fibrinogen, prothrombin and calcium, were present in normal concentrations and the thrombocytes seemed to function as in normal blood. Owren called the new component factor V, thereby disregarding Patek's and Taylor's discovery of the antihæmophilic globulin some 6 to 7 years earlier. Owren's discovery of factor V, later called proaccelerin and in its activated form factor VI or accelerin,

is unique at least in one respect. It was made by a young physician in a medical clinic, practically without laboratory facilities, and this during the difficulties of the German occupation of Norway.

At that time, Astrup[25] in Copenhagen made observations indicating that not thrombin but thromboplastin is formed during the autocatalytic reaction initiating coagulation, the active thromboplastin being derived from an inactive "prokinase." This corresponds very closely to our present-day conceptions.

Simultaneously with Owren, Quick[26], in 1943, found that a labile plasma component, assumed by him to be a part of a prothrombin complex and called prothrombin A, enters into the coagulation system. In fact Smith[27] and his colleagues of the Iowa group had already in 1939 pointed out that there is a "prothrombin convertibility factor" occurring in different concentrations in the plasma of different mammalian species. This effect of plasma was studied more closely by Seegers[28] and colleagues in 1947. As a consequence of these studies Seegers prefers to use the term accelerator globulin or plasma Ac-globulin instead of factor V or proaccelerin.

In several laboratories it was observed at the same time that an additional factor, other than calcium, thromboplastin and factor V, was essential for a complete and rapid conversion of prothrombin into thrombin. In analysing the different prothrombin preparations Owren[29] in 1947 had already drawn attention to a co-factor of proaccelerin, which was essential for coagulation. Later he called the factor proconvertin. Alexander[30,31] and colleagues in Boston, who made the most extensive study of this plasma component, named it "serum prothrombin conversion accelerator," SPCA. In 1951, this component was shown by Koller, Loeliger and Duckert[32] in Zürich to be closely related to prothrombin, being taken up by ordinary prothrombin adsorbents and strongly reduced in quantity by the action of dicoumarol. Koller named the component factor VII.

An interesting member of the series of coagulation factors is a new antihæmophilic protein. In 1947 Pavlovsky[33] in the Argentine observed that normal clotting capacity of the blood of a hæmophiliac could be restored by the blood from another hæmophiliac, suggesting that the hæmophilia of these two patients must have been caused by the deficiency of two different factors. Similar observations were made in other countries. In Switzerland, in 1950, Koller *et al.*[34] found a family with a hæmophilia-like disease, the "Moëna anomaly" which behaved in a similar manner. In 1952 Aggeler *et al.*[35], in describing a case of hæmophilia, showed that the coagulation deficiency was due to a lack of a well-defined plasma protein differing from the ordinary antihæmophilic globulin. It was called "plasma thromboplastin component, PTC."

Biggs, Douglas and Macfarlane[36–38] found in 1952 and 1953 a similar coagulation deficiency in seven patients. They coined the name Christmas-factor for the essential plasma protein and Christmas-disease for this form of hæmophilia. Not less than 9 different names have been suggested by different authors for this plasma component.

Koller[39] has recently suggested the use of numbers for the different components entering into the coagulation, fibrinogen being factor I, prothrombin factor II, thromboplastin factor III, calcium factor IV, proaccelerin and accelerin factor V and VI, convertin factor VII, the antihæmophilic globulin factor VIII and the Christmas-factor, factor IX. Koller himself added still another component, factor X, to the series. The synonyms suggested for the new coagulation factors, from 5 to 13 for each one of them, are presented by different authors of recent monographs[40–42].

In addition to the two types of hæmophilia caused by the lack of factor VIII and factor IX respectively, a third type was described by Rosenthal[43] in New York in 1953. The missing plasma component was called plasma thromboplastin antecedent or PTA. This form of hæmophilia occurred with equal frequency in males and females.

The two first mentioned forms of hæmophilia were named hæmophilia A and B by Cramer *et al.*[44] and by Soulier[45].

All the factors presented here, except fibrinogen, are active in the first phase of the coagulation. According to the modern view the following clotting factors take part in the coagulation (Fig. 2, Koller[39]).

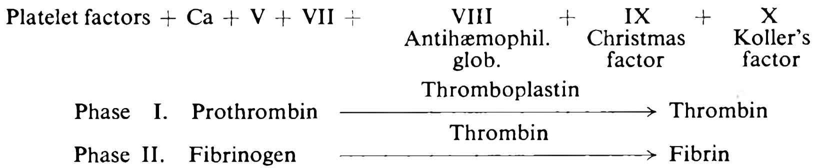

FIG. 2

SOME PROPERTIES OF THE COAGULATION FACTORS AND THEIR FATE DURING COAGULATION

Except for the peptides split off during coagulation all the fibrinogen is transformed into fibrin.

The antihæmophilic globulin (VIII) behaves in many respects like fibrinogen. It is precipitated at 25 per cent. saturation with ammonium sulphate and follows the fibrinogen in fraction I in Cohn's fractionating procedure 6 for the separation of plasma proteins. It is not adsorbed on the ordinary prothrombin adsorbents and not reduced during dicumarol treatment. As shown conclusively by Brinkhous *et al.*[46] in using hæmophilic dogs it disappears during coagulation. According to Tocantins and Seegers the serum still contains plenty of antihæmophilic globulin, although bound to a lipid inhibitor, which can be removed with ether.

The Christmas-factor (IX) on the other hand is precipitated at 35 to 50 per cent. saturation with ammonium sulphate, it precipitates in fraction IV of the Cohn fractionation, migrates with the β-globulin and is taken up by the prothrombin adsorbents. After coagulation it is found in the serum, where it is fairly stable on storage.

Factor V and VI, the Ac-globulin, is stable in the plasma only for a limited time and disappears during coagulation. It is not adsorbed on calcium phosphate, asbestos or aluminium hydroxide and is not influenced by dicumarol treatment.

Factor VII behaves almost exactly like prothrombin. It is, however, not consumed during coagulation and is fairly stable in serum.

WHAT HAPPENS DURING COAGULATION?

Hewson was fully aware of the fact that contact with foreign surfaces initiates coagulation and Alexander Schmidt spoke about the liberation of activating thromboplastic substances from the cells and tissues. Bizzozero[47] in 1882 observed that thrombocytes accumulate around the lesion, when a thrombosis is produced mechanically in the mesenteric vessels. In 1912 Bordet and Delange[48] showed that the thrombocytes liberate thromboplastic substances. Consequently these cells were considered to be the main source of the thromboplastin. In the following years the clot promoting agents contained in tissue thromboplastin were

TABLE I

THE PROBABLE SEQUENCE OF REACTIONS AND THE TIME NECESSARY FOR THE TWO PHASES OF THE NORMAL COAGULATION ACCORDING TO BIGGS[42]

	Reactions involved	Product formed	Time occupied by the reaction
Phase I	Contact with a foreign surface Platelets, antihæmophil. glob. Christmas-factor (IX) and calcium	Intermediate product of thromboplastin	2–5 min.
	Factor V and VII and calcium	Thromboplastin	8–10 sec.
	Prothrombin and calcium	Thrombin	2–5 sec.
Phase II	Fibrinogen	Fibrin	2–5 sec.

studied particularly by Chargaff and his colleagues[49]. They showed that the thromboplastin preparations from lungs, brain and other organs were high-molecular complexes or aggregates, containing protein, lipids nucleic acids and carbohydrates. Using thromboplastin labelled with radioactive phosphorus, Chargaff demonstrated that chemical combination between thromboplastin and prothrombin is improbable. With the introduction of the silicone technique[50] it became possible to study the platelet morphology and interaction of platelets. However, lately it has been found that the plasma thromboplastin is not liberated fully-formed from the thrombocytes but that it arises as a product of an interaction between plasma factors and accelerators from the platelets. The question arises to what extent the contact with foreign surfaces activates the plasma factors and to what extent the activated components react with the thrombocytes. Thus Brinkhous[51] in 1947 clearly demonstrated that a plasma factor, lacking in hæmophilia, is required for platelet utilisation during the normal coagulation. The factor was suggested by him to be a thrombocytolysin.

The interaction between the plasma factors and factors derived from the platelets has been the main topic of study during the last years. Blood, lacking some of the antihæmophilic factors, or with some platelet deficiency, has offered the best material for these studies.

In Table I and Fig. 3 the views concerning the coagulation mechanism held at present by two well-known authors, Rosemary Biggs[42] and Paul Owren[40] are summarised.

It is common knowledge that addition of brain extract, that is, "extrinsic thromboplastin," to blood or plasma causes rapid coagulation. According to Biggs, Douglas and Macfarlane[37] during normal clotting a substance forms which is highly active, much more so than the tissue thromboplastin. Thus in the presence of calcium the activators liberated from the thrombocytes react with the antihæmophilic globulin and the Christmas-factor to give an intermediate product usually called "intrinsic thromboplastin." According to Biggs, factors V and VII are, like calcium assumed to participate in the final thromboplastin formation. These parts of the coagulation process are the most time-consuming ones, the thrombin and fibrin formation like many other enzyme reactions being almost instantaneous. Biggs, Douglas and Macfarlane[38] developed a thromboplastin generation test by means of which the hæmophilias A and B and deficiencies of the thrombocytes can be recognised.

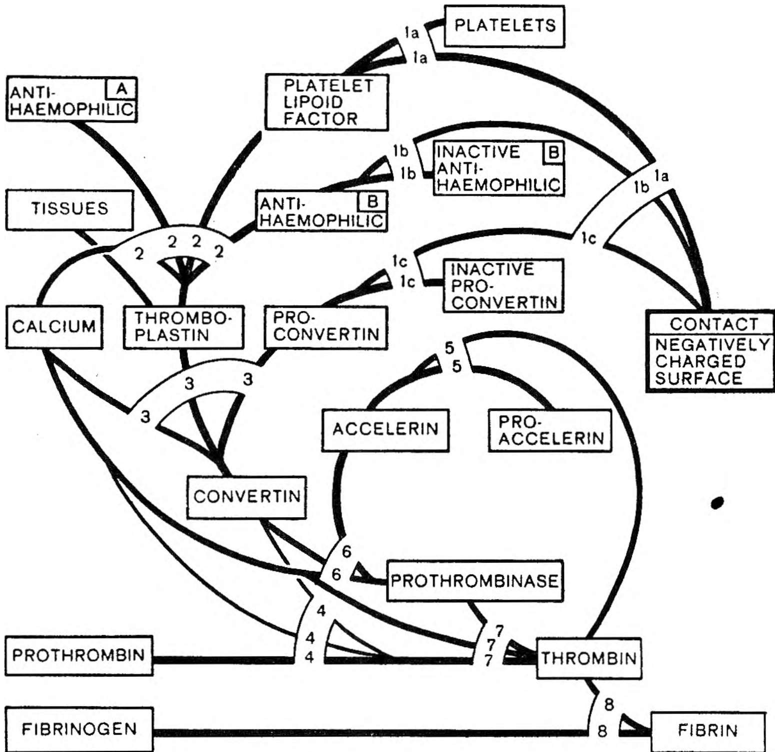

FIG. 3. Blood coagulation theory of P. A. Owren.

OWREN'S SCHEME OF COAGULATION, 1954

In Owren's scheme factor VI and VII participate together with thromboplastin in the transformation of prothrombin to thrombin. Koller and colleagues[52] like Biggs do not consider these factors necessary for the action of the plasma thromboplastin. However, the two schemes do express the same thing although in different words. Owren's prothrom-

binase is a fully correct name for the final thromboplastin because peptides are split off enzymatically from prothrombin during its activation.

As to the different factors deriving from the platelets and from the plasma, participating in the thromboplastin formation, Seegers[53] gives the following classification:

TABLE II

CLASSIFICATION OF PLATELET AND PLASMA CO-FACTORS

From the platelets:	
Platelet factor 1 ..	.. Accelerator factor (platelet-AcG)
Platelet factor 2 ..	.. Fibrinoplastic factor (thrombin-fibrinogen)
Platelet factor 3 ..	.. Threone component (thromboplastin factor)
Platelet factor 4 ..	.. Antiheparin factor
In the plasma:	
Platelet co-factor I	.. Threone component (antihæmophilia factor)
Platelet co-factor II	.. PTC (plasma thromboplastin component)

Thus not less than four factors are obtained from the thrombocytes (see also Ackroyd[54]). The two co-factors occurring in plasma, the "platelet co-factor I" or the "threone component" and the "platelet co-factor II" or Aggeler's "plasma thromboplastin component"[55], would have been easier to recognise if they had been named the antihæmophilic globulin (VIII) and the Christmas-factor (IX) respectively.

According to Seegers[56,57] the platelet factor I functions like the accelerator globulin and can in fact restore the Ac-globulin deficiency of stored, oxalated human plasma. Likewise Owren[58] recently reported about the proaccelerin activity of the platelets. Platelets from a patient with a congenital lack of proaccelerin, a case of parahæmophilia, showed only about 1/50 of the accelerator activity of normal platelets. The main interest has been concentrated upon platelet factor III, which, as found by van Crevald and Paulssen[59], reacts with the antihæmophilic globulin (VIII). Purified extracts containing this factor can restore normal coagulability in cases of thrombocytopenic purpura[60].

The physiological importance of platelets in hæmostasis is not limited to the thromboplastin formation. Morphological studies[61-63] have shown the importance of the agglutination of platelets for hæmostasis and for clot retraction[64]. The platelets also contain a vasoconstricting factor[61], probably 5-hydroxytryptamine, "serotonin"[65]. A local platelet agglutination and a vasoconstriction certainly create a mechanical hindrance to the loss of blood from smaller blood vessels. On the other hand, the clot retraction, facilitated by a platelet factor, may fill a physiological function in partially restoring the patency of recently thrombosed larger vessels (see Ackroyd[54]).

The *chemical reactions* occurring in the thromboplastin formation are completely unknown. The same applies to the activation of proaccelerin to accelerin and to the formation of convertin. Its properties indicate that it may derive from prothrombin.

The first reaction more closely studied is the transformation of prothrombin to thrombin. A prerequisite for this study has been Seegers' preparation of an electrophoretically well-defined prothrombin[66,67]. Thrombin can, as found by Seegers and colleagues[68], be formed from it

in different ways either by means of thromboplastin, or autocatalytically. In a 25 per cent. citrate solution spontaneous activation takes place. During the activation peptides containing a carbohydrate component are released. A similar proteolytic splitting takes place during the fibrin formation. In an excellent investigation, Bailey and Bettelheim[69] in Cambridge in 1952, showed that under the action of thrombin two peptides, A and B, are split off from fibrinogen, one of them, peptide B, with the phenolic hydroxyl of tyrosine conjugated with sulphuric acid[70,71]. The view until recently held by Eagle[72] and Ferguson (see Astrup[73]) that thrombin acts like a plasma protease has thereby been revived. In fact thrombin also acts on a synthetic substrate, splitting tosylarginine methyl ester. The proteolytic action of thrombin is, anyhow, quite specific, taking only one peptide bond out of 1000 in the fibrinogen molecule.

In spite of this splitting off of some peptides from the fibrinogen it is still too early to fully abandon Wöhlisch's[74] view about the denaturating influence of thrombin upon the fibrinogen during the fibrin formation.

Why Does Blood Remain Fluid in the Veins?

We can offer no explanation for this observation. The speed of flow of the circulating blood is considered to be of importance, because in bedridden persons with a greatly reduced flow thrombosis is common. Early ambulation after operation and childbirth strongly reduces the number of thrombo-embolic complications. However, in some hibernating mammals the speed of the blood flow during hibernation is extremely low. Simultaneously there is a marked increase in the number of mast cells in different organs, which could indicate a possible increased production of heparin[75]. But in the blood of most mammals, in human, horse and ox blood, there is practically no heparin to be found, which does not help us to understand the phenomenon.

There are of course other anticoagulating factors of yet unknown importance. Thus Tocantins[76] presented much evidence for the occurrence of a lipid inhibitor of coagulation (see Seeger[57], p. 85). Nilsson[77,78] has recently studied a heparin-like prothrombin inhibitor in mammalian blood.

Also, we know of some protective mechanisms against thrombosis. The first one is the almost instantaneous disappearance of thrombin from the blood, where its presence during life would be very dangerous. Thrombin is strongly adsorbed to the fibrin clot. There are furthermore in the blood strong antithrombins with an action on thrombin *in vitro* as well as *in vivo*. They have been extensively studied during the last years (Astrup[25], p. 101 and Seegers[57], p. 76). They are plasma proteins, some being activated by heparin, which in the presence of a plasma protein, the heparin co-factor, is a very strong antithrombin.

Fibrinolysis, or the dissolution of newly formed fibrin clots, is another very effective but dangerous protective mechanism. There is in the plasma a large excess of plasminogen, a proenzyme which through a yet unknown mechanism may be transformed into plasmin, a proteolytic enzyme highly active on fibrin and fibrinogen. The enzyme makes the

blood more or less inclottable because of fibrinogen deficiency. An activation of plasminogen to plasmin, causing lethal bleeding, sometimes occurs, e.g. in shocked persons, after pneumectomies or abruptio placentæ. In the last mentioned case it is more common that placental thromboplastin enters the maternal circulation, activates prothrombin in the blood and causes multiple emboli of fibrin in the pulmonary vessels. (Seegers[79] *et al.*). This part of the coagulation mechanism demonstrates the complexity of the system by means of which the blood to the benefit of life remains fluid or clots under changing conditions. There are consequently powerful mechanisms in the blood acting in opposite directions, either causing or preventing coagulation, both of them essential to life. If one of them becomes deficient life will be threatened, either by a bleeding tendency, as in hæmophilia, or by a thrombosis. The main problem, yet unsolved, is how these mechanisms counter-balance each other in the blood.

In conclusion I would like to quote Psalm 139, v. 14:—

"I will praise thee; for I am powerfully and wonderfully made: marvellous are thy works; and that my soul knoweth right well."

I think we can all agree that this is the only thing that we really know.

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PRODUCTS OF HUMAN BLOOD*

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Although blood in its entirety is often irreplaceable, each of its components has its own therapeutic function and one means of economising in the use of human blood consists of separating these and using each individually. Mechanical separation of plasma from cellular components presents no difficulties, but the protein constituents, albumin in particular, can be separated only by a methodical fractioning process like that which has been elaborated and progressively improved by the Harvard School since 1943[1].

This brief article will add little to the excellent literature recently published[2] on blood, plasma and human serum, except to add some new technical facts which are also applicable to the derivatives of blood.

Cell Constituents of the Blood

Erythrocytes, leucocytes and platelets, although they are distinct in nature and functions, have one characteristic in common—their fragility—which, up to the present, has limited their use by injection for therapeutic purposes.

Erythrocytes. The red blood cell is short-lived and fragile in nature. Its preservation outside the body is therefore difficult because of the artificial conditions to which it is subjected. To prevent the blood from coagulating, citrate, itself an abnormal constituent, is added and the red corpuscles are kept inactive and at a low temperature in the container in which they have been collected. Their metabolic rate and gaseous exchanges decrease but glycolysis persists for some time and the lactic acid which is formed reduces the pH below the physiological level. The red cell loses potassium, gains sodium and water, and the resistance of the membrane gradually becomes less.

It has been found that a mixture of citric acid, citrate and dextrose favoured the survival of erythrocytes. The citrate, which cannot pass across the membrane of the cell, exerts an osmotic pressure which counterbalances the endocellular osmotic pressure, and in this way retards hæmolysis. The dextrose allows the cell to continue its metabolic process, while citric acid acts by lowering the pH, which is known to facilitate survival. The alteration in nature of the preserved cell is not, however, due only to the internal hypertonicity: even under physiological conditions the red corpuscle finally disintegrates.

At the present time the citric acid-citrate-dextrose mixture is widely-used as an anticoagulant in the U.S.A,[3], especially when it is desired to preserve the red cells. Since low temperatures (between + 4° and + 6°C.) stop metabolism the cells are more easily preserved. Several attempts have been made to keep them at an even lower temperature, but the thawing process destroyed them. However, recent tests, in the presence

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of glycerol, are quite promising: 70 per cent. of red cells preserved at −79° C. for a period of 6 months in a 30 per cent. glycerol solution, together with 3 per cent. of trisodium citrate, have been found to survive. These conditions cannot be achieved in practice, but the technique is improving to an extent that it is now possible to envisage preservation for a period of three months at −20° C. in a medium containing citrate and a large proportion of glycerol. When the medium is returned to a normal temperature the glycerol may be removed by continuous washing of the red corpuscles in increasingly diluted solutions of glycerol, the operation taking two hours[4]. This process is still only in the experimental stage. However, transfusion laboratories have already been successful in preserving erythrocytes of rare blood groups by freezing them in a medium containing glycerol.

Plasma was employed on a large scale during the war, while the erythrocytes remained unused. This situation has changed to-day, however. The B.P. 1953 devotes a monograph to them which says they may be obtained from one or more preparations of Whole Human Blood which is not more than seven days old and each of which has been directly matched with blood of the intended recipient. The Concentrated Human Red Corpuscles must be used within 24 hours of their separation. Whether they are in the form of a puree or in suspension in an isotonic solution, their intravenous injection should be accompanied by the same precautions as are usual for whole blood. They replace freshly-drawn blood when corpuscular regeneration only is intended. Furthermore, they have more specialised applications: e.g., they are preferable to whole blood in those patients who are likely to overtax their circulatory system; they are prescribed exclusively in hæmolytic jaundice suffered by patients who have undergone a series of transfusions[5].

The red corpuscles are also prescribed for external use, in ointment or powder form, as energetic stimulants to the healing process. For this purpose, both human and animal blood has been used. Lehmann[6] has provided a formula for blood paste using fresh human blood, drawn aseptically, and citrated, to which an agar emulsion is added. It is necessary to obtain an adhesive preparation which remains moist and homogeneous and for this purpose, mucilage of agar appears to be more suitable than carbowax or polyethylene glycol. Vehicles containing greases or fatty alcohols are unsuitable.

Leucocytes[7] *and Platelets*[5,8]. The transfusion of leucocytes and platelets is still in the experimental stage. Leucocytes are rich in enzymes, and their phagocytocic properties, and the part played by certain of them in the formation and transportation of antibodies, makes them of particular interest. However, they are difficult to separate and to preserve, both because they are short-lived and because these cells are rendered fragile by virtue of their greatly hydrated protoplasm within an adhesive membrane. When they are separated by the special Cohn centrifugal machine which has the property of moistening the membrane, they are preserved in special media with a pH value of 7 with monovalent crystalloids and colloids without albumin but containing gelatin, traces

of ascorbic acid, a small quantity of sodium acetate and traces of deoxyribonuclein. Darkness is essential and the optimum temperature is $+ 4°$ C[7,9]. In spite of these precautions, not only is preservation short-lived, but when leucocytes are injected as a cream or in suspension they disappear in less than an hour and reappear fixed in the lung. It is, however, possible that the long-term effect of such therapy is favourable.

Leucocytes and platelets represent scarcely 0·4 per cent. of the blood as a whole. Of all the cellular constituents in the blood the platelets are the smallest and lightest in weight. Their origin is still much discussed, but recent findings do not seem to contradict the hypothesis that platelets are simple cytoplasmic fragments of the megakaryocyte.

Little is known of their metabolism: it seems that they have no synthetic activity. When injected, they disappear in four or five days and are fixed by the reticulo-endothelial tissue and especially by the spleen, as recent tests with ^{32}P have verified[10].

During the coagulation of the blood, the platelets play a large part in the formation of the thromboplastin and contractions of clots. On removal from the vessel, it appears that the platelets are combined with calcium; this conception makes it at least possible to understand how easily the platelets may be fixed by the cation exchange resins used to remove calcium from the blood. The blood is then incapable of coagulation. The platelets are then eluted by a large volume of saline solution or a very small quantity of a solution of sodium or calcium acetate, which implies that the calcium is associated with the platelet lipo-protein, i.e., with the thromboplastin. This process combined with centrifuging, by taking advantage of the low density factor of the platelets in silicone or plastic coated containers, has refuted the idea that platelets are fragile and immediately destroyed on removal from the blood vessels.

Protein Constituents of the Plasma

Albumin and globulins were first of all separated by the "salting-out" method, i.e., by fractional precipitation with concentrated acid salts or neutral salts. However, the distinction between euglobulins and pseudoglobulins, defined by the ammonium sulphate concentration which precipitates them, is not apparent in other tests, in particular, on electrophoresis, which separates four principal fractions of the plasma proteins, namely, albumin and α-, β- and γ-globulins, the albumin being the most mobile and the γ-globulin the least. In the normal man, the average percentage of each fraction in comparison with the total proteins in the serum is as follows[11]:

Albumin 61 $\pm$ 3 per cent., α-globulins 12·5 $\pm$ 2 per cent., β-globulins 12·5 $\pm$ 2 per cent., γ-globulins 14 $\pm$ 2 per cent.

The salting-out process, based on a single comparison test, was unselective and inapplicable in practice to the separation of blood-proteins for therapeutic purposes. During the last war it was necessary to use human blood to the best possible advantage, i.e., without waste and using the specific properties of each fraction. The process methodically worked out by Cohn and his associates at Harvard fulfils these

requirements and is still capable of being improved; to-day it is used in most blood transfusion centres in the world[12]. The five variables (ionic strength, ethanol concentration, pH value, temperature, protein concentration) give the Cohn-Oncley 6–9 method plenty of flexibility and the separated fractions are practically identical with the components revealed by electrophoresis.

Plasma is thus divided into five fractions: fraction I is separated from the citrated plasma at a temperature of $-3°$, pH value of 7·2, ionic strength of 0·14 and an ethanol concentration of 8 per cent., while the protein concentration is 5·6 per cent. This precipitate is essentially made up of fibrinogen and antihæmophilia globulin. The supernatant fluid, when cooled, brought to a pH value of 6·9, ionic strength of 0·9, protein concentration of 3 per cent., then has ethanol added in a sufficient quantity to produce a 25 per cent. concentration. The prothrombin, the γ-globulins, the *iso*-agglutinins, the plasminogen and the β-lipo-proteins then precipitate to form fractions II and III. Fractions IV and IV–4 comprise the α-globulins, the α-lipo-proteins and the β-globulins, while fraction V is mainly albumin.

However, the disadvantages of the Cohn method are the cost of installation, the slowness of the operations and the denaturing action of ethanol upon proteins. At present attention is centred upon the use of heavy metals, for example zinc, already suggested by the Cohn 10 method, which is quicker, and in particular by method 12 which acts on whole blood. The metal processes are quick and economical: they save expensive refrigeration plant and their efficiency is greater.

These methods have made it possible to separate a large number of plasma proteins or fractions where a single type of protein predominates: albumin, globulins (α, β and γ), fibrinogen, antihæmophilia-globulins, prothrombin and *iso*-agglutinins. Sometimes the techniques are sufficiently sensitive to produce associated proteins such as α- and β-lipo-proteins. Undoubtedly, the separated fractions are mixtures. They nevertheless lend themselves to therapeutic requirements.

Albumin. This constitutes 99 per cent. of fraction V of Cohn-Oncley method 6–9, but the stable plasma protein solution which Cohn obtains after extracting the α- and β-globulins and fibrinogen from plasma by the zinc method is particularly rich in albumin. Albumin plays a multiple role in plasma: it fixes and ensures the movement of numerous bodies, it contributes to nitrogenous nutrition but, in particular, its high colloidal osmotic pressure causes it to play an important part in the regulation of the hydration of the organism. Not only does it retain the fluid in the blood vessels (1 g. of albumin retains 18 ml. of fluid), but it absorbs the extra-cellular fluids at capillary level and thus prevents œdema[13,14].

However, albumin is able to transmit the virus of serum hepatitis, and an effort has therefore been made to destroy the virus without denaturing the albumin. The addition of acetyltryptophan 0·04 M and sodium caprylate 0·02 M makes it possible to heat a solution of albumin

to 64° C. at pH 7·1 to 7·3 for several hours without change[15]. Ultraviolet light may be used to sterilise the plasma if sodium caprylate is added to prevent the denaturation of the albumin and the other proteins[16].

In practice, the albumin used in therapy is available in a 25 per cent. solution in 100 ml. bottles, one bottle constituting one dose. This solution, at pH 6·8, is stabilised by tryptophan, or by 0·2 M sodium caprylate or mandelate.

The test for the official solution consists of identifying the albumin by its mobility when subjected to electrophoresis, and by checking clarity when the solution is heated to 57° C. This solution should be sterile, apyrogenic and atoxic to mice. It is stored at a low temperature.

Albumin is used principally in the treatment of hæmorrhagic shock conditions where it is of paramount importance to re-establish the blood volume: 100 ml. of a 25 per cent. solution has the osmotic value of 500 ml. of blood. The injection is followed by a blood-dilution of extravascular origin. If repeated injections of albumin are given or if there has been considerable hæmorrhage, the injection should be followed by an injection of isotonic fluid, and in serious cases it is used only as a temporary measure until such time as a whole blood transfusion can be given, the latter alone being capable of combatting anoxia.

Another use is in the treatment of serious burns where fluid is constantly leaking from denatured capillaries; here the albumin solution is diluted to 5 per cent. with isotonic saline. A further application is in the treatment of hypo-proteinæmia where the protein deficiency is primarily an albumin deficiency. Injection of albumin is prescribed in the so-called deficiency œdema, in cirrhosis and in nephrosis. The dose, given by perfusion, is 50 to 75 g. daily for an adult and 25 g. for a child. However, repeated injection may lead to anæmia or to hypoprothrombinæmia[14], while the use of large quantities or too rapid injection may create the risk of pulmonary œdema.

γ-Globulins. So many papers have been published recently on the γ-globulins[12,17,18] that no attempt will be made here either to give a definition of them (wholly electrophoretic), nor to describe their venous or placentary origin[19] leading to products of the same immunological properties[20], nor their extraction where the metal techniques[12,18,21,22] are slowly superseding the Cohn-Oncley process, nor their physical, chemical or biological nature[17,23,24]. Their entire interest lies in their antibody properties. It is due to the fact that his blood is rich in γ-globulins that the newly-born infant owes his immunity to disease during the first few months of life. In the same way, passive immunity—which is more durable in the child than the adult—corresponds to a longer average life of the injected γ-globulins and, for a given age, acquired immunity also is longer or shorter according to whether the receiver is able or not to re-use the specific functional groups with their antigenic properties.

We shall confine ourselves solely to the question of presentation of and test for the γ-globulins.

The γ-globulins when prepared give a pale yellow or brownish solution because of the presence of denatured hæmoglobin which is clear or

slightly opalescent and which may, in the course of time, leave a granular deposit. In the United States, doses are generally indicated in ml. of a 16 per cent. solution. In France, Ardry recommends a 10 per cent. solution to be prescribed in mg. of active product[12]. Antiseptics such as sodium ethylmercurithiosalicylate at 1 : 10,000 may be added to these solutions as well as a stabiliser like tryptophan, glycine, alkaline salts or sodium thiosulphate. The final solution is neutral and isotonic.

The tests are designed for inclusion in the French Pharmacopœia[25] and comprise:

1. The general tests for injectable solutions: neutrality (pH value $6{\cdot}8 \pm 0{\cdot}2$), sterility (in a thioglycolate medium), and toxicity to mice.

2. Identification and purity tests. Identification by immunological reactions (the solutions must precipitate with human anti-sera but not with animal anti-sera). Establishment of purity by electrophoretic mobility (90 per cent. of the proteins should satisfy the mobility test $-2{\cdot}8 \times 10^{-5}$) or by precipitation with ammonium sulphate at 36 per cent. saturation (the solution of proteins having previously been diluted 1 : 10 with normal saline). Under the same conditions the solution of γ-globulins must not give a precipitate with ammonium sulphate at 25 per cent. saturation (freedom from fibrinogen) while 70 per cent. of the proteins should precipitate when ammonium sulphate to 33 per cent. saturation is added.

3. Quantitative tests:

(*a*) measurement of proteins by a colorimetric or gravimetric method.

(*d*) determination of activity: for lack of anything better, standard diphtheria toxin is used. The latter, if injected alone intradermally into a guinea-pig, produces necrosis at the level of the injection site. Mix 1 ml. of a standard toxin solution with 1 ml. of one series of dilutions of γ-globulins to be titrated, and after a period of 2 hours at 37° C. and 22 hours in the refrigerator, inject 0·1 ml. of each of the mixtures into guinea-pigs weighing about 250 g. The neutral mixture should not, after 4 days, have produced necrosis at the injection site. The test for specific γ-globulins taken from hyper-immunised human serum consists of the measurement of their specific efficacy. For example, in the case of immune poliomyelitis globulin, mice inoculated with the Lansing strain are used[26].

It is impossible to give the therapeutic uses of the γ-globulins[27] in detail here. These γ-globulins, which were prepared first of all in the U.S.A. for the prevention of poliomyelitis, in preference to plasma, which is capable of transmitting homologous jaundice[28], have had a considerable vogue. It has since been found that they give only a belated and brief protection and their use, which is hazardous, is too costly[29]. Moreover, the γ-globulins are at present reserved particularly for prophylaxis in measles and in treatment by its attenuation[30,32–34], and for the prophylaxis of epidemic hepatitis[27,35]. However, specific γ-globulins are recommended in the corresponding diseases of, for example, poliomyelitis and whooping-cough.

γ-Globulins are also a major indication of a very different complaint, namely a-γ-globulinæmia, which was discovered by serum electrophoresis and which manifests itself in an extraordinary susceptibility in certain individuals to infectious diseases[36].

The γ-globulins are administered either subcutaneously or intramuscularly. The average dose in the prophylaxis of measles is given as 33 mg./kg. and 8 to 10 mg./kg. in attenuation. In the case of a-γ-globulinæmia a dose of 100 mg. is given twice monthly.

Other blood proteins used in therapy are all connected with the blood-clotting processes[2,7,14,37].

It is known that hæmophilia may be related to the absence of certain factors. Up to the present, only the antihæmophilia globulin has been used in therapy and in the form of fraction I of the Cohn method[38], but it can be obtained in a purer form[39] as an amorphous white substance obtained by lyophilisation. It is available in ampoules containing 200 mg. and is given intravenously.

Thrombin and Fibrinogen. Thrombin and fibrinogen play an essential part in blood clotting and are of great service to therapy.

Prothrombin, which was first isolated from bovine plasma may also be extracted from human blood and transformed into thrombin by the addition of placentary thromboplastin[13]. The B.P. 1953 describes a thrombin of human origin satisfying various tests: solubility in saline solutions; coagulation of citrated plasma in the absence of ionised calcium; sterility and finally activity tests. Thrombin in the United States Pharmacopeia is of bovine origin; it is measured in N.I.H. units[40]. Thrombin is used alone as a hæmostatic in buffered solution or orally[41] in hæmatemesis[42] or by injection in hæmophilic articular hæmorrhages[43], or is used in association with fibrinogen.

The B.P. describes human fibrinogen as a white powder or a friable solid. When placed in a saline solution it may coagulate spontaneously in the course of time but will coagulate immediately if thrombin is added. Its nitrogen content is between 15 and 16 per cent. 85 per cent. of this nitrogen is retained in the clot resulting from the addition of thrombin. Fibrinogen may be identified by electrophoresis (in an aqueous buffer solution of pH 8, mobility is between that of the β and the γ-globulins) and by coagulation when thrombin is added to a freshly prepared solution in saline solution. The preparation should be used immediately after reconstitution.

Fibrinogen, with the addition of thrombin at the time of use, has been widely used in America in neuro-surgery[14,37], in the treatment of burns, and in cutaneous grafting. The use of fibrin clot, formed *in situ* in operations to remove and extract stones in the kidney, is also known.

Fibrin may be obtained by the action of thrombin on fibrinogen under suitable conditions, e.g., fibrin foam, listed in the B.P. as a hæmostatic artificial sponge. Fibrin films or plates of varied shape and consistency are also used, especially in neuro-surgery. These latter preparations should fulfil special tests corresponding to the purpose for which they are required, namely, the absence of toxicity to the tissues, the possibility

of becoming flexible when in contact with salt water, the re-absorption in contact with the tissues in a given time.

Notes

The practice of blood transfusion, for a long time regarded with apprehension and as a last resort, was developed under the pressure of the needs created by the war. Now, however, a few figures will give an idea of the importance of the products of human blood. In 1953, the National Blood Service[44] issued 2000 ampoules of fibrinogen, 3695 of thrombin and 6545 of γ-globulins. In 1954, the Paris hospitals used 54,000 ampoules of thrombin and 4615 of γ-globulins*. The organisation of the preparation of blood products has been regulated by respective Governments. In Great Britain the Therapeutic Substances Regulations of 1954[45] and in France the law of July 4, 1952 and various decrees and orders for its application[46] have laid down regulations for the collection of human blood and for the separation, fractioning, preservation, inspection and distribution of products from human blood. First importance is attached to asepsis in handling the products and to the absence of any profit motive when the blood is issued.

However, there is still a need for an effort towards accurate standardisation in the direction recently attempted by Ardry with regard to the γ-globulins and, more especially, their checking and control[20].

By degrees, monographs have appeared in the official Pharmacopœias regarding the products of blood. The 1953 B.P. mentions Concentrated Human Red Blood Corpuscles, Human Fibrinogen, Human Fibrin Foam and Human Thrombin, while the U.S.P. XV lists Thrombin, Antihemophilia Globulin and Immune Serum Globulin. A plan is under consideration for including human γ-globulins in the Codex français.

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SIZE AND SHAPE OF MACROMOLECULES IN PLASMA EXPANDERS

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Although artificial "plasma expanders" were used during the first world war (gelatin[1]; acacia[2]), it was only during the second world war that their use developed. The main problem has always been, and still is, the selection of the colloidal, macromolecular component, the rest of the solution, the small molecules and ions, presenting no major difficulties. During the last fifteen years many macromolecules of different chemical types have been used in plasma expanders but it is only since 1952 that the importance of molecular weight has been realised in determining the behaviour of a macromolecule in the animal body[3-5]. The shape, structure and electric charge of a macromolecule may modify its passage through a membrane, and since the macromolecules used in plasma expanders vary not only in molecular weight but also in shape and structure care must be taken in comparing the experimental results obtained with different plasma expanders. It is the purpose of this review to examine the present state of our knowledge of the behaviour of macromolecules in the body and draw whatever conclusions are possible.

A plasma expander is used, as an alternative to whole blood or plasma, to restore the circulating blood volume after loss of blood and then maintain it by preventing loss of water into the extravascular space. The basic concept of the control of water balance by the colloidal osmotic pressure exerted across the capillary walls, due to Starling[6], has not been changed by recent work. The suggestion that more than a very small quantity of water is held in circulation by hydration of the plasma proteins is not supported by experiment. Although there is some uncertainty about the degree of hydration of proteins, Alexander and Johnson[7] have concluded that a maximum value is 0·4 g. of water per g. of anhydrous protein, whereas experiments[8] and calculations[9] have shown that 1 g. of serum albumin increases the plasma volume by about 18 ml. The hydration of the macromolecules used in plasma expanders is probably about the same as that of proteins[10] so that the effectiveness of a particular macromolecule in a plasma expander will depend largely upon the value and persistence of the osmotic pressure which it exerts across the capillary wall. This persistence depends upon (i) the rate at which the macromolecule passes through the capillary walls, decreasing the effective colloidal osmotic pressure, and (ii) the rate of its elimination from the body, either by excretion of unchanged polymer or by its degradation to small molecules which do not exert any colloidal osmotic pressure. Also of importance is the interaction of the macromolecules with tissue cells and naturally occurring molecules in the body, possibly resulting in storage of the macromolecule and impairment of normal physiological functions.

No attempt has been made to give a complete bibliography of plasma expanders. Reference will be made only to those publications bearing on the effect of size and shape of the solute particle on the functioning of the macromolecular weight component. There is a considerable "twilight" literature on the subject of plasma expanders in the reports of the U.S. Government Scientists, which are not readily available outside the U.S.A. Since references in the normal scientific literature (e.g., reference 11) to these reports show that they contain valuable information, they are referred to in this review, although the author has not read all these documents.

The macromolecules which have been used in plasma expanders are given in Table I. Most of the work of interest to this review has been carried out with dextran, polyvidone and, to a lesser extent, gelatin preparations. No reference will be made to the use of rutin[12], a low molecular weight flavone glucoside, since its mechanism of action, by decreasing the permeability of the capillary wall, is quite different from all the macromolecules of Table I.

TABLE I

MACROMOLECULES WHICH HAVE BEEN USED IN PLASMA EXPANDERS

Chemical type of macromolecule	Molecular weights used in commercial preparations		
	Average*	Range†	References
Proteins			
Casein digests			
Gelatin preparations:			
Fluid gelatin type (*a*)	$M_n = 37,000$	4% less than 10,000 M.W.	23
,, ,, (*b*)	$M_n = 50,000$	—	
Isinglass			
Oxypolygelatin	$M_w = 31,200$	35% < 10,000; 6% > 100,000	23
Globin			
Hæmoglobin			
Plasma proteins in ascitic fluid			
Polysaccharides			
Acacia			
Dextran: American type	$M_n = 42,000$	0–160,000	15
British type	—	0–450,000	16
Swedish type	$M_n = 34,000$	—	11
Methyl cellulose			
Pectin			
Synthetic polymers			
Polyvinylalcohol			
Polyvinylpyrrolidone (Polyvidone):			
American type (*a*)	$M_w = 56,000$	—	11
,, ,, (*b*)	$M_w = 33,000$	—	11
British type	—	5% < 10,000; 3% > 100,000	22
German type	$M_w = 25,000$	—	84

* M_n, number average molecular weight.
M_w, weight average molecular weight.
† The range of molecular weights has been estimated wherever possible from published information.

SIZE AND SHAPE OF MACROMOLECULES IN SOLUTION

Most of the macromolecules in Table I are not homogeneous in molecular weight, and, in order to understand the physiological action of a particular sample, it is necessary to know the distribution of molecular weights as well as the average value. These measurements will also be of value in the quality control of commercially produced plasma expanders.

Standard methods of molecular weight determination have been used and, for details, reference should be made either to some general textbook[7] or to the original papers referred to in Table II, which gives a selected bibliography of molecular weight determinations of the macromolecules used in plasma expanders. Apart from the absolute methods, the experimentally simpler determination of intrinsic viscosity is of value provided the relationship between intrinsic viscosity and molecular weight has been determined for each chemical type of macromolecule.

TABLE II

BIBLIOGRAPHY OF METHODS OF MOLECULAR WEIGHT DETERMINATION

Method	Dextran	Gelatin Preparations	Polyvidone
Absolute Methods			
Light scattering	85, 112	18	13, 25
Osmotic pressure	16	11	13, 25
Sedimentation rate	86–89	18	33
Chemical methods	90	11	
Method Requiring Calibration			
Viscosity	86, 112	91, 92	13, 25, 33

Frank and Levy[13] have, however, found that such a relationship for polyvidone is somewhat dependant upon the molecular weight range of the sample being measured. It may be that the viscosity relationships reported for other macromolecules would, on detailed experimental examination, suffer from the same defect.

Molecular weight distribution determinations appear to have been reported for only dextran, gelatin and polyvidone. Three methods have been used:

(1) The molecular weight distributions of polyvidone samples[10] and of dextran samples[14,15] have been calculated from the concentration variation of sedimentation rate.

(2) Samples of macromolecules have been divided by fractional precipitation into a series of fractions, the molecular weights of which have then been determined. This method has been applied to dextran[16] and to polyvidone[17]. Methods of fractionating gelatin have been described by Gouinlock and others[18] but were not used to determine molecular weight distributions.

(3) A variation of this last method, in which fractions are not actually isolated, is based on the turbidimetric method of Morey and Tamblyn[19]. In this method the weight of the macromolecule precipitated by a particular solvent-precipitant mixture is determined turbidimetrically, the molecular weight of the precipitate being a function of the solvent-precipitant ratio. This method has been applied to dextran[20,21] and to polyvidone[22]. Since very small samples are required this method has been used to determine the molecular weight distribution of dextran[21] and polyvidone[5] isolated from urine.

Fuhlbrigge and others[23] have determined the molecular weight distributions of various preparations of gelatin and oxypolygelatin but the methods used are not reported. The ranges of molecular weights in

some macromolecules used in commercially made plasma expanders is given in Table I.

The macromolecule in solution is hydrated and moves around with its shell of tightly bound water. Very little is known about the structure of this hydration shell for the macromolecules considered in this review but the hydration may be nearly half the dry weight of the macromolecule. The shape of the solute particle, depending very largely on the chemical type of the macromolecule, can be either a compact ellipsoid, a rigid, extended, rod-like molecule or a more or less flexible, randomly-coiled, chain-like molecule. Most of the proteins referred to in Table III are ellipsoids, held together by intra-molecular hydrogen bonding and their shapes are generally indicated by the axial ratios of the ellipsoids. Dextran [24] and polyvidone [25] have now been shown to be more or less flexible molecules, and, although axial ratios can be calculated for them, these can be very misleading since they represent a statistical mean shape of the solute particle and do not take into consideration the variations in orientation of the repeating units of the chain about the mean shape, which are continuously taking place in the solution[26]. Recently, it has been shown that gelatin in solution is a randomly-coiled molecule rather than a compact particle or a rigid rod.[18] This implies that there can be very little intra-molecular hydrogen bonding in the gelatin molecule.

Another property which may be of importance in determining the physiological action of a macromolecule is the charge on it in solution. Dextran has been shown to have no charge[27] and polyvidone is generally stated to have a very small negative charge, equivalent to about one carboxyl group per molecule[28]. Every protein molecule bears a charge depending upon its isoelectric point and the pH of the solution.

The Osmotic Pressure of Solutions of Macromolecules

The colloidal osmotic pressure of human blood is 33 cm. of water (normal range 28 to 48 cm.)[29], about one third[9] of this being due to the Donnan effect as a result of the electric charge on the proteins. Serum albumin, possessing a lower molecular weight than the rest of the plasma proteins, is normally responsible for about three-quarters of the colloidal osmotic pressure.

If it is assumed that the macromolecule has a single molecular weight, M, possesses no electric charge and behaves ideally in solution, the osmotic pressure, in cm. of water, π of a solution (concentration, c g./ml.) at absolute temperature, T, is

$$\pi c = 8{\cdot}48 \times 10^4 \times T/M.$$

The relation between molecular weight and the concentration required to give an osmotic pressure of 33 cm. is given in Figure 1 and indicates that the higher the molecular weight the greater is the concentration of the macromolecules required in a plasma expander. In practice this simple ideal model, in which there are no interactions between molecules, will not hold. In the plasma expander solution, before it is diluted by blood during an infusion, interactions between macromolecules will result in a

higher osmotic pressure, for a given concentration, than is given by Figure 1. The dilution of the plasma expander normally likely to occur during an infusion (e.g., 500 ml. to 2800 ml., an average plasma volume) will reduce the concentration of macromolecule and with it the effect on osmotic pressure of the above type of interaction. After infusion, interactions between the macromolecules and proteins become important but very little experimental data is available on the osmotic pressure of mixed solutions of proteins and macromolecules. Wales and others[30] studying dextran-albumin mixtures and Rowe[31] with dextran-serum mixtures have shown that the osmotic pressure is greater than would be expected from the protein and dextran concentrations, indicating that protein-macromolecule interactions are important.

The macromolecules generally used in plasma expanders are not homogeneous in molecular weight. Apart from the corrections due to intermolecular interaction already discussed, the total osmotic pressure exerted by the macromolecule, as measured by a perfect semi-permeable membrane, will depend upon the number average molecular weight and will be greater than the effective colloidal osmotic pressure exerted in the body across the capillary wall as a semi-permeable membrane, since the low molecular weight molecules will either pass rapidly through the capillary walls, or else be rapidly excreted. Consequently, this effective osmotic pressure, which has never been measured for any plasma substitute, will depend markedly upon the molecular weight distribution of the macromolecule used. It is important, therefore, once the effectiveness of a particular grade of macromolecule has been established, to ensure that all subsequent batches will have the same distribution of molecular weights.

It is the author's opinion, for the reasons already given, that the colloidal osmotic pressure of a plasma expander measured in an osmometer is very nearly meaningless in indicating its effectiveness. Since it is impossible to eliminate all small molecules from commercially prepared samples of macromolecules, careful consideration should be given to the possibility of increasing the effectiveness of a plasma expander by increasing the concentration of the macromolecule to such an extent that, after loss of the small molecules from the blood, the remaining macromolecules would exert an osmotic pressure comparable to that of the normal plasma proteins. Provided the osmotic pressure of the small molecules was small compared with that of isotonic saline, there should be no danger of crenation.

The two macromolecules most widely used in plasma expanders, dextran and polyvidone, do not receive any contribution to their osmotic pressure from the Donnan effect since there is only an extremely small electric charge on them[27,28]. Scatchard[3] has suggested that the ideal macromolecule for a plasma expander would have, under physiological conditions, a similar charge to serum albumin. Such a charge would reduce the concentration of polymer required to produce a given osmotic pressure, and, hence, would result in a solution of lower viscosity, a factor discussed immediately below. The charge on a molecule might also be

important in controlling the rate at which the macromolecule passed through the glomerular membrane or the capillary walls. With the exception of amino-acid polymers, no attempt is being made to develop ionised synthetic macromolecules for use in plasma expanders. The various gelatin preparations have, of course, charged molecules.

The Viscosity of Solutions of Macromolecules

The viscosity of aqueous solutions of macromolecules increases with increasing molecular weight, the exact form of the relationship depending upon the chemical type of the macromolecule. The relative viscosity of solutions of concentration given by the osmotic pressure curve of Figure 1 have been included in that figure and have been calculated assuming that the macromolecule is polyvidone. The viscosity-concentration relationship due to Fikentscher[32] and the viscosity-molecular weight relation due to Scholtan[33] have been used. It is evident, therefore, that increasing the molecular weight of the colloidal component will result in a higher relative viscosity partly due to the increased size of the solute molecule and partly to the increased concentration necessary to give the required osmotic pressure. This conclusion, although qualitatively correct, will be incorrect quantitatively since, as in the discussion of osmotic pressure, no allowance has been made for the range of molecular weights normally present in a macromolecule sample.

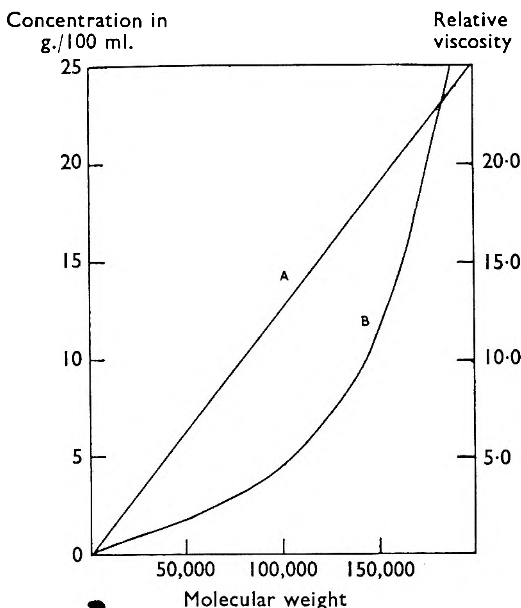

Fig. 1. Curve A. Relation between molecular weight of solute and the concentration required to give an osmotic pressure of 33 cm. The assumptions made are given in the text.

Curve B. The relative viscosity (calculated as described in text) of the solutions defined by curve A.

It is desirable that the relative viscosity of a plasma expander should not be so much greater than that of true plasma (1·8 at 38° C.[29]) as to throw undue strain on the heart. Dilution of the plasma expander during infusion will undoubtedly reduce its relative viscosity but there does not appear to have been any authoritative statement about the maximum permissible relative viscosity. Plasma expanders containing polyvidone have a relative viscosity only slightly above that of true plasma (e.g., Plasmosan; relative viscosity = 2·1) whereas those based

on dextran have a considerably higher viscosity (e.g., the solution accepted by British hospitals must have a relative viscosity between 3·0 and 6·0 at 37° C.[16]).

ELIMINATION OF MACROMOLECULES FROM THE BODY

The elimination of macromolecules from the circulating blood is of importance in connection with the duration of action of a plasma expander and with the possibility of storage in the body. The more rapid the elimination, the shorter will be the time during which an effective colloidal osmotic pressure is exerted. Absence of a mechanism of elimination must result in storage in the body.

Some macromolecules, such as dextran[34], can be completely metabolised in the body to low molecular weight molecules (in the case of dextran, to sugars and ultimately carbon dioxide), which do not exert an effective colloidal osmotic pressure and can be rapidly excreted by the kidney. It will be shown that, for each chemical type of macromolecule, there is probably a threshold molecular weight above which it is excreted by the kidney only very slowly, and, consequently, only macromolecules which are completely metabolised will be eliminated rapidly when their molecular weight is greater than this threshold value. No information has been published on the effect of molecular weight on the rate of metabolism of dextran. Of the other macromolecules used in plasma expanders, there is no agreement about the metabolism of gelatin[35,36] and the limited degradation of polyvidone, postulated by Campbell and colleagues, can but assist the renal excretion of this macromolecule[5,37].

The main route for elimination of macromolecules from the body is by excretion by the kidney. Most work on the permeability of the normal kidney nephron to large molecules has been concerned with the passage of proteins from the plasma into the urine and is summarised in Table III. There are undoubtedly difficulties in comparing these results. The different animals used may have nephrons of different permeabilities. The results with hæmoglobin may be anomalous since it has been suggested that it alters the nephron permeability[39]. The proteins used may form complexes with the plasma proteins, as has been shown to be the case with lysozyme[40]. It is broadly apparent, however, that protein molecules with a molecular weight greater than about 68,000, the molecular weight of hæmoglobin, do not pass through the normal nephron. A more detailed study indicates that other factors, which may be molecular shape and electrical charge, are significant in determining the renal excretion of macromolecules but no definite correlations are evident. Two exceptions to the simple molecular weight rule are albumin and fetuin. Albumin, the molecular weight of which is now believed to be 65,000 instead of 69,000[41], does not pass through the human nephron, whereas hæmoglobin, of molecular weight 68,000, does. Fetuin of molecular weight 51,000 is not excreted in the urine of rabbits whereas hæmoglobin with a greater molecular weight is[38]. Brant and others in a study of the renal clearance of hæmoglobin in normal and proteinuric

TABLE III

RENAL EXCRETION OF PROTEINS BY NORMAL KIDNEYS (*a*)

Protein injected into plasma	Renal excretion: Animals used	Renal excretion: Present (+) or absent (−) in urine	Molecular weight	Isoelectric (*f*) point	References: Renal excretion	References: Molecular weight	References: Isoelectric point
Lysozyme	Dog	+	15,000	10·5 (0·1)	40	97	104
Myoglobin	Dog	+	17,000	7·0	43	97	101
Ovomucoid	Dog	+	28,000	3·9 (0·1)	40	97	105
Globin	Rat	−	?34,000 or 68,000 (*c*)	7·5 (0·1)	96	103	104
Bence Jones	Dog, cat, rabbit	+	35,000	5·2 (0·02)	94	7	7
Fetuin	Man Rabbit, calf	+ −	35,000		38 38	98	
Gelatin	Dog, cat, rabbit	+	35,000 (*d*)		94	94	
β-lactoglobulin	Dog	+	37,300	5·1 (0·1)	40	97	104
Ovalbumin	Dog, rabbit, calf, rat	+	47,000	4·6 (0·1)	40, 94, 96, 93	97	104
Serum albumin	Man, dog, cat, rabbit, rat	−	65,000	4·7 (0·1)	94, 96, 93	41	104
Hæmoglobin	Man, dog, cat, rabbit	+ (*b*)	68,000	7·0	43, 94, 39, 95	101	101
Conalbumin	Dog	−	87,000	6·8 (0·1)	40	97	105
Casein	Dog, cat, rabbit	−	75,000–100,000	4·0–4·5 (0·1)	94	99	104
Serum globulin	Man, dog, cat, rabbit	−	176,000	γ = 5·8 (0·1)	94	7	104
Edestin	Dog, cat, rabbit	−	c.a. 350,000	5·5–6·0	94	100	97
Hæmocyanin helix	Dog, cat, rabbit	−	1,000,000 (*e*)	4·7	94	101	101

NOTES:

(*a*) The work of Bott and Richards[93] on amphibia has not been included in this table.

(*b*) Appears in the urine only when the plasma concentration is greater than 0·1 g.100 ml.[95].

(*c*) The literature (e.g., reference 102) on the use of globin in plasma expanders gives the molecular weight as 34,000. Since it has been shown that globin dimerises very readily, the absence of globin in the urine may indicate that the higher molecular weight of 68,000 is correct.

(*d*) Stated to include breakdown products of about 1,000 molecular weight.

(*e*) There appears to be doubt about this molecular weight since it varies with the concentration of the solution used in the measurement. Hæmocyanin may reversibly dissociate into smaller molecules.

(*f*) The ionic strength of the solution used for the measurements is given in parentheses wherever known.

human subjects concluded that the nephron is more permeable to molecules with large electric charges[42]. Measurements of the actual rates of passage of proteins through the nephron[40,43] indicate that the sharp cut-off in renal excretion at a molecular weight of about 68,000 is not real, there being a gradual decrease in excretion rate with increasing molecular weight until no detectable excretion is observed at molecular weights greater than 68,000.

Many experiments have shown that a limited re-absorption of proteins, whatever their nature, takes place in the kidney tubules, different proteins competing with each other to saturate the tubular re-absorption[44,46]. It is now believed that the absence of higher molecular weight proteins in the urine is not due to the impermeability of the glomerular membrane to these proteins but to the passage of only small quantities of them through the glomerular membrane and their subsequent reabsorption in the tubules. The evidence for this belief, based on the appearance of proteinuria when the homologous plasma protein concentration is raised above normal[47] and on other experiments, has been reviewed by Rather[48]. The evidence from protein studies is, therefore, that the glomerular membrane is permeable to all proteins, the rate of penetration decreasing, but never becoming zero, with increasing molecular weight and changing, to a lesser extent, with molecular shape and electric charge.

The very little evidence available suggests that the renal excretion of macromolecules other than protein involves simple glomerular filtration and that tubular reabsorption does not occur. Although transient deposits of dextran have been found in the tubule cells[49], Wallenius[21] has concluded, from renal clearance experiments with low molecular weight dextran, that there is no tubular re-absorption of dextran, a very small quantity of low molecular weight material merely diffusing by a purely physical mechanism, into the tubule and other cells of the nephron. Campbell and others[5] have shown that polyvidone of high molecular weight is excreted into the urine of human subjects in very low concentrations some 300 hours after an infusion. Such a low concentration would appear to be unlikely if a tubular reabsorption mechanism existed for all molecular weights of polyvidone.

The inverse relation between renal excretion rate and molecular weight has been demonstrated, by measuring the renal excretion of fractions of limited molecular weight range, for dextran[20,21,50–54], polyvidone[55,56], gelatin[57,58,113] and polyethylene glycols[59]. The results of Campbell and others[5] (see Fig. 2), who studied the variation in molecular weight distribution of polyvidone excreted by human subjects, with time after an infusion, support this relationship. Early work on dextran[20,52,53] had suggested that there might be a threshold molecular weight of about 40,000 above which it was not excreted but it seems possible that higher molecular weight dextrans have not been detected in urine owing to lack of a sufficiently sensitive analytical method. The results of Wallenius[21] (see Fig. 3) on dextran clearance rates support this conclusion, the asymptotic approach to the molecular weight axis being similar to the curves obtained with proteins. Campbell and others[5] have shown by

isolating the macromolecule from urine that polyvidone molecules with molecular weights greater than 100,000 are slowly excreted by the kidney, the rate of excretion becoming slow at a molecular weight of about 50,000.

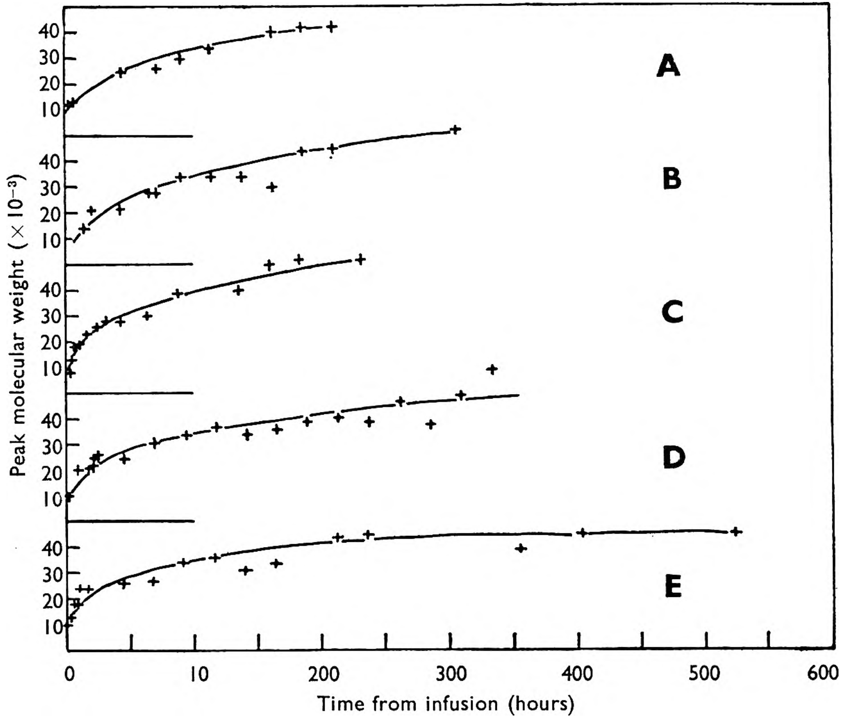

FIG. 2. Increase in peak molecular weight of polyvidone excreted by human subjects with time after an infusion (reproduced with permission from Campbell and colleagues[22]).

This latter molecular weight is supported by Warner's[60] conclusion that there is a sharp cut-off of renal excretion of polyvidone between 35,000 and 40,000 molecular weight. A more sensitive method of analysis would presumably have shown the slow excretion of higher molecular weight polyvidone. Although no actual measurements have been published of the variation of renal clearance rate of polyvidone with molecular weight, it is reasonable to assume a similar relation to that for dextran (Fig. 3).

The renal excretion studies of large molecules reported above are concerned with the normal kidney. It is well established that kidney disease can result in an increase in the glomerular permeability to macromolecules. Starling and Verney[61] have shown that oxygen lack can result in an increase in the permeability of the glomerulus to proteins. Under those clinical conditions of shock necessitating an infusion of a plasma expender, oxygen lack might well be the cause of impairment of the renal circulation so that the glomerulus might pass large molecules

more rapidly than normally. Wallenius[21] has obtained evidence of this with dextran since he detected in the urine molecules of molecular weight 57,000 for two cases of second degree burns whereas normally the maximum molecular weight detected was 46,000. Consequently, the conclusions drawn from the studies of normal renal excretion may be correct qualitatively but may be incorrect quantitatively as a result of increased glomerular permeability in cases of shock. It is, however, evident that for solute particles, size is the most important factor governing the rate of excretion, although shape and electric charge may be important. The glomerular clearance rate becomes very small with increasing molecular weight but there is no experimental evidence suggesting that it actually becomes zero so that, given sufficient time, all macromolecules circulating in the blood could pass through the glomeruli, and, in the absence of tubular reabsorption, appear in the urine. The reviewer believes that the slow excretion of very large chain-like molecules, such as dextran and polyvidone, may be the result of variations in orientation of the repeating units of the chain in solution resulting in changes in the shape of the solute particle from the statistical mean value which is normally measured. Such changes in shape could not, of course, occur with the plasma proteins without rupture of intra-molecular hydrogen bonds and, hence, denaturation.

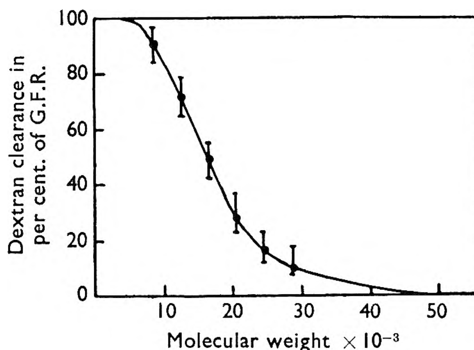

FIG. 3. The renal clearance of dextran (in per cent. of the glomerular filtration rate) and its relation to the molecular weight of the dextran molecules, in normal dogs (after Wallenius[21]).

The bearing of these results on the exact mechanism whereby macromolecules are transported across the glomerular membrane is not discussed here. Wallenius[21] has stated that it is not yet possible to determine which mechanism is correct, restricted diffusion through microscopic pores[62] or diffusion through the gel-like membranes postulated by Chinard[63].

PASSAGE OF MACROMOLECULES INTO THE LYMPH

The more rapidly do the macromolecules of a plasma expander leave the circulating blood and pass into the lymph, the more rapid is the decrease in colloidal osmotic pressure and, hence, in the effectiveness of the plasma expander. The permeability of the capillary wall to macromolecules has not been studied as fully as has that of the glomerular membrane but qualitatively one would expect similar laws to hold in both cases. Quantitatively, however, there might be very large differences. Forker and others[64], using radiotracer methods, have shown that exchange of plasma proteins between blood and lymph is complete in less

than one hour but no measurements were made on the variation of the rate with molecular weight. Grotte and others[54], using fractions of Swedish dextran of different molecular weights, have shown that molecules with molecular weights as great as 205,000 pass into the lymph, the rate decreasing with increasing molecular weight. More recently Martin[16] has reported experiments (Fig. 4) which show that diffusion into the lymph can be reduced by use of higher molecular weight dextran. Martin[16] and Wasserman and Mayerson[65] have shown that dextran below about 40,000 molecular weight passes rapidly into the lymph. No similar data are available for polyvidone, but, from the work of Fresen and Weese[66] who made a histological examination of lymph nodes and other tissues after infusions of polyvidone, it can be inferred that molecules with molecular weights as great as 400,000 pass into the lymph and that the rate decreases with increasing molecular weight. Little and Wells[67] have shown that molecules of gelatin pass through the capillary wall less readily than do plasma proteins.

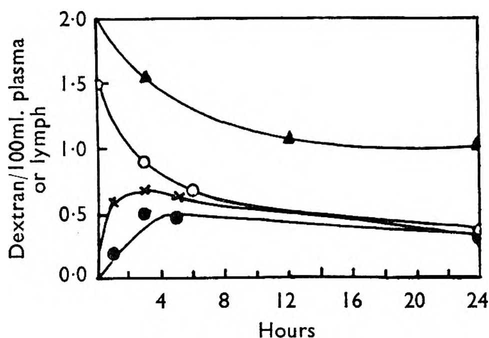

FIG. 4. Rate of decay of dextran fractions A and D from the plasma and their diffusion into the lymph (after Martin[16]). Dose 20 ml./kg. intravenous. Average of normal rabbits.

	Weight average mol. wt.
Fraction A. O—O, plasma, ×—×, lymph	45,000
Fraction D. ▲—▲, plasma, ●—●, lymph	120,000

VARIOUS INTERACTIONS WITH MACROMOLECULES

It is essential that any compound used in a plasma expander should not interfere with the normal functioning of the body. It is well established that macromolecules sometimes form complexes with other macro molecules, such as proteins, and with ions. In most cases no formal chemical bonds are formed, the complex being held together by electrostatic or van der Waals' forces. The present discussion has been limited to those interactions of macromolecules in which the effect of molecular weight is known. For example, the vast literature on the use of polyvidone as a drug delaying agent or as a detoxifying agent will not be referred to since no proper examination of the effect of molecular weight has been made.

It is well established that macromolecules foreign to the blood interact with erythrocytes producing an increase in their sedimentation rate (dextran[68–70,111]; gelatin[69]; oxypolygelatin[71]; polyvidone[69,70,72]) and, in sufficiently high macromolecule concentration, aggregation of the cells which clinically may produce some anæmia[73]. The most detailed work

on the influence of molecular weight has been carried out by Thorsen and Hint[69] who showed that the erythrocyte sedimentation rate increases with increasing molecular weight with dextran and gelatin, and that the minimum molecular weight which will produce aggregation is 59,000 for Swedish dextran and 18,000 for gelatin. These authors also proved that the presence of low molecular weight dextran would prevent, or reduce, the effect of higher molecular weight dextran. Hummel and Hamburger[74] suggest that the minimum molecular weight for aggregation is about 35,000 for polyvidone. In clinical practice, no bad effects have been reported with the grades of dextran, gelatin and polyvidone at present used in plasma expanders.

There is evidence that the antigenicity of dextran depends upon molecular weight. Dextrans of various origin have been shown to be antigenic in man[75] and recently Glynn and others[76] have shown that dextran of high molecular weight has a greater antibody response than has low. Dextran molecules of molecular weight about 5000 were non-antigenic but produced a precipitate with antisera. Smaller molecules acted as non-precipitating haptens. Similar antigenic reactions have not been reported for gelatin preparations or for polyvidone.

Although it has been established that polyvidone and dextran do not interact sufficiently strongly with the plasma proteins to interfere with their electrophoresis[77], it has been proved[78] that polyvidone, acacia and all dextrans except the lowest molecule weights all form insoluble complexes at 4° C. with fibrinogen. At 37° C. no insoluble complexes were formed. No ill effects due to the formation of fibrinogen complexes have been reported in the use of plasma expanders, but a fuller study of this problem seems desirable.

The storage in the body of the macromolecular component of a plasma expander is of importance as a possible source of interference with normal physiological functions although such interference has never been found in cases where storage of macromolecules has been reported. Experiments involving massive doses of the macromolecule can be disregarded since these conditions of high concentration are never likely to be met in normal clinical use of plasma expanders. Stenger and Müller[79] have concluded that the ability of the body to store macromolecules is mainly of academic interest. Storage in the tissue of a macromolecule might well depend upon molecular weight since the deposition of a water soluble molecule in tissue will require the formation of some kind of complex with the tissue molecules. It has been shown by many authors[53,80] that only temporary storage of dextran occurs, mainly in the liver, spleen, kidney and lymph nodes. It would appear that no strong complex formation is associated with the storage of dextran and that its temporary presence in various cells may be due to a mere physical diffusion into them[21]. If this is correct it would appear that increase in the molecular weight of the dextran would reduce the quantity temporarily stored in the cells. The evidence about the storage of polyvidone is conflicting[37,72,79,81,72] and may partly be due to the use of different grades of the polymer since it has been demonstrated[64,83] that increase in molecule

weight increases the possibility of storage. The reviewer believes that a careful, critical examination of the methods used for the detection of polyvidone in tissue cells is necessary before this problem can be finally solved. No information has been published on the question of storage of gelatin preparations.

The Ideal Macromolecule for a Plasma Expander

In this review an attempt has been made to show the importance of solute particle size and shape in governing the behaviour of macromolecules in the body. Although many more experimental studies would be necessary to complete our knowledge of this subject, it should be possible, at least in principle, to determine the essential properties of the ideal macromolecule for a plasma expander, and, hence, to select both the chemical type and molecular weight range most suitable. When the ideal macromolecule has been finally designed, economic manufacture of it might well prove impossible so that some compromise would be necessary.

The major difficulty in the selection of the macromolecule is the determination of the duration of effectiveness required for a plasma expander. It would appear ideal for the macromolecule to remain in the circulating blood and not to pass into lymph, thereby reducing the osmotic pressure. It would then be eliminated at such a rate that the colloidal osmotic pressure decreased as the osmotic pressure due to the plasma proteins returned to normal. Although it is obvious that an exact balance between the osmotic pressures of the added macromolecules and of the plasma proteins would be an impossibility, an approximation might be arrived at from a knowledge of the time taken for the regeneration of plasma proteins. Unfortunately, most published information on regeneration times in human subjects is for cases of malnutrition. Wallace and Sharpey-Schafer[106] have shown that in human subjects not suffering from shock, regeneration of the plasma proteins is virtually complete in 3 to 90 hours, but no similar studies have been made on human subjects who are in a severely shocked condition through loss of blood. Ebert and colleagues[107] have shown that in dogs, severely shocked after considerable loss of blood which was replaced by an equal volume of isotonic saline, regeneration of proteins was extremely slow. More recently Semple[108], also working with dogs, replaced 45 per cent. of the blood with a dextran solution, and found that protein regeneration was fairly rapid. This last author concluded that dextran did not retard protein regeneration but there does not seem to be any evidence whether the addition of a macromolecule actually assists regeneration. Kozoll and others[109] (pectin), Feigan and others[110] (oxypolygelatin) and Gropper[11] (dextran) have obtained evidence that these macromolecules may actually stimulate the appearance of proteins from some store in the body, thereby producing a greater effect in maintaining plasma volume than can be explained simply by the osmotic pressure produced by the infusion. If this should be proved correct, the fundamental approach to the development of a plasma expander becomes even more difficult and experimental studies

would be required on the effect of molecular weight of different macromolecules on protein regeneration. It must be admitted that, at the present time, our basic knowledge is quite inadequate for an attempt to be made to develop a plasma expander by the suggested *a priori* method.

An alternative approach, and the one actually used in practice, is to determine the clinical effectiveness of different plasma expanders containing macromolecules of different chemical type and molecular weight range. Another difficulty arises, the differences between the requirements of various users which result in differences of medical opinion. Some require a fairly short acting plasma expander for use in cases of hæmorrhagic shock where there has been a single loss of blood[81] whereas others require a longer acting plasma expander which can be used in cases of severe burns, where there is a prolonged loss of fluid from the burn surface[50]. This review is not the place to discuss this question but it appears to the reviewer that the different plasma expanders available to-day may each be of value in the particular application most suited to it.

Our knowledge of the behaviour of macromolecules in the body is very incomplete, and, even when further experimental studies have completed this knowledge, the ideal macromolecule for use in a plasma expander would probably have properties very similar to those of serum albumin, normally responsible for the greater part of the control of fluid balance. This would suggest that the use of serum albumin itself might simplify the development of plasma expanders, provided that it could be obtained in large quantities in a form suitable for long-term storage. The present developments in large-scale fractionation of plasma proteins and in the preparation of despecified sera may be an indication that, in the future, artificial molecules such as dextran, oxypolygelatin and polyvidone, may be replaced by serum albumin in plasma expanders.

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RESEARCH PAPERS

SALTS OF DECAMETHYLENE-BIS-4-AMINOQUINALDINIUM ("DEQUADIN")*, A NEW ANTIMICROBIAL AGENT

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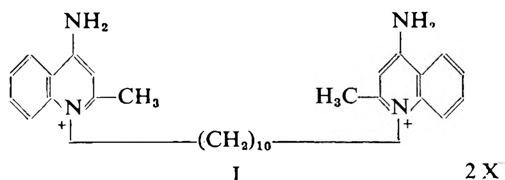
THE presence of high antimicrobial activity in polymethylene bis-*iso*-quinolinium (BIQ) salts[1] led to the synthesis and examination of a large number of related compounds, including the corresponding bis-quinolinium (BQ) salts[2] and many derivatives of both these series. Among these, bis-4-aminoquinaldinium (BAQD) salts showed broad activity and relatively low toxicity and the decamethylene member (I, BAQD 10, Dequadin) was selected for more extensive biological investigation



MATERIALS AND METHODS

Compounds. The iodide, chloride, nitrate and acetate of Dequadin were used, their approximate solubilities in water at 25° C. being: iodide, 1 in 3500; chloride, 1 in 200; nitrate, 1 in 400; acetate, 3 in 4. Other members of the BAQD series (see Table I) were iodides. Methods of preparation and chemical properties of this series will be described elsewhere. The synthesis of the BIQ and BQ iodides (Table I) has already been described[1,2]. The following salts of other antibacterial agents were used: aureomycin hydrochloride, benzalkonium chloride, potassium benzylpenicillin, chlorhexidine acetate, domiphen bromide and streptomycin sulphate. Substances may be referred to below by the name of the active acid or base, but weights given are those of the salt. In most tests cetrimide was used for reference.

Strains in vitro. The microbial species used and the code numbers of some strains are shown in Table II. Both strains of *Proteus vulgaris* (L.H. 13 and 14) and the 12 penicillin-resistant strains of *Staphylococcus aureus* (L.H. 1 to 12) were recently isolated at the London Hospital. Strains not numbered in Table II had the following origins: staphylococci, streptococci and salmonellas were isolated from domestic animals at the Royal Veterinary College, London, or the Agricultural Research Council

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TABLE I

ACTIVITIES OF POLYMETHYLENE-BIS-*iso*QUINOLINIUM (BIQ), QUINOLINIUM (BQ) AND -4-AMINOQUINALDINIUM (BAQD) IODIDES AGAINST FOUR BACTERIAL SPECIES IN PEPTONE WATER. READINGS AFTER 5 DAYS AT 37° C.

Series and number of carbons in chain	Geometric mean M.I.C. in µg./ml.			
	Staph. aureus (CN491)	*Myco. phlei* (NCTC.525)	*P. vulgaris* (L.H.14)	*Ps. pyocyanea* (NCTC.8203)
BIQ 10	50	100	>100	>100
BIQ 11	50	20·4	>100	>100
BIQ 12	12·5	3·13	>100	>100
BIQ 13	5·00	2·50	>100	>100
BIQ 14	0·77	0·70	>100	>100
BIQ 15	0·44	0·63	>100	>100
BIQ 16	0·12	0·88	100 – >100	>100
BIQ 17	0·20	0·99	50	>100
BIQ 18	0·22	1·10	25	>100
BIQ 19	0·16	1·40	8·89	100 – >100
BIQ 20	0·49	3·13	6·25	100 – >100
BQ 10	17·7	50	>100	>100
BQ 11	12·5	25	>100	>100
BQ 12	3·97	4·42	>100	>100
BQ 13	2·50	3·54	>100	>100
BQ 14	0·38	0·78	>100	>100
BQ 16	0·18	0·88	100	>100
BQ 18	0·44	1·10	19·6	100
BQ 20	0·63	3·13	12·5	70·7
BAQD 8	0·67	1·97	>100	100 – >100
BAQD 10	0·35	1·66	63·0	67·3
BAQD 12	0·35	0·88	35·4	70·7
BAQD 14	0·39	0·79	35·4	84·1
BAQD 16	0·67	1·69	70·7	>100
BAQD 18	0·99	3·95	70·7	>100
BAQD 20	2·48	70·7	>100	>100

Field Station, Compton; *Actinomyces dermatonomus* was isolated from sheep at the Animal Diseases Research Association, Edinburgh; fungi were from St. John's Hospital for Diseases of the Skin, except *Trichophyton verrucosum* which was isolated at the London School of Hygiene and Tropical Medicine; *Bacterium coli* and *Bacillus subtilis* were isolated at Ware.

Growth inhibition in vitro. Tube dilution tests were performed in a medium of 1 per cent. peptone (Difco) in water containing 0·5 per cent. dextrose and 0·5 per cent. sodium chloride, adjusted to pH 7·2. For *Actinomyces dermatonomus* 1 per cent. beef extract (Lab-Lemco) was added, for *Streptococcus pyogenes* CN10, 10 per cent. horse serum was added after autoclaving and *Mycobacterium tuberculosis* was grown in Dubos' medium with 4 per cent. bovine albumin. Drugs were serially diluted 1 to 2 and, after autoclaving, inoculated with suspensions of bacteria, adjusted to give approximately 50,000 organisms/ml. test medium. With the exception of *Myco. tuberculosis*, which was grown for 7 days, 18 hour cultures of bacteria were used for preparing inocula. After incubation at 37° C., growth was read by eye at 24 hours and 5 days, except for *Myco. tuberculosis*, which was read at 14 and 28 days. Results were expressed as the minimal inhibitory concentration (M.I.C.). For detailed studies of growth inhibition by Dequadin, *Staph. aureus* CN491 was used.

Plate tests were used for certain nutritionally exacting organisms. *Neisseria catarrhalis* and *Str. pneumoniæ* were grown on 10 per cent.

horse blood agar and *Hæmophilis influenzæ* on 10 per cent. boiled rabbit blood agar. Drugs were incorporated in the plates, whose surfaces were sown with approximately 10^8 organisms per plate. Methods of antifungal test were similar to those previously described[2].

Bactericidal action. 5 ml. quantities of nutrient agar in 50 ml. conical flasks were seeded with 0·5 ml. of an 18 hour dextrose peptone water culture of *Staph. aureus* CN491 and incubated overnight at 37° C.

TABLE II

INHIBITORY ACTIVITY OF DEQUADIN IODIDE *in vitro*. IN BRACKETS, NUMBER OF STRAINS

Microbial species	Strain No.	Geometric mean M.I.C. in μg./ml. at			
		24 hours	5 days	14 days	28 days
Actinomyces dermatonomus	—	0·63	0·63	—	—
Bacterium coli	—	6·87	11·9	—	—
Bacillus subtilis	—	2·50	2·50	—	—
Corynebacterium diphtheriæ	NCTC 3989	0·31	0·35	—	—
Hæmophilus influenzæ	NCTC 8468	17·7	25·0	—	—
Mycobacterium phlei	NCTC 525	1·54	1·66	—	—
Myco. tuberculosis	H37	—	—	2·14	2·69
Neisseria catarrhalis	NCTC 4103	1·25	2·5	—	—
Pseudomonas pyocyanea	NCTC 8203	41·0	67·3	—	—
Proteus vulgaris (2)	L.H. 13 & 14	50·0–59·5	63·0–70·7	—	—
Salmonella dublin	—	25·0	50·0	—	—
Salm. typhi	NCTC 5758	2·70	6·80	—	—
Salm. typhimurium	—	25·0	50·0	—	—
Staphylococcus aureus	CN 491	0·32	0·35	—	—
Staph. aureus (2)	—	0·63	0·63–0·88	—	—
Staph. aureus, penicillin-resistant (12)	L.H.1 to 12	0·16–0·63	0·31–0·63	—	—
Streptococcus agalactiæ	—	0·63	0·88	—	—
Str. dysgalactiæ (2)	—	0·44–1·25	0·63–1·25	—	—
Str. fæcalis	850	1·66	2·79	—	—
Str. pneumoniæ	NCTC 7465	2·5	5·0	—	—
Str. pyogenes	CN 10	1·10	1·10	—	—
Str. pyogenes (3)	—	0·31–0·88	0·44–1·25	—	—
Str. uberis (2)	—	0·79–1·25	0·99–2·50	—	—
Vibrio choleræ	Madras 48210	4·26	5·36	—	—
Candida albicans	—	—	—	4·47	—
Microsporum canis	—	—	—	1·67	—
Trichophyton mentagrophytes	—	—	—	2·39	—
T. rubrum	—	—	—	0·59	—
T. verrucosum	—	—	—	1·67	—

Growth was washed off and shaken for 10 minutes with glass beads in 16 ml. water per culture flask. 5 ml. of this suspension, diluted as required, were thoroughly mixed with 5 ml. drug solution in buffered saline and maintained at 20° C. for 1 hour. The mixture was then diluted in 2 per cent. bovine bile ("Bacto-Oxgall") in water and 1 ml. samples plated out in 10 ml. nutrient agar to obtain viable counts.

Local therapeutic action. 0·1 ml. of an overnight broth culture of *Staph. aureus* 663, diluted 1 to 100 in 5 per cent. mucin in saline, was injected intraperitoneally (I.P.) into male white mice weighing 9 to 12 g. Half an hour later drugs were injected I.P. or subcutaneously (S.C.). The number of mice surviving was counted 10 days after inoculation and the ED50 and its standard error was estimated by the method of Miller and Tainter[3]. In experiments with *Str. pyogenes* CN10 a 6 hour culture diluted 1 to 1 million in blood broth was used.

Toxicity. In acute tests in mice, deaths were counted 7 days after treatment and the LD50 and its standard error estimated by the method of Miller and Tainter[3], with results obtained in 10 or more animals at

each dose level. In subacute tests, drugs were administered once daily, except on Saturdays and Sundays. In rabbits, saline solutions of Dequadin chloride were instilled in one of each pair of eyes, or creams containing Dequadin were applied to the shaved skin. Creams were applied also to the skin of hairless mice. In a chronic toxicity test, groups of 10 young female Wistar rats, maintained on M.R.C. diet No. 41, received Dequadin chloride at 0·05 and 0·01 per cent. of their drinking water. Rats were weighed weekly and at the end of the test period blood examinations were performed by conventional methods and tissues prepared for histological examination.

RESULTS

Antimicrobial Action in vitro

Screening. The results of comparative bacteriostatic tests of BIQ, BQ and BAQD iodides are expressed in Table I. High activity against *Staph. aureus* was present in all series, being particularly marked in BIQ 16, 17, 18 and 19, BQ 16 and BAQD 10, 12 and 14. Activity against *Myco. phlei* was also general, being highest in the neighbourhood of each tetradecamethylene member. *P. vulgaris* and *Pseudomonas pyocyanea* were most sensitive to the longest chains of the BIQ series and to BAQD 10, 12 and 14. For reasons discussed below, BAQD 10 was selected for further study.

TABLE III

SYNERGISM OF DEQUADIN IODIDE AND CHLORAMPHENICOL AGAINST *Staph. aureus* CN491 IN PEPTONE WATER AFTER 5 DAYS AT 37° C.

Per cent. drug in mixture		Geometric mean M.I.C. in µg./ml.
Dequadin	Chloramphenicol	
100	0	0·3
28	72	1·2
13	87	2·0
9	91	3·0
6	94	4·0
0	100	13·2

Antimicrobial spectrum. All bacteria and fungi tested were sensitive to Dequadin iodide (Table II). It inhibited Gram-positive bacteria more readily that Gram-negative, and mycobacteria and fungi to an intermediate degree. The strains of penicillin-resistant staphylococci shown in Table II to be sensitive to Dequadin were also found to be sensitive to BIQ 16. As expected, the chloride, nitrate and acetate of Dequadin showed comparable activity to the iodide.

TABLE IV

EFFECT OF SERA ON THE INHIBITION OF *Staph. aureus* CN491 BY ANTIBACTERIAL AGENTS IN PEPTONE WATER AT 37° C.

Antibacterial	Percentage Serum added to medium	M.I.C. in µg./ml. at 24 hours	M.I.C. in µg./ml. at 5 days
Dequadin chloride ..	None	0·31	0·31
	rabbit 10	0·31	0·63
	bovine 10	0·63	0·63
	horse 10	0·63	0·63
	human 10	0·31	0·63
	human 50	0·63	0·63
Cetrimide	None	0·63	0·63
	human 10	6·25	12·5
Benzalkonium chloride	None	0·31	0·63
	human 10	6·25	6·25
Domiphen bromide ..	None	0·31	0·31
	human 10	1·56	3·13
Chlorhexidine acetate ..	None	0·25	0·25
	human 10	2·5	5·0

Effect of inoculum size. When the inoculum was increased a hundred-fold to

about 5×10^6 organisms per ml. test medium, five times as high a concentration of Dequadin was needed to inhibit staphylococcal growth.

Synergism. In mixtures, Dequadin and chloramphenicol were roughly additive in action (Table III). A similar effect was also obtained with binary mixtures of Dequadin with aureomycin, penicillin, streptomycin and cetrimide.

Antagonism. The effects of sera on the potencies of Dequadin and some other antibacterial agents in current use are expressed in Table IV. Among these, Dequadin alone was not appreciably antagonised.

The results of exploring possible antagonists of Dequadin are expressed in Table V. For bactericidal tests, bovine bile was chosen as antagonist, because it combined activity with ease of handling. The degree of antagonism of Dequadin by evaporated milk shown in Table V was observed also with two bovine strains of staphylococci.

TABLE V

ANTAGONISM OF BACTERIOSTATIC ACTION OF DEQUADIN AGAINST *Staph. aureus* CN491 IN PEPTONE WATER AT 37° C.

Material added to medium	Per cent.	M.I.C. in µg./ml. at 24 hours	M.I.C. in µg./ml. at 5 days
None		0·31	0·31
Egg yolk	10	0·31	1·25
Evaporated milk	10	25	25
Vegetable lecithin	0·1	25	50
Egg lecithin	0·1	6·25	6·25
Bovine bile	0·2	2·5	2·5
Bovine bile	1	3·13	6·25
Bovine bile	2	6·25	12·5
"Lubrol W"	10	1·56	3·13

Bactericidal. The bactericidal action of Dequadin nitrate was examined in 7 experiments, summarised in Table VI. In the conditions of these, 400 µg./ml., Dequadin and 200 µg./ml. cetrimide killed virtually all organisms. At 100 µg./ml., each drug killed more than 99·99 per cent. organisms. These experiments showed that the two drugs possessed bactericidal activity of a similar order.

TABLE VI

BACTERICIDAL ACTIONS OF DEQUADIN NITRATE AND CETRIMIDE ON *Staph. aureus* CN491 IN BUFFERED SALINE AT pH 7·2 FOR 1 HOUR AT 18° C.

Concentration of drug in µg./ml.	Mean no. of survivors/ml.	
	Dequadin	Cetrimide
500	<10	—
400	<10	—
250	2×10^2	<10
200	4×10^3	—
100	10^3	8×10^3
50	10^7	6×10^6
25	—	2×10^7
5	—	10^{12}
None	10^{14}	10^{14}

Local Therapeutic Action

Staph. aureus. Using an intraperitoneal inoculum adjusted to kill all control mice, the therapeutic action of Dequadin given intraperitoneally was compared with those of BAQD 12 iodide and cetrimide by the same route, and with Dequadin subcutaneously. The results of these experiments, involving 500 mice, are expressed in Figure 1. 4 mg./kg. Dequadin chloride, I.P., prevented death in all of 30 animals. Higher doses showed some toxicity. A similar picture was seen with BAQD 12. On the other hand, tolerated doses of Dequadin, S.C., or cetrimide, I.P., failed to protect more than one or two individuals.

Str. pyogenes. In similar experiments with streptococci a total of 388 mice was used. 0·9 mg./kg. Dequadin chloride, I.P., prevented death in all of 16 animals treated. Its ED50 was 0·26 ± 0·04 mg./kg. and its

LD50 in these infected animals was 11·0 ± 0·1 mg./kg. Dequadin S.C., and cetrimide I.P., were both ineffective.

Toxicity

Acute. During screening of the iodides in Table I, the acute I.P. toxicities of BAQD 10, 12 and 14, BIQ 20 and BQ 20 were compared in mice. The LD50 values (mg./kg.) were: BAQD 10, 20·9 ± 2·9; BAQD 12, 15·1 ± 1·1, BAQD 14, 18·9 ± 2·1; BIQ 20, 2·8 ± 0·2; BQ 20, 1·8 ± 0·2.

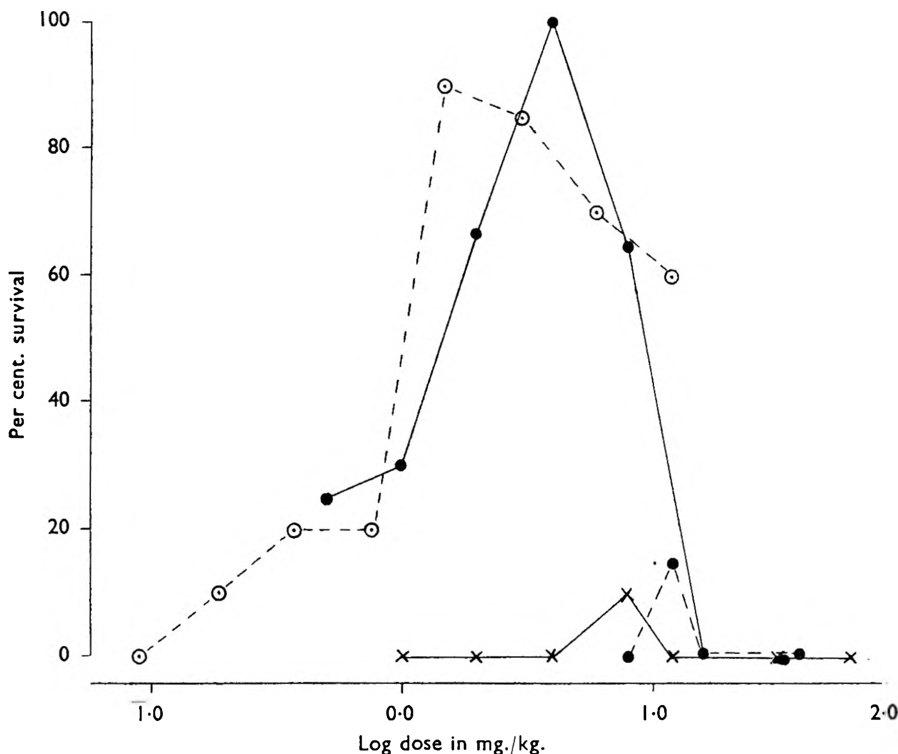

FIG. 1. Protection of mice by polymethylene-bis-4-aminoquinaldinium salts (BAQD 10 and 12). Drugs administered intraperitoneally (I.P.) or subcutaneously (S.C.) 30 minutes after I.P. inoculation of staphylococci.

⊙- - -⊙ BAQD 12 I_2,I.P. ●- - -● BAQD 10Cl_2,S.C.
●——● BAQD 10 Cl_2,I.P. ×——× Cetrimide,I.P.

In mice, the acute LD50 values (mg./kg.) of Dequadin chloride were: S.C., 70 ± 6·6; intravenous, 1·9 ± 0·2. After intravenous administration of Dequadin, mice appeared to die of respiratory paralysis.

Administered orally in 5 per cent. suspension in water, 2 g./kg. Dequadin chloride failed to kill any of 20 mice; while the same dose of the very soluble Dequadin acetate killed 6 of 20 animals.

In a series of intradermal injections in rabbits a solution containing 40 μg. Dequadin chloride/ml. saline caused erythema and induration in

5 of 8 injection sites and 20 μg. evoked the same reaction in 4 of 14 sites. 320 μg. cetrimide/ml. saline caused reactions in 5 of 8 sites and 160 μg. in 2 of 7 sites.

Subacute. Solutions containing 2 mg. Dequadin chloride/ml. saline appeared to be without effect on the eyes of rabbits when instilled daily over a period of 2 weeks. 0·4 per cent. Dequadin chloride or iodide in

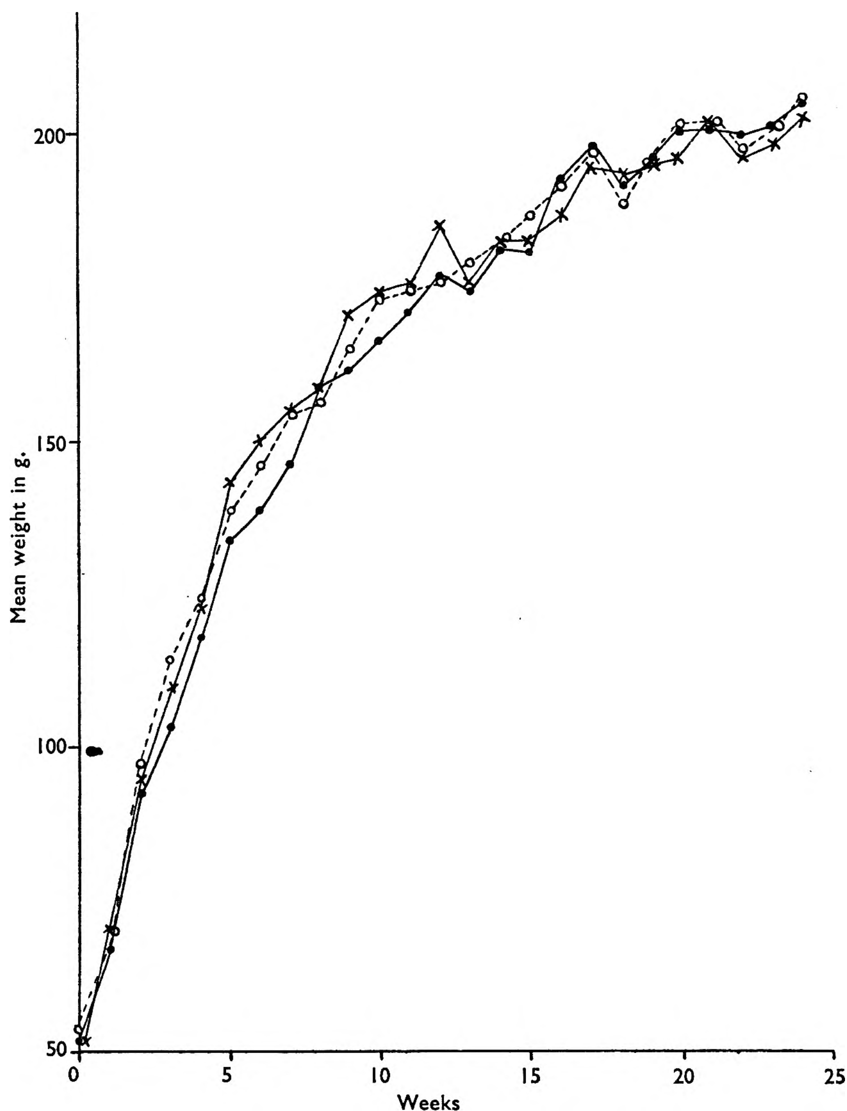

FIG. 2. Absence of growth inhibition in young rats by Dequadin administered in drinking water.

○- - -○ 0·05 per cent. Dequadin chloride
×——× 0·01 per cent. ,, ,,
●——● No addition

creams were applied daily over a period of 4 weeks to the shaved skins of rabbits without evoking any reaction. Similar experiments with the chloride were performed in hairless mice with the same result. As the skin reaction of hairless mice to toxic agents was unknown, chloroform was applied daily to an area of skin. Within 24 hours after the second application a sharp reaction, consisting of erythema, induration, and brownish pigmentation appeared. Some necrosis followed.

TABLE VII

MEAN VALUES OBTAINED FROM BLOOD EXAMINATION OF RATS RECEIVING DEQUADIN CHLORIDE IN THEIR DRINKING WATER FOR 26 WEEKS. L = LYMPHOCYTES; M = MONOCYTES; N = NEUTROPHILS; E = EOSINOPHILS; B = BASOPHILS.

Per cent. drug in water	Per cent. hæmoglobin	Erythrocytes per $mm^3 \times 10^6$	Leucocytes per $mm^3 \times 10^3$	Differential counts per cent.			
				L & M	N	E	B
0·05	103	8·0	9·8	75·05	21·90	3·00	0·05
0·01	101	7·7	9·3	70·15	25·40	4·40	0·05
Untreated controls	100	7·8	10·5	75·45	20·30	4·25	0·00

Chronic. All rats receiving 0·05 per cent. Dequadin in their drinking water for 26 weeks survived; but one rat in the group receiving 0·01 per cent. drug died during the 5th week for unknown reasons. Compared with control animals, rats receiving Dequadin in their drinking water showed no depression of growth (Fig. 2). Blood examinations (Table VII) showed no substantial difference between treated and control rats. Histological examination of sections of brain, stomach, small and large intestines, liver, thyroid, spleen, kidney, heart, lung and ovary of all rats showed no pathological effects attributable to Dequadin.

Discussion

The activities of BIQ 10, 11, 12, 13, 14, 16, 18 and 20 against *Staph. aureus*, *Myco. phlei* and *Ps. pyocyanea* have been examined previously[1]. The present results with these compounds and species, given in Table I, confirm previous findings.

The peaks of activity against the bacteria in Table I occurred at similar positions in the BIQ and BQ series. On the other hand, in the BAQD series, the peaks against *Staph. aureus*, *P. vulgaris* and *Ps. pyocyanea* occurred at shorter chain lengths. This might be because the amino groups in the 4-position on the BAQD rings take over the role of the quaternary nitrogen atoms in the BQ and BIQ series as points of attachment to certain receptors in the bacterium.

In Table I, BAQD 10, 12 and 14 were roughly equivalent in activity to BIQ 20 and BQ 20; but the BAQD compounds were relatively less toxic and more easily prepared. In the BAQD series the decamethylene member was chosen for further study, because it could be most readily supplied. In view of comparative tests and of their structural similarity, it seems unlikely that the general properties of BAQD 12 or 14 differ greatly from those of BAQD 10.

One of the most striking features of Dequadin *in vitro* was the absence of appreciable antagonism by sera, even by 50 per cent. human serum in peptone water (Table IV). Ten per cent. of human serum antagonised the other antibacterials tested by a factor of about 10. For chlorhexidine, this factor was somewhat higher than that reported for rabbit serum[4], although our end-point with human serum was comparable to that obtained with rabbit serum by Davies, Francis, Martin, Rose and Swain. The difference between the two results lies in the relatively greater activity of chlorhexidine in peptone water than in the brain-heart infusion used by these authors.

In mice infected I.P. with virulent cocci, Dequadin was effective by the I.P., but not by the S.C. route. We concluded that its therapeutic action was essentially local. This and other observations described above led to the view that clinical trial of Dequadin as a local chemotherapeutic agent was justified. Results in limited trials of the chloride have so far been encouraging.

It is well known that many heterocyclic polymethylene bis-quaternary ammonium salts possess neuromuscular blocking activity. The paralysis of mice receiving Dequadin intravenously suggested that this drug was no exception, and this suggestion was borne out in an experiment on the tibialis preparation of the cat.

SUMMARY

1. The inhibitory activities of polymethylene-bis-*iso*quinolinium (BIQ), -quinolinium (BQ) and -4-aminoquinaldinium (BAQD) iodides, in which chain-length ranged between 8 and 20 methylene groups, were compared against *Staph. aureus*, *Myco. phlei*, *P. vulgaris* and *Ps. pyocyanea*.

2. Decamethylene-bis-4-aminoquinaldinium (BAQD 10, Dequadin) salts inhibited growth of all microbial species used, which included Gram-positive, Gram-negative and acid-fast bacteria, and fungi.

3. In binary mixtures, Dequadin was additive in bacteriostatic activity with aureomycin, chloramphenicol, penicillin, streptomycin and cetrimide.

4. Dequadin was not antagonised by serum, but was antagonised to varying degrees by bile, milk and lecithin.

5. When suspensions of *Staph. aureus* were exposed to 100 μg./ml. Dequadin for 1 hour at 20° C., more than 99·99 per cent. organisms were destroyed.

6. Dequadin protected all mice against death when administered intraperitoneally 30 minutes after they had been inoculated intraperitoneally with lethal bacterial suspensions. With streptococci, 0·9 mg./kg. and, with staphylococci, 4 mg./kg. were fully effective. Subcutaneously, Dequadin was only feebly active.

7. Iodides of BAQD 10, 12 and 14 showed approximately equal intraperitoneal toxicities in mice, which were lower than those of BIQ 20 and BQ 20.

8. In mice, the acute subcutaneous and intravenous and in rabbits the acute intradermal toxicities of Dequadin were determined.

9. Dequadin failed to exhibit toxic effects when administered subacutely to the eyes and skin of rabbits or to the skin of hairless mice, and chronically to rats in their drinking water.

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THE STRUCTURE OF THE ROOT, ROOTSTOCK AND STEM-BASE OF *RAUWOLFIA VOMITORIA* AFZ.

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The use of the whole root and the selected alkaloids of *Rauwolfia serpentina* Benth. for the treatment of hypertension and various psychiatric conditions has prompted the investigation of other members of the Apocynaceæ for similar active constituents. One such species which is now employed commercially as a source of reserpine is *Rauwolfia vomitoria* Afz., a bush or tree sometimes attaining a height of about 10 m. and of common occurrence in tropical Africa from the Guinea coast to Mozambique. This plant, which possesses many local names[1], has been employed for its verminicidal and insecticidal properties as well as for the treatment of a wide variety of ailments[1,2,3,4,5]. Some of the therapeutic effects of the water-soluble constituents of the drug have been studied by Raymond-Hamet[6,7]; it can act as an emetic and drastic purgative when administered orally.

In 1943, Paris[8] isolated from the root bark of *R. vomitoria*, ajmaline, *iso*ajmaline, small quantities of ajmalicine and ajmalinine and probably serpentinine, five alkaloids contained in *R. serpentina*[9,10]. The roots also contain alstonine[11], two new alkaloids raumitorine and sederine described by Janot *et al.*[12] and reserpine[13].

The structure of *R. serpentina* root and rhizome has been fully investigated by Wallis and Rohatgi[14], several other species have been described by Youngken[15] and *R. parakensis* from Malaya by Wan[16]. No complete histological investigation of *R. vomitoria* root appears to have been published; a brief unillustrated description is given by Paris[8] in which the histology of the root is stated to be analogous to *R. heterophylla* and Pichon[17] includes the species in his classification of the genus based on floral structure. In view of the commercial importance of *R. vomitoria* and the need for differentiating between the common species of this genus a study of the structure of the root and attached rootstock and stem-bases has been undertaken and the results presented below.

Plant Material

The following material was utilised in this investigation:

1. Roots and aerial parts collected in October, 1954, by Dr. W. S. S. Ladell, Oshodi, Nigeria.

2. Roots, twigs and leaves obtained from the Ivory Coast in 1949 and presented by Professor R. Paris, Faculté de Pharmacie de Paris.

3. Various commercial samples obtained from a number of geographical sources and made available by Riker Laboratories,Ltd., England, and Riker Laboratories Inc., Los Angeles, U.S.A.

RAUWOLFIA VOMITORIA AFZ.

MACROSCOPY

The roots occur in cylindrical or flattened, slightly tapering and occasionally branched pieces up to 30 cm. in length and 5 cm. in diameter. Segments 0·5–1·5 cm. in diameter and cut into short lengths form the bulk of commercial samples (Fig. 1 : A).

The outer surface consists of a greyish brown cork, often deeply longitudinally furrowed or rubbed smooth. If present, the outer cork has a soft texture and is easily removed as irregular flakes by rubbing, thereby exposing the smooth light brown surface of the outer cortex. Small roots and rootlets which occur in some samples are seldom found attached to the larger roots but their remains appear at intervals as oblique protuberances or stumps.

With the exception of the small brittle roots the drug is extremely tough, eventually breaking with a fracture splintery in the wood and short in the bark.

A smooth transverse surface of a root exhibits a narrow pale brown bark, rarely exceeding 3 mm. in thickness in the dried material but swelling considerably on soaking in water. The buff or yellowish coloured finely radiate porous wood, showing in the larger pieces distinct growth rings, forms the majority of the drug.

When dry the root is odourless. The cork is practically tasteless, the phloem and outer cortex very bitter and the wood slightly bitter.

Occasional rootstocks with attached stem-bases occur in commercial samples and will be described below.

MICROSCOPY

In the following description the symbols R, T and L designate measurements in the radial, tangential and longitudinal planes respectively. Wherever possible, ranges of size have been obtained from a wide variety of material but nevertheless they cannot be considered as absolute.

The transverse sections of roots of different girth show different appearances depending chiefly on the degree of development of stone cell layers within the secondary phloem. These differences range from a few isolated groups of stone cells in some small roots to interrupted concentric bands in some of the larger roots. Figure 1 : B–E.

The radially arranged cork cells appear in transverse sections as alternating zones of flattened, unlignified suberised cells, 3–4 layers in radial depth and larger lignified cells from one to about 120 layers in radial depth. The largest of the latter cells in any one stratum usually occur immediately interior to the nonlignified zones (Fig. 2: G). For the unlignified cells R = 5 to **7** to **11** to 14 μ, T = 9 to **25** to **36** to 53μ and L = 14 to **29** to **40** to 46 μ and for the lignified cells R = 14 to **32** to **50** to 208 μ, T = 9 to **28** to **40** to 53 μ and L = 7 to **22** to **40** to 53 μ. Some young roots, and older roots deprived of most of their cork, may show only lignified cork cells. In surface view the cork cells appear polygonal and tangentially sectioned, polygonal or elongated (Fig. 2: J).

The phellogen consists of thin walled, radially flattened cells and is followed by the phelloderm possessing about 5 to 16 layers of cells

according to the size of the root. The cells near the phellogen often occur in radial rows but the inner ones are generally displaced and oval or much flattened, with intercellular spaces. R = 3·6 to **14** to **29** to 43 μ, T = 8·0 to **36** to **72** to 130 μ and L = 22 to **36** to **49** to 72 μ. Some of the cell walls are thickened with cellulose and others lignified forming single

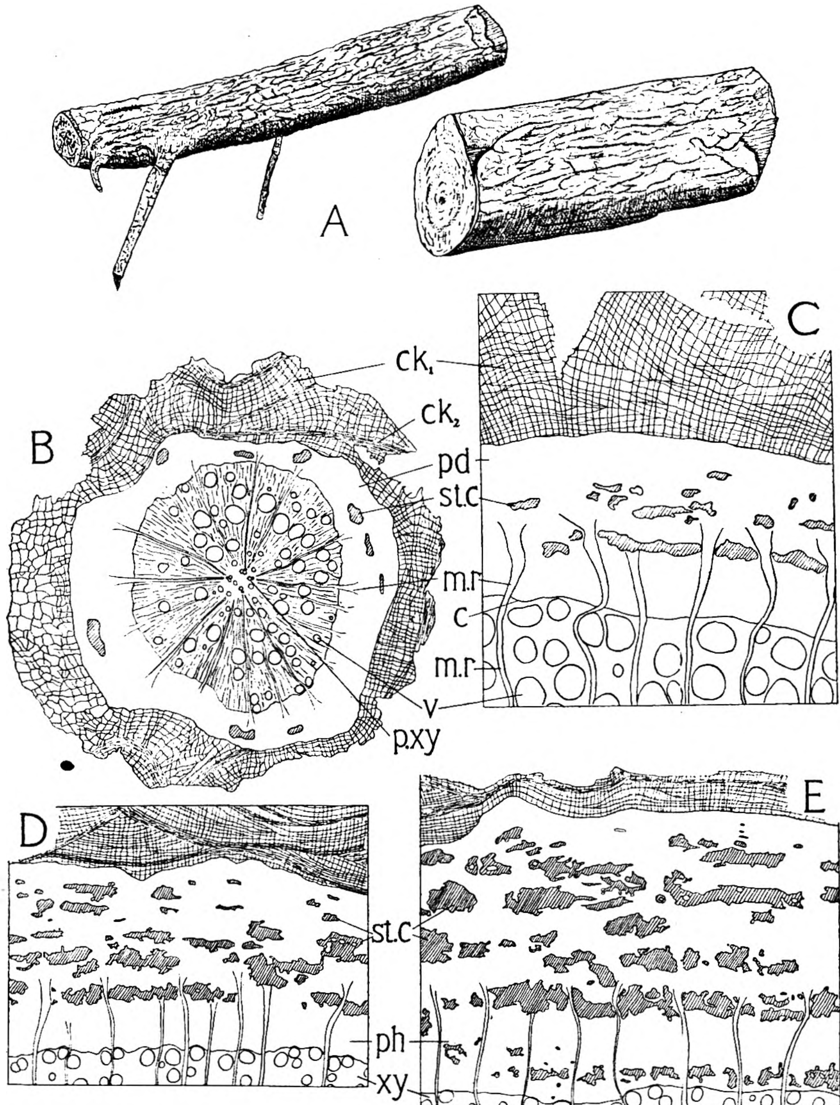

Fig. 1. *Rauwolfia vomitoria* Afz. Root. A, roots taken from a commercial sample × 1. B–E, general diagrams of transverse sections of roots. B, 1 mm. diameter × 50. C, 8 mm. diameter × 50. D, 2·5 cm. diameter × 25. E, 4 cm. diameter × 25. *c*, cambium; ck_1, lignified cork cells; ck_2, unlignified cork cells; *m.r*, medullary rays; *pd*, phelloderm; *ph*, phloem; *p.xy*, primary xylem; *st.c*, stone cell groups; *v*, wood vessels; *xy*, xylem.

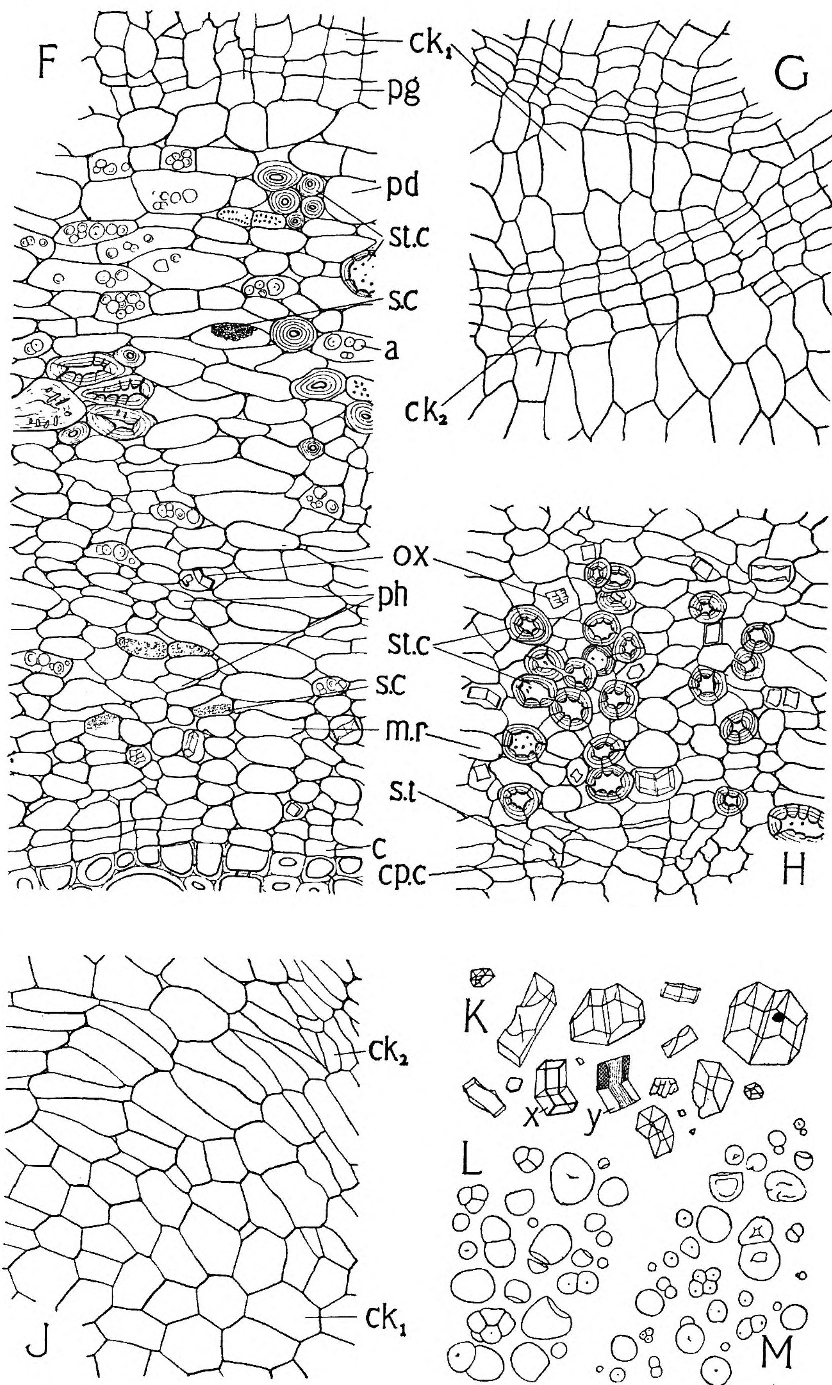

FIG. 2. *Rauwolfia vomitoria* Afz. Root. F–H, transverse sections of the bark × 200. F, root diameter 6 mm.; G, cork cells; H, inner phloem, root diameter 2·0 cm. J, tangential longitudinal section of cork × 200. K, isolated crystals of calcium oxalate × 320. L, wood starch × 320. M, phelloderm and phloem starch × 320. *a*, starch grains; *c*, cambial cells; ck_1 lignified cork cells; ck_2, unlignified cork cells; *cp.c*, companion cells; *m.r*, medullary rays; *ox*, calcium oxalate crystals; *pd*, phelloderm; *pg*, phellogen; *ph*, phloem; *s.c*, secretory cell; *s.t.* sieve tube; *st.c*, stone cells; *x*, twin crystal as seen in chloral hydrate mountant; *y*, the same crystal viewed in polarised light.

or small groups of stone cells (Fig. 2: F; 3: N). Individual stone cells may be isodiametric, tangentially or longitudinally elongated and in the latter case often arranged end to end. R = 11 to **22** to **36** to 45 μ, T = 18 to **36** to **72** to 115 μ and L = 11 to **32** to **50** to 180 μ. The isodiametric stone cells have relatively thinner walls than the elongated structures and sometimes contain calcium oxalate prisms. All the sclereids possess conspicuous pits in their walls. Scattered crystals of calcium oxalate occur in the phelloderm either as twin or single prisms or smaller irregular masses. Starch is present chiefly as single rounded grains, possessing hila as central points or single or star shaped clefts and generally showing a well marked maltese cross when examined in polarised light. Some of the larger grains appear somewhat gelatinised and fail to show the polarisation effect. Individual grains are 1 to **7** to **11** to 20 μ in diameter. 2 to 4 compound grains are also present and these sometimes split to give individual plano-convex or angular grains (Fig. 2: M).

In the smaller roots the secondary phloem consists of sieve tubes, companion cells, phloem parenchyma, secretion cells, medullary rays and isolated groups of stone cells. In larger, older roots there is often an outer non-functioning secondary phloem with up to about five discontinuous bands of stone cells and an inner region in which the sieve elements are clearly discernible. The structure of the phloem is shown in Figure 2: F, H (Fig. 3:O–R).

The heterogeneous rays are 1 to 3, occasionally 4 to 5 cells wide and are composed of groups of small cells, often with wavy walls, R = 25 to **32** to **36** to 49 μ, T = 11 to **18** to **22** to 25 μ and L = 11 to **14** to **22** to 36 μ, supported in longitudinal rows of deeper cells, R = 11 to **14** to **25** to 36 μ, T = 22 to **25** to **29** to 32 μ and L = 25 to **53** to **72** to 90 μ. (Figure 3: O, P). The rays pass through gaps in the stone cell layers and due to the tangential elongation of individual cells become funnel shaped near the outer bark. R = 14 to **22** to **32** to 36 μ, T = 36 to **52** to **75** to 96 μ. Occasional cells in the vertical extensions of the rays may be lignified.

Calcium oxalate occurs abundantly throughout the secondary phloem in the medullary rays and other parenchymatous cells; it is best studied in the longitudinal sections in which long rows of crystals are evident. The crystals take the form of small irregular structures and larger monoclinic prisms. The latter occur singly or as geniculate twins with the twinning occurring on one of the hemi-pyramid faces. The crystal shape is often complicated by pseudomorphism and in chloral hydrate mounts, often rendered difficult to observe due to the deceptive appearance of many of the twin crystals. The effect is illustrated in Figure 2: K. In certain positions the crystals are not always apparent when viewed in polarised light but the twin crystals can usually be detected due to their bicolouration at certain orientations. Length of the well formed prisms = 9 to **15** to **22** to 36 μ, breadth = 7 to **11** to **14** to 18 μ.

A conspicuous feature of the outer phloem is the numerous irregular stone cell groups up to about five cells in radial thickness and 13 cells in depth. Individual stone cells vary greatly in size and shape ranging

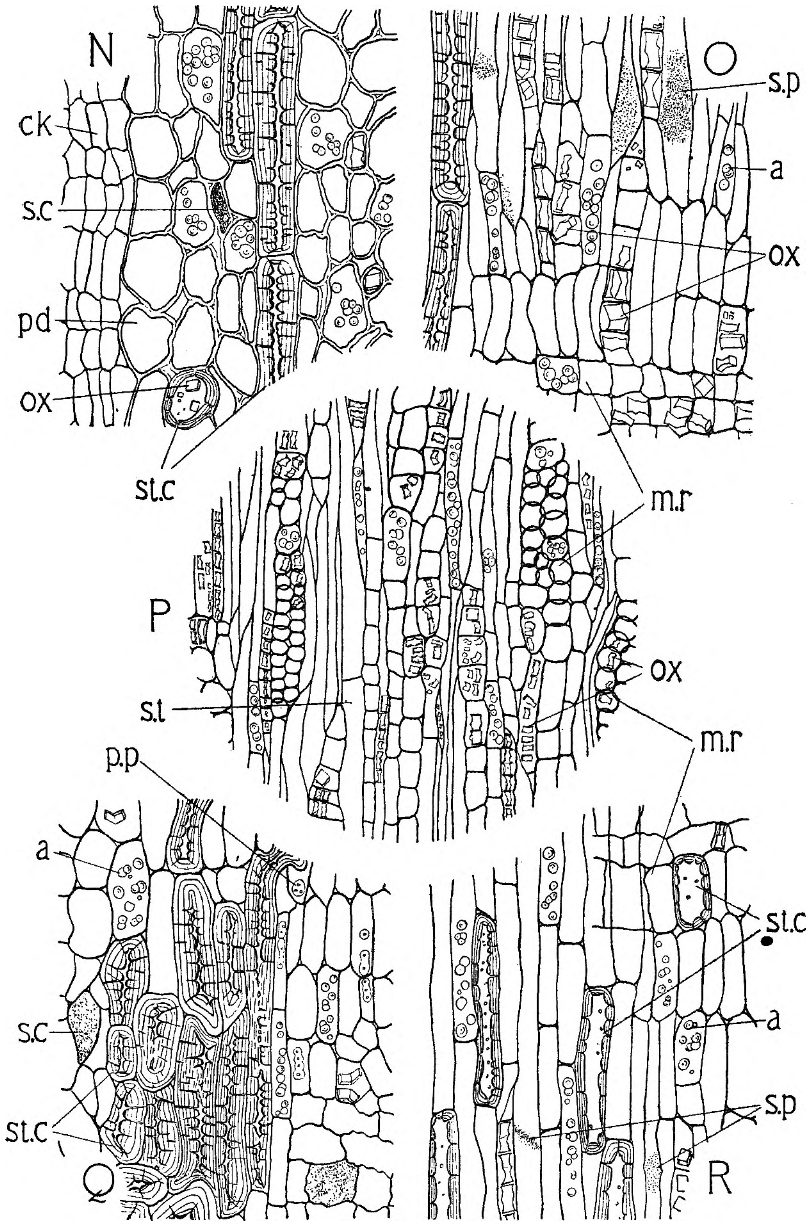

FIG. 3. *Rauwolfia vomitoria* Afz. Root. Longitudinal sections of the bark. N, radial section of outer tissues, root diameter 1·3 cm. O, radial section of inner phloem, root diameter 1·3 cm. Both × 200. P, tangential section of phloem, root diameter 6·0 mm. × 85. Q, radial section of stone cell group of nonfunctioning phloem, root diameter 2·0 cm. R, radial section of inner phloem, diameter 2·0 cm. Both × 200. *a*, starch grains; *ck*, cork; *m.r*, medullary rays; *ox*, calcium oxalate crystals; *pd*, phelloderm; *p.p*, pits in walls of parenchymatous cells; *s.c*, secretion cells; *s.p*, sieve plate; *s.t*, sieve tube; *st.c*, stone cells.

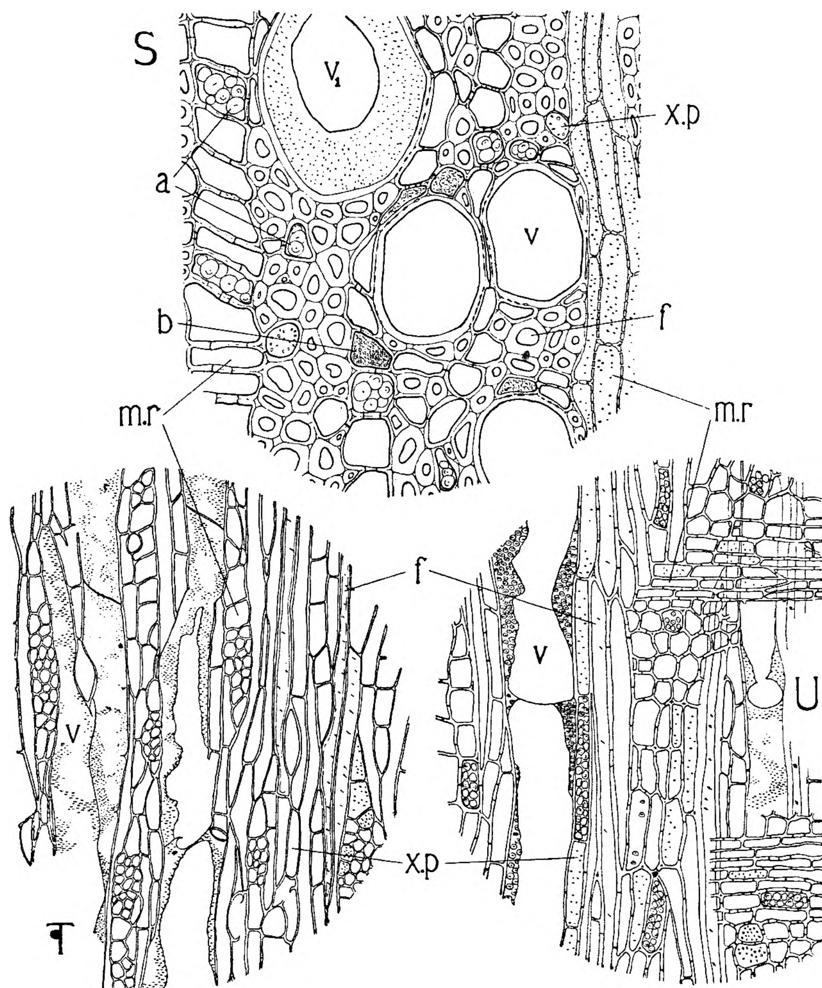

FIG. 4. *Rauwolfia vomitoria* Afz. Root. Secondary wood. S, transverse section × 200. T, tangential longitudinal section × 85. U, radial longitudinal section × 85. *a*, starch; *b*, resinous material; *f*, fibres; *m.r.*, medullary rays; *v*, vessel; v_1, vessel showing perforation plate; *x.p*, xylem parenchyma.

from isodiametric to elongated and irregular structures attached to lignified protuberances. The stratified cell walls are unevenly thickened and bear conspicuous funnel shaped pits. Cells isolated by Schultze's macerating fluid had the following measurements, length = 28 to **71** to **198** to 288 μ, breadth = 14 to **21** to **36** to 56 μ (Figures 2: F; 3: A; 5: X). Incomplete stone cells, often with lignin deposited on one wall only, may occur adjacent to the stone cell groups. Other cells of the phloem parenchyma show on their walls pits surrounded by oval areas of thickening. Occasional stone cells contain starch grains and others crystals of calcium oxalate. Occasionally the lignified walls of the stone cells fit so

closely to the enclosed crystals that in the macerated material, cells with cavities of the exact form of the crystals can be seen (Fig. 5: X).

Secretory cells are not numerous but occur scattered throughout the secondary phloem; their contents appear as amorphous masses when stained with iodine, sudan III or tincture of alkanna (Fig. 2: F; 5: Y).

Variable amounts of starch occur in the phloem. Some samples possess practically none, and others, appreciable quantities in the outer secondary phloem with longitudinal rows of grains in the narrow phloem elements (Fig. 3: O–R). In size and shape the starch grains resemble those of the phelloderm.

The secondary xylem is completely lignified and composed of medullary rays, vessels, fibres and wood parenchyma (Fig. 4: S, T, U). The vessels occur solitary or in pairs and in transverse section are rounded or somewhat radially elongated. R = 36 to **82** to **126** to 180 μ, T = 36 to **72** to **108** to 134 μ. Numerous alternately arranged, bordered pits occur in the vessel walls, the latter often separating into longitudinal strips on maceration (Fig. 5: V). Transverse or oblique perforation plates occur at intervals of about 135 to 540 μ. Yellow or orange tyloses, staining red with phloroglucinol and hydrochloric acid, are rare but may occur in vessels near the centres of roots. Other nonfunctioning vessels may be occluded by brown amorphous material.

In transverse section the apotracheal wood parenchyma occurs in short uniseriate rows; viewed longitudinally the cells are arranged end to end with irregularly pitted cross walls. The anticlinal walls may possess longitudinal rows of bordered pits, depending on the nature of adjacent cells (Fig. 5: W). R = 10 to **18** to **25** to 38 μ, T = 10 to **18** to **25** to 36 μ and L = 52 to **72** to **108** to 150 μ.

The heterogeneous medullary rays consist of one or two, less commonly three groups of small cells, R = 14 to **32** to **41** to 111 μ, T = 10 to **14** to **21** to 29 μ and L = 10 to **14** to **21** to 36 μ embedded in uniseriate longitudinal rows of larger cells, R = 14 to **21** to **36** to 72 μ, T = 14 to **24** to **36** to 42 μ and L = 32 to **36** to **72** to 126 μ. In longitudinal sections the smaller cells are often nearly circular in outline with small intercellular spaces and occur in groups of 3 to 42 cells in rays of up to 25 larger cells in depth. All the walls are heavily pitted. The rays seldom exceed three cells in width (Figs. 4: S, T, U; 5: W).

The medullary ray and wood parenchyma cells are usually packed with starch grains similar in form to those of the bark, but on an average larger in size with a higher proportion of angular and compound grains. Diameter of single grains = 1 to **4** to **18** to 30 μ (Fig. 2:L). In addition to starch some of these cells possess material staining a pale pink-brown with sudan III.

Numerous wood fibres occur associated with the other wood elements and in transverse section appear as rounded or irregularly outlined angular structures with thick lignified walls. Isolated fibres may be irregular, spindle shaped or arcuate in outline with oblique slit-like pits. Some tracheids and intermediate fibre-tracheids with bordered pits are

also present. Fibres with swollen ends or centres are of frequent occurrence and in macerated material often break across the enlarged thin walled areas. Length = 200 to **540** to **1080** to 1485 μ, breadth = 6 to **18** to **22** to 32 μ (Fig. 5: Y).

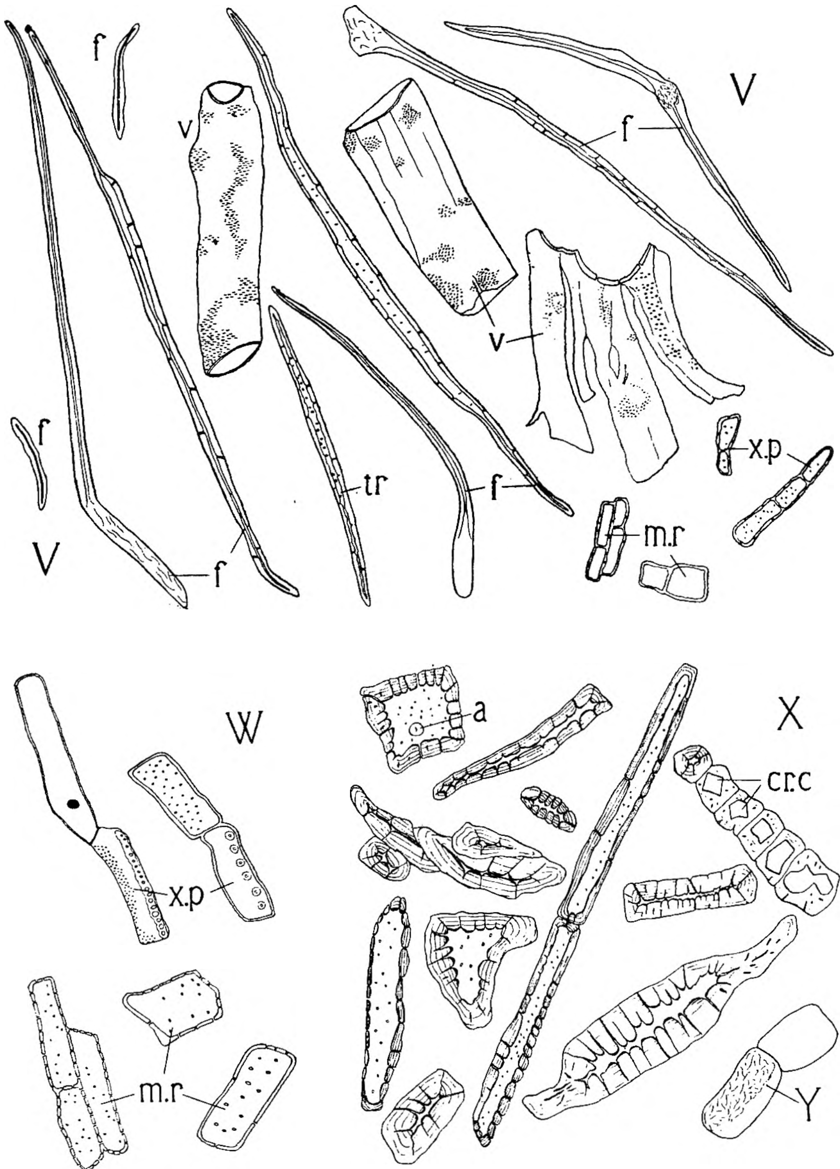

FIG. 5. *Rauwolfia vomitoria* Afz. Isolated elements of the root. V, wood elements × 85. W, ditto × 200. X, stone cells × 200. Y, secretion cell × 200. *a*, starch; *cr.c*, cavities formerly occupied by crystals; *f*, xylem fibres; *m.r*, medullary ray cells; *tr*, tracheid; *v*, vessel; *x.p*, xylem parenchyma.

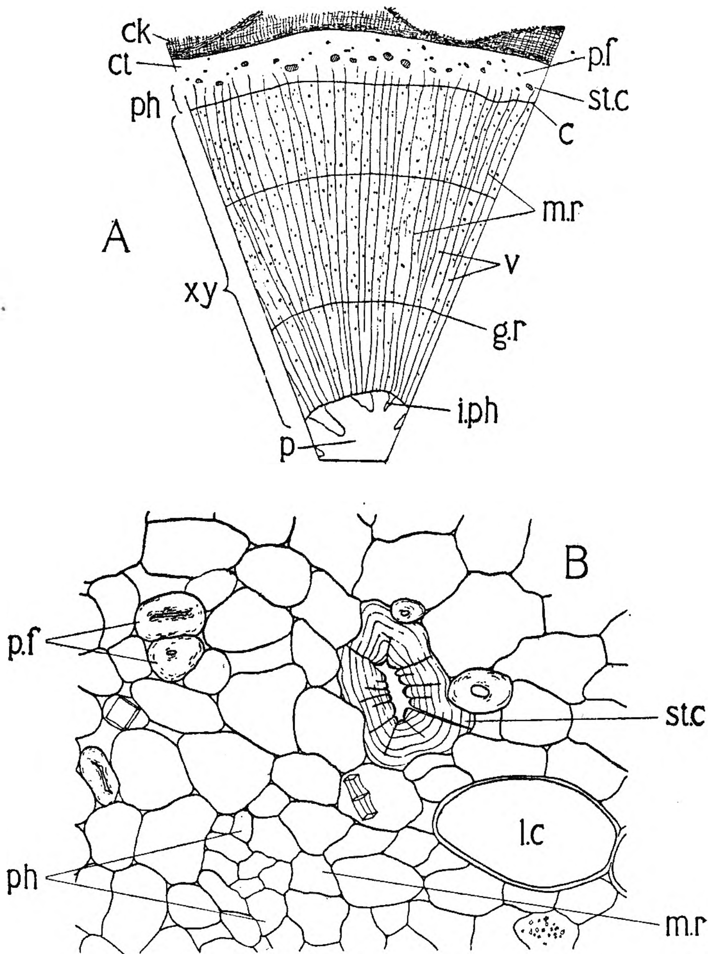

FIG. 6. *Rauwolfia vomitoria* Afz. Stem base. A, transverse section general diagram × 15. B, transverse section in the region of the pericycle × 200. *c*, cambium; *ck*, cork; *ct*, cortex; *g.r*, growth ring; *i.ph*, internal phloem; *l.c*, latex canal; *m.r*, medullary ray; *p*, pith; *p.f*, pericyclic fibres; *ph*, phloem; *st.c*, stone cells; *v*, wood vessels; *xy*, xylem.

ROOTSTOCK AND STEM-BASE

Pieces of rootstock with attached stem-bases occasionally occur in the drug and macroscopically they resemble the roots but possess a small central pith. In the samples examined, the rootstocks measured 2 to 3 cm. in diameter and the aerial stem-bases 0·5–2·0 cm. in diameter but much larger sizes must be possible. The cork of the stem-bases is much smoother than that of the roots and does not easily crumble. Well marked growth rings occur in the very hard, compact, finely radiate wood. The taste is bitter, and as with the root, aqueous decoctions show a marked blue fluorescence and give precipitates with the common alkaloid reagents.

Histologically, the rhizome and stem-bases show certain differences from the root. In all three the cork cells are similar and may exhibit the typical stratified structure of lignified and unlignified layers. In the stem-base the stratification may be absent with only a few layers of one type of cell; in the rhizome, up to seven alternating bands have been observed. Generally, however, the amount of cork is much less than on the root.

Beneath the cortex, consisting of about five layers of tangentially elongated phelloderm cells and the remains of the primary cortex is an incomplete ring of highly refractive, thick walled unlignified pericyclic fibres occurring either singly or in small groups often associated with stone cells (Fig. 6: A, B). These fibres are particularly numerous in the stems and in transverse sections they appear either circular or oval in outline with a small, often flattened lumen; fibres isolated from a 5 per cent. potassium hydroxide solution macerate show stratified walls with oblique pits and at intervals, enlargements of the fibre walls. Length = up to at least 6·5 mm., breadth = 14 to **18** to **32** to 54 μ and at the swellings, 36 to 90 μ (Fig. 8: F).

Stone cells and calcium oxalate are of the same size, shape and distribution as in the roots. The occurrence of crystals within the stone cells is quite common.

The cortex and outer phloem are permeated by latex canals seen in transverse section as circular or ellipsoidal structures with yellow walls (Fig. 6: B). R = 50 to 110 μ, T = 110 to 340 μ. Isolated by an alkali maceration, these canals vary considerably in length (Figure 8: H), and slight pressure on the coverslip of a temporary mount will extrude globules of granular appearing latex from their broken ends.

The wood is much harder than the root wood due to the absence of large vessels and a correspondingly larger number of fibres. Small wood vessels with bordered pits measure 18 to **33** to **48** to 70 μ in diameter and vessels with spiral thickenings occur in the protoxylem (Fig. 7: C). Viewed in tangential longitudinal sections, the heterogeneous medullary rays usually consist of one or two groups of up to 70 small cells with one or two longitudinal rows of larger cells. Individual cells of the rays have similar measurements to those of the root and the maximum width of a ray is about eight cells (Fig. 7: *D*).

Occasional cells of the medullary rays and wood parenchyma contain granular contents staining brown with iodine and others contain single and compound starch grains.

A small central core about 1 mm. in diameter consists of a ring of collapsed internal phloem groups and the central pith (Fig. 8: E). The pith is mainly parenchymatous with a few slightly lignified cells arranged in longitudinal rows, latex tubes similar to those of the bark and calcium oxalate crystals. A few stone cells, 11 to 72 μ in length, occur either isolated or in small groups at the periphery of the internal phloem (Fig. 8: G).

The Powdered Root

The principal features of the powdered root are:

1. Large quantities of lignified tissue derived from the secondary

xylem consisting of fragments of relatively thin walled vessels with bordered pits, libriform fibres, fibre-tracheids with bordered pits, isodiametric and elongated pitted medullary ray cells and elongated wood parenchyma cells. The two latter are usually packed with starch grains.

2. Isodiametric, elongated or irregularly shaped stone cells occurring either singly or in groups. A few may contain calcium oxalate crystals or starch grains.

3. Thin walled yellow cork cells of two types—lignified and radially compressed unlignified cells.

4. Thin walled cellulosic elements derived from the phelloderm and phloem, usually much broken but sometimes containing calcium oxalate crystals, starch grains and resinous material.

5. Crystals of calcium oxalate in the form of single or twin monoclinic prisms, irregular masses, aggregates and irregular structures.

6. Numerous rounded, oval, plano-convex and concavo-convex single starch grains about 1 to **4** to **18** to 30 μ in diameter. (Occasional samples possess practically no starch).

7. Yellowish irregular masses of amorphous material derived from the lumina of older vessels.

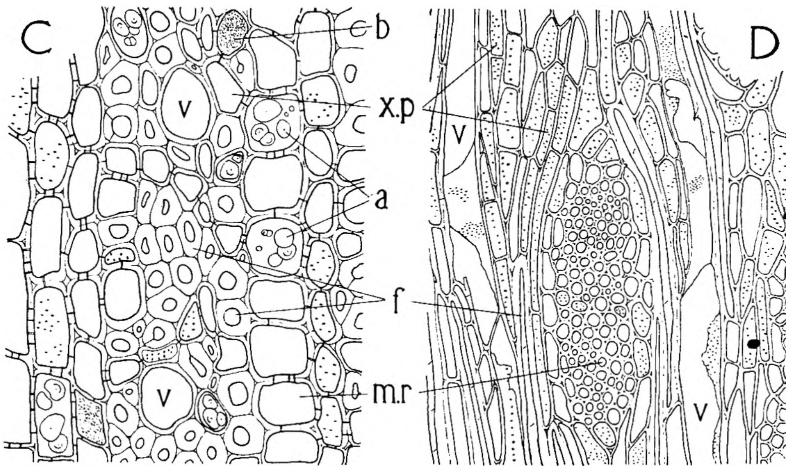

FIG. 7. *Rauwolfia vomitoria* Afz. Stem base. Wood. C, transverse section × 200; D, tangential longitudinal section × 85. *a*, starch grains, *b*, resinous material; *f*, fibres; *m.r*, medullary rays; *v*, vessel; *x.p*, xylem parenchyma.

Discussion and Summary

The structure of *R. vomitoria* root is such that it can readily be distinguished from *R. serpentina* root in both the whole and powdered conditions. The main differences lie in the composition of the woods and in the presence or absence of stone cells.

Most other species of *Rauwolfia* examined to date, differ macroscopically from *R. vomitoria* and can be distinguished by microscopical sections in which the type of cork, phloem modifications and wood structure are

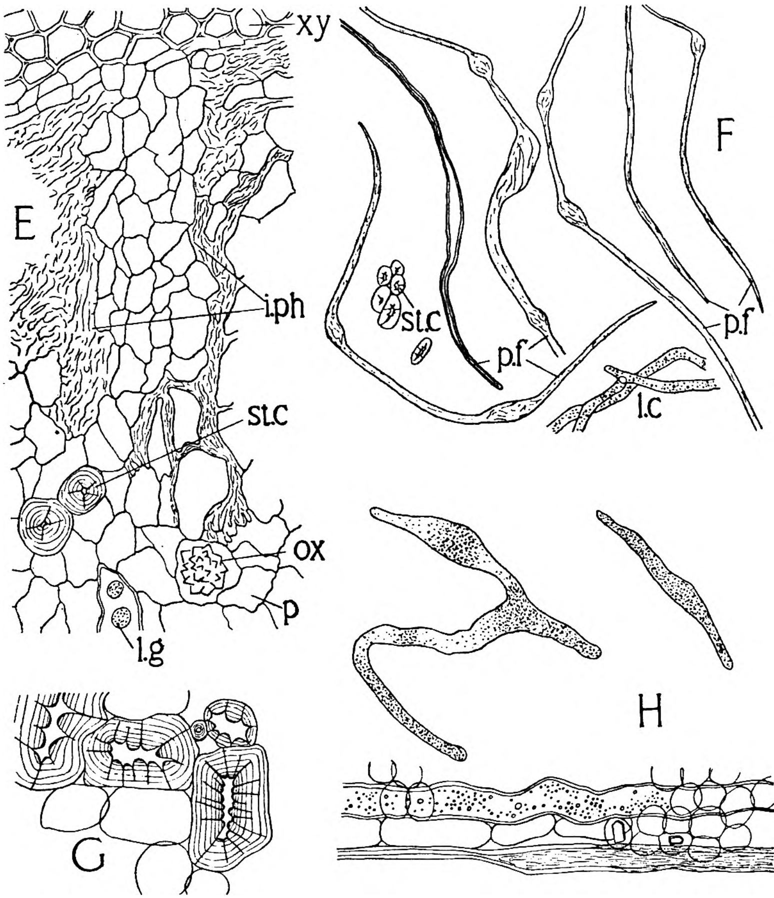

FIG. 8. *Rauwolfia vomitoria* Afz. Stem base. E, transverse section of the pith and internal phloem × 200. F, isolated elements from the bark × 45. G, isolated stone cells from the pith × 200. H, latex vessels from the phloem, lower diagram with attached pericyclic fibre × 85; *i.ph*, internal phloem; *l.c*, latex canal; *l.g*, latex globule; *ox*, calcium oxalate crystal; *p*, pith; *p.f*, pericyclic fibres; *st.c*, stone cell; *xy*, xylem.

particularly useful. These species present some difficulty when in the ground condition since wood structure is not obvious and the presence or absence of alternating zones of lignified and unlignified cork cells may be difficult to ascertain. The micromeasurements available [15] show certain differences from those of *R. vomitoria* and on further investigation, may provide an additional tool for the differentiation of co-generic species.

The general structure of *R. vomitoria* root and rootstock appear typical of the genus, in so far as it has been examined. The presence of latex canals in the stem structures is characteristic of the Apocynaceæ as a whole, although they do not occur in the rhizome of *R. serpentina*.[14] One feature of interest is the almost complete absence of starch in a few of the commercial samples examined. Since no pre-treatment of the

material was obvious it is possible that the starch content of the living roots undergoes some natural variation.

The principal histological characters of the drug are summarised below:

1. CORK. A light spongy layer composed of alternating zones of lignified cells and radially flattened, unlignified cells; easily removed as powdery flakes due to the rupture of the larger fragile elements.

2. PHELLODERM. Chiefly parenchymatous, contains starch grains and calcium oxalate. Isodiametric or longitudinally elongated stone cells occur singly or in small groups.

3. PHLOEM. Larger roots possess an outer nonfunctioning secondary phloem with numerous stone cell groups arranged in interrupted tangential rows. An inner secondary phloem consists of sieve tubes, companion cells, phloem parenchyma and secretory cells arranged between the medullary rays. Longitudinal rows of calcium oxalate crystals and starch grains are common.

4. XYLEM. A tough lignified secondary wood comprises the majority of the drug and numerous relatively large vessels give the wood a porous structure. Most vessels possess walls with bordered pits, towards the centre of the root they may be occluded with yellowish brown material. Libriform, thick walled fibres are numerous together with fibre-tracheids and some tracheids. The heterogenous lignified medullary rays and the lignified wood parenchyma contain single and compound, often angular, starch grains and sometimes resinous material.

5. ROOTSTOCK AND STEM-BASE. These differ from the root in the following respects. An incomplete ring of isolated, or small groups of, unlignified pericyclic fibres occurs between the cortex and phloem. The wood is very dense and exhibits no large vessels. A central, almost unlignified zone up to 1 mm. in diameter, consists of a ring of internal phloem groups, a few stone cells and pith. Latex canals occur in the pith and bark.

The author's thanks are due to Dr. G. E. Trease and Miss M. E. Brown who made photomicrographs of the general characters of a wide range of roots.

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THE UNRELIABILITY OF THE OFFICIAL ASSAY FOR TABLETS OF GLYCERYL TRINITRATE

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The monograph of the current Pharmacopœia on Tablets of Glyceryl Trinitrate prescribes limits of 81–121 per cent. for the declared glyceryl trinitrate content, that a tablet containing 1/130 grain (approximately 0·5 mg.) shall be dispensed or supplied when no strength is specified, and an assay reading as follows:—"Weigh and powder 20 tablets. Mix an accurately weighed quantity of the powder, equivalent to about 1 mg. of glyceryl trinitrate, with 5 ml. of glacial acetic acid, shake continuously for one hour, and filter. Mix 1 ml. of the filtrate with 2 ml. of phenoldisulphonic acid, stir well, and allow to stand for 15 minutes. Add about 8 ml. of water, make alkaline with strong solution of ammonia, cool to about 20° C., dilute to 20 ml. with water, and filter if necessary. Compare, under similar conditions in a suitable colorimeter, the colour of this solution with the colours of solutions containing known quantities of potassium nitrate which have been treated in an exactly similar manner. Each gram of potassium nitrate is equivalent to 0·7487 g. of $C_3H_5O_9N_3$. Calculate the weight of glyceryl trinitrate in each tablet of average weight." The following directions are given in the B.P. for the preparation of phenoldisulphonic acid—"Heat 3 g. of phenol with 20 ml. of sulphuric acid on a water-bath for 6 hours; transfer the resulting liquid to a stoppered vessel."

The above method is basically that published by Meek[1] in 1935, which was a development of an original suggestion made by Scoville[2] in 1911. The main differences are the use of potassium nitrate as standard in place of the silver salt and glyceryl trinitrate itself, and the comparison of the colours of the test and standards in a "suitable colorimeter" in preference to Meek's use of Lovibond yellow units. It has been our experience that the results given by the official assay do not always agree with those given by the U.S.P. assay so we decided to subject both methods to a detailed study. An additional object was to discover if it was possible to use a spectrophotometric standard in the official assay. There appeared to be two main obstacles to this objective, firstly a possible variation in the colour produced by different batches of phenoldisulphonic acid reagent and secondly, the importance attached by Meek to the temperature at which the colour was measured; he reported that values obtained by him at 25° C. were 10 per cent. higher than they were at 15° C. We found that if we made the reagent exactly as the B.P. specifies, i.e., 20 ml. at a time, there was no significant difference in the results obtained when experiments were repeated using different batches of reagent, and secondly that there was no variation between the values obtained at different temperatures. We were unable to reproduce Meek's 10 per cent. difference even over the range of 10–30° C.

Two minor variations were made in the official assay; we used the equivalent of four tablets with 10 ml. of glacial acetic acid which enabled us conveniently to perform a triplicate assay from each extraction and we made up the final volume of the solution immediately before the determination of the absorption to 25 ml. instead of 20 ml. When the intensity of the colour was plotted against the logarithm of the concentration the relation appeared to fit the Beer-Lambert equation.

THE SPECTROPHOTOMETRIC CHARACTERISTICS OF THE NITRATE STANDARD

The direction in the B.P. assay that the standard and test are to be treated in an exactly similar manner is very important; we stress this point as we have seen it stated in text-books[3,4] that the standard may be prepared by taking silver nitrate solution to dryness with the subsequent addition of phenoldisulphonic acid reagent. It is imperative that the colour of the standard be developed in the presence of glacial acetic acid otherwise it will be far too intense. Our examination of the standard comprised a determination of the values obtained by the use of potassium and silver nitrates in both the presence and absence of glacial acetic acid and also by the employment of a standard acetic acid solution of glyceryl trinitrate itself. In addition to the magnitude of the absorption at $\lambda_{max.}$ we were interested in the reproducibility and the spectrophotometric characteristics of the curves obtained from the different standards used; for comparison purposes the curve having an E value of 1·000 at $\lambda_{max.}$ was calculated for each standard.

In the official assay the equivalent of two tablets corresponding to approximately 1 mg. of glyceryl trinitrate, is extracted with 5 ml. of glacial acetic acid; one ml. of the filtrate is used for colour development, hence the standard required is the colour given by 0·2 mg. of glyceryl trinitrate in 1 ml. of glacial acetic acid. The solution of glyceryl trinitrate used for standard determinations was assayed by both the official method of the B.P.C. for solution of glyceryl trinitrate and the U.S.P. assay for tablets after suitable adjustment; the solution used by us had a strength of 0·95 per cent., both methods of assay agreeing with this figure. All figures given in this paper concerning this solution however, are recalculated to those expected from an exactly 1·0 per cent. solution. When potassium nitrate is used as standard it can be calculated from theoretical considerations that a solution containing 0·2672 mg. of potassium nitrate per ml. is required; for silver nitrate the concentration should be 0·4500 mg. per ml. The values obtained for these standard solutions are given in Table I, and it will be seen that standards prepared by the evaporation of potassium or silver nitrates to dryness with the subsequent addition of glacial acetic acid before colour development, agreed very well with that obtained from glyceryl trinitrate itself. The value for glyceryl trinitrate was 0·646, for silver nitrate 0·649 and for potassium nitrate a little lower at 0·638. If glacial acetic acid was omitted during colour development the values obtained were significantly higher at 0·727 for potassium nitrate and 0·738 for silver nitrate. The characteristics of the absorption curves originating from the use of these different standards are given in Table II; it is evident

TABLE I

STANDARD NITRATE E VALUES AT $\lambda_{max.}$ (405 Mμ) OBTAINED FOR GLYCERYL, POTASSIUM AND SODIUM NITRATES

Glyceryl trinitrate						
	0·651	0·623	0·642	0·637	0·645	0·645
	0·647	0·657	0·660	0·647	0·649	0·645
	0·631	0·642	0·645	0·639	0·669	0·651
	Mean value 0·646 ± 0·005 (P = 0·95)					
Potassium nitrate						
(*a*) Glacial acetic acid present						
	0·642	0·639	0·642	0·639	0·637	0·640
	0·650	0·633	0·631	0·648	0·633	0·623
	Mean value 0·638 ± 0·005 (P = 0·95)					
(*b*) Glacial acetic acid absent						
	0·730	0·726	0·737	0·728	0·735	0·707
	0·737	0·732	0·722	0·735	0·714	0·726
	Mean value 0·727 ± 0·006 (P = 0·95)					
Silver nitrate						
(*a*) Glacial acetic acid present						
	0·638	0·639	0·658	0·656	0·656	0·644
	0·650	0·638	0·654	0·640	0·658	0·654
	Mean value 0·649 ± 0·005 (P = 0·95)					
(*b*) Glacial acetic acid absent						
	0·758	0·745	0·747	0·742	0·738	0·756
	0·728	0·717	0·738	0·725	0·733	0·747
	Mean value 0·738 ± 0·008 (P = 0·95)					

that the characteristics are practically identical for all three sources of nitrate whether acetic acid is present or not; the effect of acetic acid in the case of both inorganic nitrates is to depress the value of the absorption obtained without alteration of its characteristics. The wavelength of maximum absorption is well defined at 405 mμ in each case. It is suggested that, using the data in Table I, we can fix a standard for the official assay; if the equivalent of two tablets containing in each 0·5 mg., i.e., a total glyceryl trinitrate content of 1 mg. is subjected to the B.P. assay and the final dilution is to 25 ml. instead of 20 ml. then an E value of 0·646 at 405 mμ should be obtained, if the glyceryl trinitrate content is exactly 0·5 mg. per tablet. A close examination of the data in Table I reveals a

TABLE II

CHARACTERISTICS OF THE VARIOUS ABSORPTION CURVES OBTAINED

Wavelength, mμ	Glyceryl trinitrate	Potassium nitrate	Potassium nitrate + acetic acid	Silver nitrate	Silver nitrate + acetic acid
355	0·715	0·671	0·650	0·661	0·675
360	0·529	0·515	0·506	0·510	0·512
365	0·544	0·532	0·530	0·531	0·532
370	0·612	0·603	0·602	0·601	0·600
375	0·693	0·679	0·682	0·677	0·679
380	0·773	0·763	0·768	0·761	0·764
385	0·850	0·840	0·848	0·840	0·841
390	0·916	0·908	0·912	0·902	0·909
395	0·962	0·960	0·961	0·955	0·960
400	0·991	0·993	0·989	0·990	0·991
405	1·000	1·000	1·000	1·000	1·000
410	0·982	0·988	0·983	0·984	0·987
415	0·945	0·947	0·947	0·949	0·954
420	0·884	0·890	0·886	0·892	0·895
425	0·813	0·818	0·813	0·818	0·824
430	0·725	0·733	0·726	0·730	0·741
435	0·640	0·647	0·641	0·643	0·655
440	0·550	0·550	0·546	0·549	0·562
445	0·463	0·460	0·459	0·461	0·476
450	0·384	0·379	0·399	0·377	0·392
455	0·313	0·307	0·306	0·306	0·319
460	0·251	0·243	0·245	0·241	0·258
465	0·201	0·192	0·195	0·192	0·207
470	0·158	0·148	0·152	0·148	0·161
475	0·126	0·114	0·120	0·114	0·131

certain variation between replicates and although this variation is small, it is as well to realise that it exists; as normal routine we took the mean of triplicate determinations.

The U.S.P. Assay

It is very useful in an investigation of this type to have available an entirely independent method of assay and we have used the official assay of the U.S.P. for checking our results. In this method 100 tablets are used for each test; after ethereal extraction of the glyceryl trinitrate and subsequent hydrolysis the nitrate is reduced to ammonia which is estimated by the usual distillation into standard acid—a blank on the reagents being subtracted. We have carried out tests using this assay and obtained practically 100 per cent. recoveries.

The B.P. Assay

It was the study of the curves obtained on various samples of trinitrin tablets and the comparison of their characteristics with those of the curves obtained from the different standards that led us to examine the assay in detail. We observed that the characteristics of the curves obtained from the great majority of tablets were very different from those exhibited by the standards and it was an explanation of these differences that we sought. It is always useful in spectrophotometry to keep a check on the shape of a curve by calculating two ratios one on either side of the maximum absorption; in this case we made much use of the ratios of the absorptions at 375 mμ and 430 mμ to that at the maximum at 405 mμ. Upon investigation we found that the absorption curve obtained on a typical sample of tablets could be influenced by a number of factors. One of the more obvious ones was the effect of incomplete filtration; we found that the fat derived from the cocoa contained in the base was the main cause of cloudy solutions, the difficulty being eliminated when experiments were carried out on tablets made with a fat free base. In the official assay, unless great care was taken with the filtration, solutions were obtained which although clear, were not as bright as the standard solutions. The absorption due to the suspended fat was uniform in character (i.e., its magnitude was constant at different wavelengths) as distinct from the more usual general absorption encountered in spectrophotometry. This fact was illustrated by further filtration of such cloudy solutions, resulting in a constant decrease in the absorptions at three wavelengths 375, 405, and 430 mμ. It was a common cause of gross disagreements between replicates. We found that the use of a Whatman No. 42 filter paper, the rejection of the first 5 ml. of the initial filtrate, followed by refiltration of the remainder through the same filter into a clean beaker, gave solutions which were both bright and clear. It was however apparent that even when this source of error was eliminated, the characteristics of the absorption curve were still not the same as those of the standard. The next step was the investigation of any possible blank given by the reagents and also by the base in the absence of glyceryl trinitrate. The blank given by the reagents was approximately 0·018 at 405 mμ and it is

of interest that the same value was obtained for the B.P.C. base when the cocoa was omitted from it. The different values obtained for what may be termed the various "background" absorptions are given in Table III. In the B.P. assay the proportion of solid matter to acetic acid in the initial extraction, assuming the weight of an average trinitrin tablet to be 0·3 g., is 0·6 g. to 5 ml., or in our case 1·2 g. to 10 ml. of acid. It will be seen that where the B.P.C. base was used without granulation, there was little difference in background absorption over the range 0·6 to 2·4 g.; one set of figures refers to a sample of fat extracted base and was not significantly different from the remainder, showing that an efficient filtration procedure was being employed. In the case of the granulated base the doubling of the normal weight did seem to result in somewhat higher absorptions.

TABLE III

"BACKGROUND" ABSORPTION

	E value at		
	375 mμ	405 mμ	430 mμ
(a) Reagents	0·021	0·018	0·015
(b) Ungranulated B.P.C. chocolate base; 10 ml. of glacial acetic acid plus the stated weight of base			
0·6 g.	0·032	0·025	0·023
1·2 g.	0·034	0·021	0·018
1·2 g. (defatted)	0·038	0·023	0·020
2·4 g.	0·038	0·022	0·020
(c) Granulated B.P.C. chocolate base; 10 ml. of glacial acetic acid plus the stated weight of base			
0·6 g.	0·045	0·031	0·024
1·2 g.	0·052	0·030	0·029
1·2 g. (defatted)	0·041	0·028	0·024
2·4 g.	0·079	0·045	0·040
(d) B.P.C. base with cocoa omitted	0·022	0·018	0·017
(e) Base composed entirely of cocoa	0·049	0·032	0·027

The absorptions obtained when the base was composed entirely of cocoa were similar to the others. It is to be emphasised that even though the figures given are the mean values obtained from six replicates, they are not exact figures capable of showing fine shades of difference; the broad conclusion that we have reached is that there exists a background irrelevant absorption of the order of 0·030 at 405 mμ. As the blank on the reagents (which is unaltered for a lactose-sucrose base) is 0·018 and is of course present in the standard, the net irrelevant absorption due to the cocoa of the base is of the order of 0·012, which corresponds to an over-estimate of approximately 2 per cent. and for all practical purposes can be ignored.

Another factor we encountered was the presence of vanillin in some tablets. This is quite permissible in the official base; in the concentrations normally employed its absorption commenced at around 390 mμ and rose steeply into the ultra-violet region, thus having the effect of greatly lifting the absorption on the shortwave side of the maximum. It had however no effect upon the absorption value at the maximum itself. The net result of the presence of vanillin was that after all the normal corrections for the usual background absorptions had been made the ratio of the absorption at 375 mμ to that at 405 mμ was still very much above the normal.

To sum up we may say that the normal absorption curve is composed of at least two components and possibly four. The two main components are the absorption due to the glyceryl trinitrate colour complex, and the background general absorption due to the base and the reagents, estimated quantitatively at approximately 0·030 at 405 mμ. The other two possible components are the avoidable uniform absorption due to the effects of cocoa fat, which can be removed by efficient filtration, and lastly the frequently encountered "lift" in the absorption on the short-wave side of the maximum due to the presence of vanillin. Although the vanillin if present has no effect on the value of the absorption at 405 mμ in a spectrophotometric assay, the possibility is to be borne in mind that if an absorptiometer is used for measurement which uses a filter with a comparatively wide band width then the presence of vanillin might influence the assay.

Recovery Tests

The data obtained by us on recovery tests are summarised in Table IV. In part I of the Table the percentage recoveries at different glyceryl trinitrate levels are given. It will be remembered that if in the assay the

TABLE IV

RECOVERY TESTS

I. Effect of different bases upon the percentage recoveries at different potency levels.	
(*a*) Ungranulated B.P.C. chocolate base.	
In each case 1·2 g. of base plus 10 ml. of glacial acetic acid containing:—	
1·0 mg. trinitrin	Recovery 86·9 per cent.
1·5 mg. ,,	,, 90·2 ,, ,,
2·0 mg. ,,	,, 88·9 ,, ,,
3·0 mg. ,,	,, 90·7 ,, ,,
(*b*) Powdered granulated B.P.C. chocolate base.	
In each case 1·2 g. of base plus 10 ml. of glacial acetic acid containing:—	
1·0 mg. trinitrin	Recovery 92·8 per cent.
1·5 mg. ,,	,, 91·3 ,, ,,
2·0 mg. ,,	,, 91·8 ,, ,,
3·0 mg. ,,	,, 92·2 ,, ,,
(*c*) Fat extracted powdered granulated B.P.C. chocolate base.	
In each case 1·2 g. of base plus 10 ml. of glacial acetic acid containing:—	
1·0 mg. trinitrin	Recovery 98·4 per cent.
1·5 mg. ,,	,, 94·8 ,, ,,
2·0 mg. ,,	,, 96·2 ,, ,,
3·0 mg. ,,	,, 94·3 ,, ,,
II. Effect of variations in the weight of base upon the percentage recovery when the potency level is kept constant.	
(*a*) 1 mg. potency level.	
1 mg. of trinitrin in 10 ml. of glacial acetic acid plus a weight of base as follows.	
0·6 g.	Recovery 96·1 per cent.
1·2 g.	,, 92·8 ,, ,,
2·4 g.	,, 76·8 ,, ,,
(*b*) 2 mg. potency level.	
2 mg. of trinitrin in 10 ml. of glacial acetic acid plus a weight of base as follows.	
0·6 g.	Recovery 91·0 per cent.
1·2 g.	,, 91·8 ,, ,,
2·4 g.	,, 77·3 ,, ,,
III. Effect of the presence of cocoa upon the percentage recovery.	
(*a*) Base without cocoa.	
1·2 g. of base plus 10 ml. of glacial acetic acid containing:—	
1 mg. trinitrin	Recovery 99·0 per cent.
2 mg. ,,	,, 98·9 ,, ,,
(*b*) Base composed entirely of cocoa.	
1·2 g. of cocoa plus 10 ml. of glacial acetic acid containing:—	
1 mg. trinitrin	Recovery 50·0 per cent.
2 mg. ,,	,, 50·2 ,, ,,

equivalent weight of four tablets was taken then 10 ml. of glacial acetic acid were used for extraction and this is the normal 2 mg. level for the usual 0·5 mg. tablets: the weight of base taken in this case was 1·2 g. In experiments tabulated in part I of Table IV we took in each case 1·2 g. of base but varied the amount of trinitrin included in the 10 ml. of glacial acetic acid added. It will be seen that the recoveries obtained with the ungranulated base are slightly lower than those given by the granulated base; this suggests that the degree of subdivision of the powder may affect the recovery. There was a significantly lower recovery with the normal base than with one which had been freed of its fat content; this seems to indicate that the fat plays some part in the percentage recovery obtained. The recovery for the B.P.C. granulated base used remained constant at about 92 per cent. which means that the official method underestimates the true glyceryl trinitrate content by approximately 8 per cent. Furthermore if the filtrates obtained are not bright and clear a further error is introduced which helps to compensate this underestimation. This explains why it is possible for a whole series of assays to be done in which the B.P. and U.S.P. methods are compared and a somewhat fortuitous agreement obtained—normally the U.S.P. assay should always be higher than the B.P. figure. In experiments summarised in part II of Table IV we studied the effect of the weight of base present, i.e., the size of the tablet. It appeared that if the tablets were one half normal size there was not a great difference in the percentage recovery, but if they were twice the normal size there was a significant drop in the percentage recovery. Finally in part III of Table IV the effect of the percentage of cocoa in the base is illustrated. The easiest way to do this was to prepare a base containing no cocoa and also to use cocoa itself as base. It will be obvious from the data obtained that the cocoa is a major cause of the failure to obtain good recoveries; if there was no cocoa present the recovery was almost complete, but if the base was composed solely of cocoa this fell to somewhere in the region of 50 per cent.

Application of the Method to Official Tablets

We carried out analyses of a number of tablets using the data obtained and we summarise the results below.

(*a*) Freshly made tablets using the actual base previously studied; manufactured to contain approximately 1/100 grain (0·65 mg.) of trinitrin. U.S.P. assay 0·67 mg. found: B.P. assay uncorrected E value 0·806, equivalent to a trinitrin content of 0·62 mg. per tablet which on correction by multiplying by 100/92 gives 0·68 mg. per tablet.

Another batch of tablets* with the same base containing approximately 1/130 grain (0·50 mg.) trinitrin. U.S.P. assay 0·49 mg.: B.P. assay 0·45 mg. uncorrected and 0·49 mg. corrected.

A third batch of tablets declared to contain 0·50 mg. with an unknown base. U.S.P. assay 0·34 mg.; B.P. assay 0·31 mg. and 0·34 mg. corrected.

* This batch of tablets analysed again 4 months later gave U.S.P. assay 0·44 mg.; B.P. assay 0·39 mg. uncorrected, and 0·42 corrected. A further correction (see later) for "bound" trinitrin gave a final figure of 0·44 mg.

The problem seemed to be solved but the following examples showed that this was not the case.

(*b*) Tablets with unknown base declared to contain 1/130 grain (0·50 mg.). U.S.P. assay 0·39 mg.; B.P. 0·29 mg. and 0·32 mg. corrected. A recovery test on this particular sample gave 93·4 per cent. of the amount added, i.e., the normal expected recovery. We investigated this sample further by attempting a soxhlet extraction with ether, followed by careful evaporation of the ether and determination of trinitrin by the B.P. assay; we expected a somewhat lower figure by this method because of the possible loss of trinitrin when evaporating the ether; the result obtained was approximately 10 per cent. below that obtained by the B.P. method and it appeared that ethereal extraction still failed to remove sufficient trinitrin to account for the U.S.P. figure. Another experiment which we tried on this particular sample was to grind the powdered tablets in a mortar with the glacial acetic acid before shaking. This failed to increase the previous figure obtained by the B.P. assay. There appeared therefore to be a portion of the trinitrin that was not removed in the B.P. assay but was recovered in the U.S.P. assay.

In none of our experiments on the B.P. method so far had we tried the addition of water followed by ether extraction which is part of the U.S.P. process. We therefore modified the B.P. assay by washing the residue on the filter paper after the acetic acid filtration with about 200 ml. of glacial acetic acid, suspended it in water, extracted with ether and then found that the "missing" trinitrin could be recovered in this way. We calculated the total trinitrin content as follows: the "free" trinitrin gave an E value of 0·376. This we multiplied by 100/92 to compensate for its distribution on extraction between the base and the acetic acid. We assumed that washing the residue with 200 ml. of acetic acid would remove the remaining adsorbed "free" trinitrin; the base then suspended in water was extracted with ether and after careful evaporation of the latter the residue was subjected to the B.P. assay and gave an E value of 0·090. From this we subtracted the reagent blank of approximately 0·020, so that our final E value was $0{\cdot}376 \times 100/92$ (i.e., 409), plus $0{\cdot}070 = 0{\cdot}479$. This corresponded to 0·37 mg. per tablet.

(*c*) Another sample assayed at 0·47 mg. per tablet by the U.S.P. method and at 0·31 mg. per tablet by the B.P. method, increased by correction to 0·36 mg. per tablet. The original E value was 0·400 and 0·435 corrected; the ethereal extract from aqueous suspension gave a further E value of 0·136. Subtraction of the reagent blank decreased this to 0·116 and this figure added to the original E value gave a total of 0·551, corresponding to 0·43 mg. per tablet. It is interesting that in this last example a portion of the U.S.P. extract which gave 0·47 mg. per tablet gave only 0·42 mg. per tablet when subjected to the B.P. assay, showing that there is some loss when the trinitrin in ethereal solution is freed from ether by evaporation.

In Table V we have selected 13 samples at random from some 50 samples of commercial trinitrin tablets on retail sale recently analysed by us[5] and it gives a good illustration of the magnitude of error involved in the use of the B.P. assay without modification. The mean trinitrin content of the

samples in the table is according to the B.P. assay about one half what it should be whereas on correction this rises to approximately two thirds. It is of interest that the mean value for the trinitrin content of the 50 samples analysed by a modified B.P. assay was approximately 65 per cent. while a U.S.P. assay carried out on 100 tablets made up of two tablets taken from each of the 50 samples gave a trinitrin content of 67 per cent.

TABLE V

COMPARISON OF THE RESULTS OBTAINED BY THE OFFICIAL AND MODIFIED ASSAYS WHEN APPLIED TO COMMERCIAL SAMPLES OF TRINITRIN TABLETS STATED TO CONTAIN 1/130 GRAIN OF MEDICAMENT

Sample No.	Gross E value at 405 mμ (*a*)	Percentage of trinitrin calculated from (*a*)	Gross E value × 100/92	Residual E value less 0·020 allowance for reagent blank (*c*)	Corrected E value (*b*) plus (*c*)	Percentage trinitrin calculated from corrected E
1	0·529	82	0·575	0·009	0·583	90
2	0·241	37	0·262	0·121	0·383	59
3	0·338	52	0·367	0·007	0·374	58
4	0·341	53	0·371	0·091	0·462	72
5	0·245	38	0·266	0·126	0·392	61
6	0·291	45	0·316	0·076	0·392	61
7	0·343	53	0·373	0·067	0·440	68
8	0·242	37	0·263	0·165	0·428	65
9	0·310	48	0·337	0·053	0·390	60
10	0·370	57	0·400	0·030	0·430	67
11	0·329	57	0·358	0·038	0·396	61
12	0·341	51	0·371	0·069	0·420	65
13	0·434	67	0·472	0·071	0·533	81
	Mean value 52					Mean value 67

CONCLUSIONS

A comparison of the assay procedures of the B.P. and the U.S.P. has shown that there is always the danger with the former method of a serious underestimate owing to its failure to extract the "bound" trinitrin: whether or not the U.S.P. assay itself estimates the total trinitrin is a question we are unable to answer. There are a number of unsatisfactory aspects of the assay of the trinitrin content of the official tablet. It has been reported by Stephenson and Humphreys-Jones[6] that after storage for many years there is always some 30 per cent. of the original trinitrin content remaining in the tablets—this is most unusual as one would expect the trinitrin content to fall eventually to zero. The fact that from a medical standpoint there is no recorded report that we can trace of trinitrin tablets in practice failing to perform the function for which they were given makes the whole subject of the chemical assay suspect.

The following conclusions are based upon the work reported in this paper but we do feel that there is need for much more work to be done before a truly satisfactory method can be established.

1. The use of glacial acetic acid to remove the trinitrin in one extraction from the powdered tablets results in an approximate recovery of 92 per cent. of the "free" trinitrin. This is only a very general statement and the figure is greatly influenced by the size of the tablet and the percentage of cocoa in the base employed.

2. Even if we assume that a suitable correction is possible for the above there is still the problem of the "bound" trinitrin, the amount of

which may vary considerably. We have little information on this subject but it may be that the age of the tablet, the formulation of the base and probably other unknown factors are involved. It may well be that the B.P. assay can be modified as regards the extraction to provide a satisfactory assay; if so the use of a spectrophotometric standard will increase its accuracy.

3. We feel that for the present the use of the U.S.P. assay or some modification thereof is far more satisfactory than the present B.P. assay which has been shown to be unreliable.

SUMMARY

1. A comparison of the assay procedures of the B.P. and U.S.P. has shown that there is a danger of low results with the former owing to its failure to extract "bound" trinitrin.

2. Use of glacial acetic acid to remove the trinitrin in one extraction from powdered tablets results in an approximate recovery of 92 per cent. of the "free" trinitrin. The figure is influenced by the tablet size and percentage of cocoa in the base.

3. The use of a suitable correction factor is affected by the unknown and very variable amount of "bound" trinitrin.

4. The U.S.P. assay or a modification is considered more satisfactory than the B.P. method.

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ABSTRACTS OF PAPERS PUBLISHED IN OTHER JOURNALS

CHEMISTRY

ANALYTICAL

Barbiturates in Pharmaceuticals, Determination of. F. A. Rotondaro. (*J. Assoc. off. agric. Chem., Wash.*, 1955, **38**, 809.) An examination is made of the different extraction procedures available and one extraction method using chloroform is recommended. Samples containing 100–150 mg. of the barbiturate if a gravimetric or a volumetric determination is to be made, or 15–30 mg. if the determination is to be made spectrophotometrically are dissolved in water, acidified with hydrochloric acid and extracted with chloroform. The chloroform layer is then passed successively through four separating funnels; the first contains sodium bicarbonate solution, the second and third 0·1N sodium hydroxide, and the fourth, water. Acidification of the bicarbonate solution in the first funnel yields any acidic decomposition products and acidic materials such as saccharin, aspirin, salicylic acid, and benzoic acid. The pure barbituric acid derivative is retained in the alkali and water washes. These are bulked and analysed spectrophotometrically, titrimetrically with silver, bromine or alkali, gravimetrically, or chromatographically. Results are given for the analysis of typical barbiturate samples. R. E. S.

Calciferol, Colorimetric Determination of. J. Büchi and H. Schneider. (*Medd. Norsk farm. Selsk.*, 1955, **17**, 87.) A detailed study of the Schaltegger method (*Helv. chim. acta*, 1946, **29**, 285) for the colorimetric determination of calciferol led to the following recommendation for an improved method: 1·00 ml. of a solution of 100 μg. of calciferol in thiophen-free benzene is placed in a 20-ml. test tube with a ground-in air condenser, and treated with 2 ml. of freshly prepared (0·15 per cent.) cumin aldehyde solution in benzene. The mixture is diluted with 4 ml. of benzene, and treated with 3 drops of perchloric acid reagent. The solution is heated on the water bath for 1½ minutes in the dark, then kept for 7 minutes in the dark, allowed to cool, and treated with 3 ml. of glacial acetic acid: 9 minutes after the heating the colour is determined in a 1 cm. cell, using filter No. 12 (550 mμ). The colour intensity is somewhat decreased by the presence of hydroquinone, but not by pyrogallol. The perchloric acid reagent used is prepared by adding 0·6 ml. of perchloric acid (60 per cent.) to a mixture of 2ml. of acetic anhydride and 2·5 ml. of glacial acetic acid. The solution is protected from moisture by a calcium chloride tube, and warmed at 95° to 100° C. for 30 minutes. G. M.

Cyanide, Thiocyanate and α-Hydroxynitriles, Determination of. R. B. Bruce, J. W. Howard and R. F. Hanzal. (*Analyt. Chem.*, 1955, **27**, 1346.) A method is described, based on the conversion of cyanide and thiocyanate into cyanogen bromide which is subsequently allowed to react with benzidine in pyridine to give an intense red colour. Air is drawn rapidly through a mixture of the sample and 20 per cent. trichloroacetic acid and into a receiving tube containing 0·1N sodium hydroxide. One drop of saturated bromine water is added to the contents of the receiving tube followed by 0·20 ml. of arsenous acid solution to remove the excess bromine. The vapours of bromine above the solution are blown off with a stream of air, 3·6 ml. of a pyridine-benzidine

mixture is then added and the red colour is allowed to develop for 15 minutes before being measured spectrophotometrically at 532 mμ; the concentration is determined from a previously prepared calibration curve. Thiocyanate is determined in the residual mixture in the aeration tube after removal of the cyanide, the contents being filtered and treated as for cyanide. α-Hydroxynitriles under alkaline conditions liberate hydrocyanic acid and this is used as a basis for the analytical procedure. In the presence of free cyanide and thiocyanate, the cyanide is determined as described above. After removal of cyanide by aeration, 10 per cent. sodium hydroxide is added to the sample in trichloroacetic acid and it is allowed to stand for 5 minutes to hydrolyse the α-hydroxynitrile. The mixture is then acidified again by the addition of trichloroacetic acid the liberated cyanide being determined as before. In experiments on cyanide and thiocyanate added to plasma 98 to 102 per cent. were recovered; 89 to 91 per cent. of cyanide was obtained from glycolonitrile and lactonitrile; recovery results of the same order were also obtained for lactonitrile added to plasma in the presence of added cyanide and thiocyanate. R. E. S.

Glucose in Invert Sugar, Iodimetric Determination of. S. O. Ericksson. (*Farm. Revy*, 1955, **54**, 441, 456.) The oxidation of glucose by alkaline iodine is dependant on the dilution, temperature, alkalinity and excess of iodine in the solution. Larger reaction volume and smaller amounts of sodium hydroxide than those previously recommended substantially reduce the oxidation of lævulose during the time necessary for quantitative oxidation of the glucose. Changes in the lævulose resulting from the action of sodium hydroxide and of bicarbonate-hydroxide buffer affect the determination, and have been studied. In the assay of invert sugar solutions changes in the lævulose are produced during the inversion of sucrose and influence the rotation of the solution and, to some extent, the determination of the glucose. The method recommended for the determination of glucose in invert sugar is as follows: 10 ml. of a 2 per cent. solution of invert sugar is treated with 30 ml. of 0·1N iodine solution and 75 ml. of 0·05M sodium hydroxide solution; 20 minutes after the addition of the alkali, the mixture is acidified with 10 ml. of 5N hydrochloric acid, and the excess of iodine is titrated back with 0·1N thiosulphate. A blank experiment is carried out similarly with 10 ml. of water. G. M.

Morphine, Polarographic Determination of. J. Holubek. (*Pharm. Zentralh.*, 1955, **94**, 347.) A method used for the determination of morphine in poppy capsules is based on the polarographic determination of 2-nitrosomorphine. Under the conditions used, with large excess of nitrous acid, the nitrosomorphine is partially converted to chinitrol and morphic acid, so that it is essential to standardise the conditions of the assay, and to standardise the method using the same conditions. For the assay, 3 g. of the dried and powdered material is extracted with 100 ml. of N hydrochloric acid, the mixture is filtered, and the extraction is repeated with a further 100 ml. of hydrochloric acid. The combined filtrates are made up to 250 ml, and to 5 ml. of this solution is added 2 ml. of N sodium nitrite solution. After exactly 5 minutes 3 ml. of potassium hydroxide (20 per cent.) is added and 7 drops of gelatin solution (0·5 per cent.). The morphine is then determined by polarograph. Results agree well with those obtained colorimetrically. G. M.

(—)-Noradrenaline and (—)-Adrenaline, Polarographic Measurement of. J. Henderson and A. Stone Freedberg. (*Analyt. Chem.*, 1955, **27**, 1064.) (—)-Adrenaline and (—)-noradrenaline were converted by iodate oxidation to

iodoadrenochrome or iodonoradrenochrome and aliquots of the reaction solutions were measured polarographically. Half-wave potentials for iodoadrenochrome and iodonoradrenochrome were $E_{1/2} = +0{\cdot}03$ volt and $E_{1/2} = +0{\cdot}02$ volt, respectively, in 0·1M acetic acid-acetate buffer, pH 4·52, 0·01 per cent. in gelatin. Polarographic measurements on aliquots containing the equivalent of about 20 μg. of amine gave diffusion currents from which the amount of adrenaline or noradrenaline could be calculated by referring to standard curves. A linear relation between concentration and diffusion current was found for 1 to 50 μg. quantities of adrenaline or noradrenaline; the error of the method was 5 to 10 per cent. Adrenaline and noradrenaline could not be measured individually in the same solution, but by using a paper chromatographic method for separation prior to polarographic analysis, eluates containing these amines were collected, converted to the iodo derivatives in the same test tubes and analysed polarographically. Coloured or fluorescent substances normally found troublesome in other methods for the measurement of catechol amines, did not interfere. R. E. S.

Opium, Assay of, by Paper Chromatography. A. B. Svendsen, E. D. Aarnes and A. Paulsen. (*Medd. Norsk farm. Selsk.*, 1955, **17**, 116.) 2·5 g. of opium is rubbed down with 5 ml. of concentrated acetic acid and the mixture filtered through sintered glass (3G3) and the filter is then washed with 5 per cent. acetic acid to a total volume of 50 ml. 25 ml. of this solution is made alkaline with ammonia and shaken out repeatedly with chloroform-*iso*propanol (3 + 1). The solvent is evaporated off, and the residue is dissolved in 5 per cent. acetic acid to a volume of 25 ml. For the chromatography a mixture of butanol, toluene, acetic acid and water (20:10:3:9) is used, the R_F value for morphine being about 0·15. A number of spots with differing quantities of morphine are applied to the paper, and one strip of the chromatogram is cut out and developed with Folin and Ciocalteu reagent. The corresponding areas of the other strips are then cut out for the determination, each spot being treated with 5 ml. of diluted reagent (1·5 ml. of Folin and Ciocalteu reagent diluted to 25 ml. with water). After 2 hours 5 ml.of sodium hydroxide solution (10 per cent.) is added and after a further hour the extinction is measured at 700 mμ. There is good agreement between the results obtained and assays by the Swiss official method. G. M.

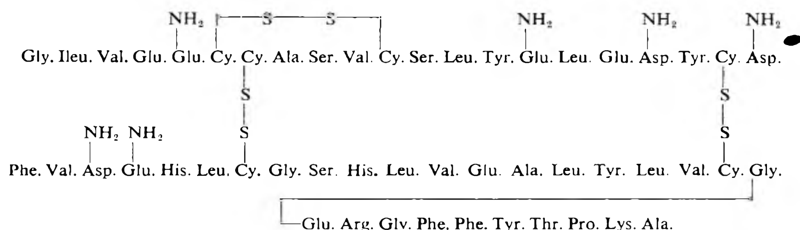
***Rhamnus frangula,* Glycoside Content of.** H. Mühlemann and H. Schmid. (*Pharm. Acta Helvet.*, 1955, **30**, 363.) A method of isolation of a mixture of reduced genuine glycosides, in acetylated form, from *Rhamnus frangula* bark is described. This mixture consists of varying amounts of glucofrangulinanthranol acetate and a bimolecular glucofrangulindihydrodianthranol acetate, resembling the sennosides described by Stoll. On storage of the drug, the bimolecular form is converted into glucofrangulinanthrone and finally into glucofrangulin. A simultaneous hydrolysis to frangulin, its reduced forms and corresponding aglucones is probable, but could not be confirmed. The bioside content of the drug decreases on storage. G. M.

Silicon, Colorimetric Determination of. E. J. King and B. D. Stacy. (*Analyst*, 1955, **80**, 441.) The removal of phosphate by basic ferric acetate precipitation prior to the determination of silica has been critically investigated; it was found that the method could give inaccurate results unless strict control of the pH were observed owing to (*a*) incomplete removal of phosphate, or (*b*) removal of

silica with phosphate. Both phosphate and silica were found to couple with molybdate in weak acid, but only the silicomolybdate complex was reduced in strongly acid solutions (Milton, *J. Appl. Chem.*, 1951, **1**, Suppl. Issue No. 2126); following this principle a new procedure for the determination of silica in biological material was developed, in which phosphate was left in solution. Iron, in amounts greater than those occurring in tissue, did not interfere. Two methods were evolved to overcome interference in urines with high phosphate concentrations by precipitation of ammonium phosphomolybdate; either phosphate was partly or completely removed by precipitation with calcium hydroxide, or ammonia and urea were removed with nitrous acid and sodium molybdate was used to couple with silica. The new colorimetric procedure gave good agreement with gravimetric analyses, and good recoveries of added silica were obtained with tissue, blood and urine. The procedure likewise gave results in good agreement with gravimetric methods when applied to small samples of mineral dusts. Details of the method are given together with tables of comparative results for silica analyses by different methods. R. E. S.

ORGANIC CHEMISTRY

Insulin, Disulphide Bonds, and Structure of. A. P. Ryle, F. Sanger, L. F. Smith and R. Kitai. (*Biochem. J.*, 1955, **60**, 541.) Experiments are described which were undertaken to determine the specific linking of the various half-cysteine residues of polypeptide fractions A and B (obtained by the action of performic acid) into the disulphide bonds which join the two fractions together in the intact molecule. Insulin was subjected to partial hydrolysis with chymotrypsin, with a crude pancreatic extract and with acid under conditions such that the disulphide bonds remained intact. This was followed by fractionation of cysteine peptides, oxidation of the latter to cysteic acid peptides, and finally identification of the resulting amino-acids after fractionation by paper ionophoresis. The structure of the cysteine peptides obtained in this way indicated the distribution of the dipeptide bonds in insulin, giving the complete structure for insulin.



J. B. S.

Insulins of Pig and Sheep, Structure of. H. Brown, F. Sanger and R. Kitai. (*Biochem. J.*, 1955, **60**, 556.) The structures of pig and sheep insulins have been determined by oxidative degradation into the respective glycyl (fraction A) and phenylalanyl (fraction B) chains. Each of these fractions was treated with proteolytic enzymes and the resulting large peptides were separated by paper ionophoresis, and finally subjected to complete hydrolysis. Certain of the large peptides were also partially hydrolysed to smaller peptides which were readily separated and identified by chromatography on paper, and which also were representative of all the residues in the insulin chains. In this way it was shown

that the phenylalanine chains have the same amino-acid sequence in pig, sheep and cattle insulins. The glycyl chains show a small variation in the three insulins, the three residues in the 8, 9 and 10 positions being Ala. Ser. Val. in cattle insulin, Thr. Ser. Ileu. in pig insulin, and Ala. Gly. Val. in sheep insulin.

J. B. S.

BIOCHEMISTRY

BIOCHEMICAL ANALYSIS

Glycyrrhetinic Acid in Urine, Estimation of. V. M. van Katwijk and L. G. Huis in 'T Veld. (*Rec. Trav. chim. Pays-Bas*, 1955, **74**, 889.) Using the method described, 20 mg. of glycyrrhetinic acid can be estimated quantitatively in a 24 hour sample of human urine. For the extraction, take 400 ml. of the sample, add 40 ml. of 25 per cent. hydrochloric acid, 200 ml. of chloroform and reflux for 10 minutes to hydrolyse any readily saponifiable esters. Cool, remove the chloroform extract and re-extract the aqueous layer twice by shaking with 150 ml. of chloroform in a separating funnel. Evaporate the combined chloroform extracts under reduced pressure and dissolve the residue in a measured volume (10 or 20 ml.) of 96 per cent. ethanol. The extract is purified chromatographically. A quantity of the extract corresponding to 1/5th of the 24 hour sample is taken, the solvent is evaporated and 50 ml. of benzene is added to the residue. 7 g. of floridine earth are brought into a chromatographic column 15 mm. in diameter and moistened with benzene. The benzene solution of the urinary extract is poured over the column, and elution carried out as follows.

3 × 50 ml. benzene (eluates 2–4).
2 × 50 ml. benzene + 0·1 per cent. ethanol (eluates 5–6).
10 × 50 ml. benzene + 0·5 per cent. ethanol (eluates 6–16).
4 × 50 ml. benzene + 1·0 per cent. ethanol (eluates 17–20).
6 × 50 ml. benzene + 2·0 per cent. ethanol (eluates 21–26).

For the spectrophotometric estimation 5 ml. of each eluate is pipetted into a 10 ml. flask, the solvent is carefully evaporated and the residues are dissolved in 96 per cent. ethanol. The extinctions of these solutions are determined at 248 mμ. No glycyrrhetinic acid could be demonstrated in the urine of patients who received orally 1·33, 2·28 and 2·5 g. from which it is concluded that less than 2 per cent. of orally administered doses are excreted as such or in the form of a salt or an easily saponifiable ester.

G. F. S.

α-Keto-acids, 1:2-Diamino-4-nitrobenzene as a Reagent for. K. W. Taylor and M. J. H. Smith. (*Analyst*, 1955, **80**, 607.) A paper-chromatographic method is given for the separation and detection of α-keto-acids in blood and urine, based on the use of 1:2-diamino-4-nitrobenzene as a reagent for α-keto-acids. Blood was added as quickly as possible to freshly prepared 5 per cent. w/v metaphosphoric acid, set aside for 10 minutes at room temperature, centrifuged and the supernantant liquid removed; 3 ml. of 0·2 per cent. w/v 1:2-diamino-4-nitrobenzene in 0·66N hydrochloric acid was then added and the mixture allowed to stand for 12 to 16 hours. After extraction with ethyl acetate the nitroquinoxalinols were extracted into 5 per cent. w/v sodium carbonate solution together with a small amount of unused reagent which was removed by washing with ether. The carbonate phase was then adjusted to pH 4 with 10N hydrochloric acid and re-extracted with ethyl acetate. The combined ethyl acetate extract was evaporated to dryness at a temperature not exceeding 40° C., the dry residue dissolved in acetone and chromatographed

for about 8 hours using descending chromatography with a solvent mixture consisting of 5 parts of ethanol, 8 parts of *n*-pentanol and 6 parts of ammonia. The nitroquinoxalinols appeared as yellow spots which faded rapidly on removal from the solvent system; the spots were eluted with 30 per cent. aqueous ethanol and optical densities after filtration measured at 280 mμ. Results are given for R_F values and ultra-violet absorption spectra of the nitroquinoxalinols of pyruvic acid and α-ketoglutaric acid. A number of compounds normally present in blood and urine were examined for possible interference with the method but this did not occur; α-ketoglutaric and pyruvic acids were detected in 15 blood and urine specimens. Results are given for normal blood levels of pyruvic and α-ketoglutaric acids and for recovery experiments using known amounts of keto-acids added to blood; from 67 to 77 per cent. of added pyruvic acid and from 77 to 100 per cent. of added α-ketoglutaric acids were recovered. R. E. S.

Lead in Urine, Determination of. W. M. McCord and J. W. Zemp. (*Analyt. Chem.*, 1955, **27**, 1171.) Lead, in an acid solution and in the presence of excess potassium iodide, is extracted quantitatively by methyl *iso*propyl ketone, followed by extraction from the ketone into an aqueous sodium hydroxide solution and development of colour with dithizone using the method of Snyder (*Analyt. Chem.*, 1947, **19**, 684). A first extraction is performed in the presence of sodium or potassium cyanide at pH 9·5 to 10·0 and interfering ions are limited to stannous tin, thallium, and bismuth; stannous tin and thallium were ignored, the bismuth being removed by an acid chloroform-dithizone extraction into a buffer solution at a pH of 3·4. An aqueous solution of the lead was then adjusted to pH 11·5 with ammoniacal cyanide solution and extracted with dithizone solution, the lead as lead dithizonate being quantitatively transferred to the chloroform layer. A low, but constant, concentration of unreacted dithizone in the chloroform layer improved the accuracy and reliability of the results as compared with lower pH methods; the high pH extraction also permitted the use of a standard dithizone solution and eliminated the necessity for titrimetric extraction. It was found that the range of sensitivity of the method, 0 to 70 μg. of lead using a 22 mm. cell was sufficient for the determination of lead in mild chronic lead poisoning. Satisfactory recoveries were obtained for quantities of lead up to 35 μg. added to urine. R. E. S.

Magnesium in Serum, Estimation of. A. J. Smith. (*Biochem. J.*, 1955, **60**, 522.) A colorimetric method is described for the routine estimation of magnesium in serum. Calcium can also be estimated in the same sample of serum. To 2 ml. of serum add 1 ml. of saturated ammonium oxalate. Mix, allow to stand for 30 minutes, centrifuge, add 2 ml. of the supernatant to 8 ml. of distilled water and add 10 ml. of 10 per cent. trichloroacetic acid solution. Mix, and after 10 minutes filter through Whatman No. 42 filter paper. Take 10 ml. of the filtrate and add 3 ml. of 8 per cent. v/v ammonia and 5 ml. of buffer (prepared by dissolving 6·75 g. of ammonium chloride A.R. in distilled water, adding 57 ml. concentrated ammonia A.R. and making up to 1000 ml. with distilled water giving a pH of 10·1). Mix and add 2 ml. of a dye solution (0·1 per cent. eriochrome black T in absolute methanol). Determine within 5 minutes the extinction at 520 mμ in a spectrophotometer and compare with a parallel series of standard solutions of magnesium acetate containing 0, 4, 8, and 16 μg. Mg to which are added 0·4 ml. of saturated ammonium oxalate, 10 ml. of 10 per cent. trichloroacetic acid, the volume made up to 20 ml., filtered and 10 ml. of the filtrate treated in the same way as the serum filtrate. The method is accurate to ±5 per cent. G. F. S.

PHARMACY

NOTES AND FORMULÆ

Ascorbic Acid, Decomposition of Solutions. F. F. Jensen. (*Dansk Tidsskr. Farm.*, 1955, **29**, 125.) Aqueous solutions of ascorbic acid (2 per cent.) were prepared with varying concentrations of dissolved oxygen, i.e., oxygen-free, in equilibrium with air (6·20 ml. per litre), and an intermediate concentration. After heating for prolonged periods at 90° and 100° C., determination of the ascorbic acid showed that there was no difference between the three series. Similar results were obtained at other pH values ranging from 2·55 to 9·90. The less acid solutions showed a considerable drop in pH. Decomposition was at its slowest at pH 6·30. When, however, similar solutions were heated in ampoules containing a considerable volume of air, the decomposition was more rapid at pH 6·30 than at 2·55. Catalysts are not of significance in the oxygen-free reaction. It was not found possible to demonstrate the presence of 2-ketogulonic acid as the result of this reaction. It was observed, however, that after heating solutions of ascorbic acid at pH 1·10, considerable pressure was produced in the ampoules, due to the formation of carbon dioxide. The reaction involved has not yet been elucidated. G. M.

Morphine Injection, Stability of. E. Gundersen and J. Mørch. (*Dansk Tidsskr. Farm.*, 1955, **29**, 181). The decomposition of morphine to oxydimorphine in solution was followed by means of the vanillin reaction, as used by Foster, MacDonald and Whittet (*J. Pharm. Pharmacol.*, 1950, **2**, 673). The absorption maximum however appears to be at 640 mμ, not at 600 mμ as previously reported. It is important to use pure vanillin. Discolouration of the morphine solution was measured at 500 mμ, as these solutions show no maximum or minimum. The presence of glycerol does not interfere. The results confirm the conclusions of previous workers, and show that there is no appreciable decomposition after sterilisation for 20 minutes at 120° C. (pH = 4·1), although there is a slight discolouration, which may be prevented by the addition of sulphite. Various samples of 2 per cent. morphine injection, having an age of 10 years, showed from 1·6 to 4·4 per cent. decomposition. G. M.

PHARMACOLOGY AND THERAPEUTICS

Adrenaline and Noradrenaline in Tissues. K. Montagu. (*Nature, Lond.*, 1955, **176**, 555.) A study of the distribution of noradrenaline in rats does not support the hypothesis that its concentration can be correlated with the density of adrenergic nerve fibres. Two and a half hours after the subcutaneous injection of insulin there is a significant increase in the adrenaline catechol ratio in the heart, kidney, liver, diaphragm and leg muscles. There are significant increases in the absolute concentration of adrenaline in heart and kidney, and a significant increase in noradrenaline in the heart. Six weeks after bilateral demedullation, and 3 to 4 weeks after bilateral adrenalectomy, there is a reduction in the adrenaline concentrations of heart and kidney. In the kidney, noradrenaline is reduced, but in the heart it is increased. In demedullated and adrenalectomised rats, insulin does not increase significantly the adrenaline concentration of either heart or kidney. The altered effect of insulin on the concentration of adrenaline and noradrenaline, brought about by operation, is very significant

for heart muscle. The changes seem to depend on medullary secretion, while the presence of high concentrations of adrenaline and noradrenaline in heart and kidney, some weeks after adrenalectomy, suggests an ability of the rat to synthesise, or store the amines, in these organs. G. F. S.

Butylamine, A Substituted, (L1935), Release of Histamine by: Comparison with Compound 48/80. W. Feldberg and J. Lecomte. (*Brit. J. Pharmacol.*, 1955, **10**, 254.) L 1935, an equimolecular mixture of 3-(4′-hydroxyphenyl)-3-(4″-hydroxy-3″-methylphenyl)-1-methylpropylamine and its dehydro form, caused a release of histamine from perfused skin flaps of the cat's hind legs and from the perfused hind quarters of the rat. Weight for weight it was 10 to 12 times less active than compound 48/80. Injected intravenously into cats, the drug caused a fall in blood pressure after a latent period of 20 to 40 seconds, characteristic of the histamine liberators. Tachyphylaxis resulted from repeated injections. On the isolated guinea-pig ileum L 1935, added to the bath in high concentration, caused a transient contraction and after washing out, the ileum showed increased spontaneous activity and decreased sensitivity to histamine and acetylcholine. Similar effects were obtained with 48/80; the two substances were equiactive on the ileum. G. P.

Capsaicin and Analogues, Pharmacological Actions of. C. C. Toh, T. S. Lee and A. K. Kiang. (*Brit. J. Pharmacol.*, 1955, **10**, 175.) Intravenous injection of capsaicin into cats under chloralose anæsthesia caused apnœa, which was either abolished or greatly reduced by cooling the vagi to 9 to 10° C. Any residual apnœa disappeared with further cooling to 2 to 3° C. The site of action was traced to receptors in the lungs, probably pulmonary stretch receptors. The drug also caused a fall in blood pressure and heart rate, abolished by cutting the vagi; receptors in the coronary circulation were probably involved. With injections of capsaicin into the carotid sinus circulation, apnœa and a fall in blood pressure were again observed and were greatly reduced by cutting the sinus nerve. Sensitisation of the baroreceptors of the carotid sinus was suggested as causing the vasodepressor action. After injection into the splanchnic circulation a rise in blood pressure was obtained, abolished by removal of the superior and inferior mesenteric plexuses. The drug acted on undetermined receptors in the skeletal muscles causing hyperpnœa and variable effects on the blood pressure when injected close-arterially into the muscle circulation. These effects were absent after section of the nerve supplying the muscle. Capsaicin contracted the isolated guinea-pig ileum, which rapidly became tachyphylactic to it, but the drug had no effect on the rat uterus or perfused rabbit ear vessels. The analogues of capsaicin, vanillylamine and vanillyl acetamide had little or no pharmacological activity. Vanillyl-*n*-decoylamide, on the other hand, had similar activity to capsaicin. G. P.

Carboline Unsymmetrical Bis-quaternary Hypotensive Agents, Pharmacology of. T. B. O'Dell, C. Luna and M. D. Napoli. (*J. Pharmacol.*, 1955, **114**, 306.) Study of a group of carboline unsymmetrical bis-quaternary derivatives has shown many of them to be potent hypotensive agents having primarily a "central" site of action with varying degrees of peripheral effects. The hypotensive action in the anæsthetised dog was biphasic, an initial rapid fall with recovery in twenty minutes, followed by a more sustained secondary fall. Only the initial fall was prevented by atropine or vagotomy. In the series, increase of the methylene chain to more than three decreased the duration of action. Variations in the small or the large (carboline) cationic heads caused pronounced

differences in hypotensive action. The more potent compounds were effective by intramuscular injection or following injection into the small intestine. In the series there was very little correlation between hypotensive activity and sympathetic ganglion blockade, tested on the nictitating membrane of the cat. The most potent ganglionic blocking compound was the *n*-methyl tetrahydro-harman derivative. Toxic doses of the compounds caused death from repiratory depression and increase in the length of the methylene chain increased toxicity *In vitro*, on the isolated rabbit and guinea-pig ileum, the compounds showed varying degrees of blockade of acetylcholine, but they did not antagonise the vasodepressor action of acetylcholine in the dog. The vasopressor action of adrenaline was not blocked, but was potentiated by compounds causing ganglionic blockade. Some of the compounds caused dilatation and fixation of the pupils in male cats and humans. Several of the compounds blocked nicotine convulsions in mice. In unanæsthetised dogs large intravenous doses caused transient staggering, shivering and sometimes collapse. Mice and monkeys showed ptosis and became sleepy. G. F. S.

Catechol Amines Injected in Man, Amine Oxidase in the Inactivation of. U. S. von Euler and B. Zetterström. (*Acta physiol. scand.*, 1955, **33**, Suppl. 118, 26.) There was no significant difference between the percentage of adrenaline, noradrenaline and corbasil (3 : 4-dihydroxynorephedrine) excreted in the free form in the urine during the two hour period following their subcutaneous injection into healthy adults. Very little of any of the amines was excreted in the conjugated form. Since corbasil, although being unaffected by amine oxidase, is inactivated to about the same extent as adrenaline and noradrenaline, it may be inferred that some other enzyme system must play a significant part in the destruction of circulating catechol amines. M. M.

Cortisone and ACTH; Treatment of Blood Disorders. (*Brit. med. J.*, 1955, **2**, 455.) This is the Third Report to the M.R.C. by the Panel on the Hæmatological Application of ACTH and Cortisone, previous reports having been submitted in 1952 and 1953. Dosage tended to be higher in this than in the two previous series, most patients receiving at least 200 mg. of cortisone a day in their initial course of treatment. With the increased availability of cortisone tablets for oral administration the number of patients treated with cortisone rather than ACTH steadily increased. Twenty-eight further cases of acquired hæmolytic anæmia were treated, making a total of 48 cases treated since 1951. Of this number partial or complete response was obtained in 39 (81 per cent.). When a favourable response occurred this was always apparent within 2 weeks of starting an adequate dosage of cortisone. Eight patients were given continuous treatment with cortisone for at least 6 months, the dose requirement varying from 50 to 200 mg. a day. In all these patients a relapse could be induced promptly by stopping cortisone or reducing the dosage. In 7 of the patients there were no apparent toxic effects from the treatment; the eighth patient developed a gastric ulcer. Of 26 new cases of idiopathic thrombocytopenic purpura complete responses were obtained in 12 and partial responses in a further 10; these results were more favourable than those reported in 1952 (partial or complete responses in 11 out of 16) or in 1951 (partial or complete responses in 8 out of 14). This was probably due to the use of larger doses. Of 8 cases of non-thrombocytopenic purpura 6 failed to respond in spite of adequate dosage. No favourable response to cortisone was noted in 16 patients with aplastic anæmia or in 2 with refractory anæmia. In acute leukæmia the

administration of ACTH or cortisone induced temporary remissions in approximately half the patients treated (34 remissions in 49 children, and 22 remissions in 52 adults) and compared favourably with other forms of treatment. Remissions, however, were not of long duration, and few patients survived for more than a year. S. L. W.

Cortisone and Aspirin in the Treatment of Rheumatoid Arthritis. (*Brit. med. J.*, 1955, **2**, 695.) This is the second Report by the Joint Committee of the Medical Research Council and Nuffield Foundation on Clinical Trials of Cortisone, ACTH, and Other Therapeutic Measures in Chronic Rheumatic Diseases. The report relates to the second year of treatment of the 58 adult patients who completed the first year's treatment as described in the committee's first report. Dosage was determined by the physician in charge in accordance with each patient's need. The doses of cortisone in use at the end of the year were from 25 to 125 mg. per day, 17 of the 26 patients receiving either 75 or 100 mg. per day. The doses of aspirin were from 2 to 6·7 g. per day, 13 of the 20 patients receiving either 4 or 5 g. per day. Treatment of one patient in each group had to be discontinued during the year because of severe side-effects and 10 patients were not receiving treatment at the end of the year. Appraisal was based on joint tenderness, range of movement in the wrist, strength of grip, manual dexterity, hæmoglobin level, blood sedimentation rate, X-ray studies of the hands and feet, and clinical assessment. The results showed remarkable similarity between the two treatment groups, and in some cases they were even closer than at the end of the first year; in particular, the previously reported advantage of the cortisone group in respect of hæmoglobin level and sedimentation rate had disappeared. The X-ray investigation, by three independent observers, was a new measure of assessment; the aspirin group showed more erosion but the difference was not statistically significant. Side effects occurred in 19 patients of the cortisone group and 12 of the aspirin group. In the cortisone group the most frequent were œdema of the ankles, moon-face, depression, euphoria and obesity, and in the aspirin group, were nausea, dyspepsia or anorexia, tinnitus and œdema of the ankles.

H. T. B.

Demecolcine (Desacetylmethylcolcine) Intravenously in Acute Gout. W. C. Kuzell, R. W. Schaffarzick and W. E. Naugler. (*Arch. intern. Med.*, 1955, **96**, 153.) Demecolcine (Colcemid), an alkaloid from the meadow saffron, differing from colchicine structurally in the replacement of the acetyl by a methyl radical, was given intravenously to a series of 20 patients with acute gout. The patients ranged in age from 42 to 83 years and the known duration of gout from 1 to 45 years. Only acute attacks were treated. Of the 20 patients, 15 enjoyed complete remission within 48 hours after the intravenous administration of 1 to 4 mg. of demecolcine, and 4 others obtained partial amelioration of symptoms. The only undesirable side reaction was the occurrence of diarrhœa in 2 patients. In contrast with intravenous colchicine, there was a marked absence of venous irritation. S. L. W.

β-Dimethylaminoethyl-2-methylbenzhydryl Hydrochloride (B.S. 5930) in the Treatment of Parkinsonism. R. O. Gillhespy and A. H. Ratcliffe. (*Brit. med. J.*, 1955, **2**, 352.) This compound, belongs to the class of substance which antagonises both histamine and acetylcholine, of which the best known example is diphenhydramine hydrochloride. It has about the same toxicity in mice as

diphenhydramine. The compound was tried in the treatment of 67 patients suffering from Parkinsonism, and a clinical improvement was noted in 39 cases. The results were considered good or excellent in 31 cases (46 per cent.). Patients were given 150 mg. of the compound daily divided into three 50-mg. doses. In some cases the amount was increased to 250 mg. daily. Above that dose signs of intolerance developed, including restlessness, slight blurring of vision and nausea, but there were few unpleasant side-effects if the daily dose was kept below 250 mg. The authors conclude that B.S. 5930 compares favourably with other drugs for the treatment of the Parkinson syndrome and is a most useful addition to the range at present available. A new method of carrying out clinical trials of this type is described and its advantages discussed.

S. L. W.

***p*-Hydroxybenzoic Esters, Action of, Against *Candida albicans*.** T. Sabalitschka, H. Marx and U. Scholz. (*Arzneimitt.-Forsch.*, 1955, **5**, 259.) Esters of *p*-hydroxybenzoic acid have already been used with some success in cases of monialisis, and the authors now report the results of experiments *in vitro* on the action against the causative organism—*Candida albicans*, Robin. Six strains of the organism were used. The most effective compounds were the butyl esters, preferably in admixture. The action of the methyl ester was less marked, and the difference was more noticeable at low temperatures (20° C.) than at 37° C. At 37° C. the effect of the esters is only slightly decreased by the presence of bouillon.

G. M.

Iron by Intramuscular Injection in Infancy. W. Gaisford and R. F. Jennison. (*Brit. med. J.*, 1955, **2**, 700.) More than 100 infants with various types of anæmia were treated with intramuscular injections of Imferon, an iron-dextran complex containing 50 mg. of iron per ml. The injections were given deeply, into the upper and outer quadrant of the buttock, either daily, or alternate days or weekly, the dosage being calculated from the formula

Fe required in mg. = 1·31 × (19 − Hb mg.) × wt. in lb.

There was marked and rapid hæmatological and clinical improvement, the average rate of response being from 1·4 to 1·5 per cent. of hæmoglobin per day. Provided the dosage calculated to be equivalent to the iron deficit is not exceeded, no unpleasant reactions are produced and discomfort is of short duration. Although not a substitute for oral iron as a routine the preparation is valuable for the treatment of severely anæmic infants or those who cannot tolerate, or are resistant to, iron by mouth.

H. T. B.

Magnesia and Alkaline Carminatives in Infancy. R. D. G. Creery. (*Brit. med. J.*, 1955, **2**, 178.) Of 200 apparently healthy infants aged from 6 months to 3 years, 184 (92 per cent.) had at some time been given alkaline mixtures by their mothers. Magnesia had been given to 158 (79 per cent.), generally for regulation of the bowels, and 133 (66·5 per cent.) had received carminatives, usually for "wind". Both magnesia and carminatives had been given to 107 (53·5 per cent.). Often only an occasional teaspoonful had been given but in a considerable number of cases the mixtures had apparently been persisted with for prolonged periods. Although not actively harmful (alkali reserve levels were always within normal limits), this habit of dosing infants with alkalis is meddlesome and unnecessary and should be discouraged. The medicaments are often given for symptoms which exist only in the imagination of the mother. The frequent resort to magnesia is due to the obsession that infants should

defæcate daily. The crying and fretfulness often attributed to "wind" may well be due to unsatisfied hunger. One of the most popular of the proprietary carminative mixtures contains about 4 per cent. of ethanol which, acting as a mild soporific, may possibly serve to allay hunger and induce sleep. S. L. W.

Methiodides of Dimethylaminobenzoylhydroxamic Acids, Antagonists of Dyflos. A. Funke, G. Benoit and J. Jacob. (*C. R. Acad. Sci., Paris*, 1955, **240**, 2575.) The isomeric dimethylaminobenzoylhydroxamic acids, *o*, *m* and *p*, were obtained in the form of their sodium salts by the action of hydroxylamine on the corresponding esters. The hydrochlorides were prepared by acidifying with hydrochloric acid and the quaternary ammonium compounds by the action of an excess of methyl iodide on the sodium salts. The effect of these compounds was investigated in mice. In a series of protective tests in which the quaternary salts were administered 15 minutes before an injection of 6 mg./kg. of dyflos in water, the coefficient—dose killing 50 per cent./dose protecting 50 per cent.—was better than for nicotine hydroxamic acid methyl iodide. The administration of atropine with the *m*-compound enabled almost complete protection to be obtained. In the curative tests the compounds merely retarded onset of death, although a certain proportion of the animals lived when given atropine as well. Again the best results were obtained with the *m*-isomer. G. B.

Morphine, Mechanism of Pituitary-Adrenal Activation by. R. George and E. L. Way. (*Brit. J. Pharmacol.*, 1955, **10**, 260.) Morphine and (+)- and (−)-methadone all caused a significant fall in the ascorbic acid content of the adrenal glands of rats in a dose of 1/5 LD50, but not with a dose of 1/15 LD50. In hypophysectomised animals these doses had no effect on the adrenal ascorbic acid. After bilateral adrenal demedullation, on the other hand, much lower doses of the three analgesics caused an appreciable depletion. Pretreatment with cortisone did not prevent depletion of ascorbic acid by morphine, aspirin or adrenaline, but reduced the severity of depletion. Nalorphine inhibited depletion by morphine and by (−)-methadone, but not by (+)-methadone or aspirin. This may suggest that different receptors or sites are involved in the mediation of the depleting action, possibly in the hypothalamus. G. P.

Nitrofurantoin; Clinical and Laboratory Evaluation. B. A. Waisbren and W. Crowley. (*Arch. intern. Med.*, 1955, **95**, 653.) *In vitro* studies of the antibacterial activity of nitrofurantoin show that it has a wide antibacterial spectrum, is bactericidal, and does not readily invoke resistant mutants. It was found particularly effective against *Micrococcus pyogenes* and *Proteus* and ineffective against *Pseudomonas æruginosa*. Sixty patients with infections of the urinary tract were treated with nitrofurantoin, in an average daily dose of 300 to 400 mg. The results were satisfactory in 27 cases, unsatisfactory in 12 cases and indeterminate in 21 cases; 14 of the 27 cases in whom treatment was effective had received prior unsuccessful therapy with antibiotics. Nine of the 60 patients had difficulty in tolerating nitrofurantoin owing to nausea and vomiting, but in only 1 case was it necessary to discontinue treatment. In none of the patients was there any evidence of rectal irritation or of depression of hæmopoiesis due to nitrofurantoin, nor were skin rashes or other manifestations of hypersensitivity encountered. Nitrofurantoin did not cause diarrhœa in any of the patients. Judged as a whole the clinical results obtained were satisfactory and equal to those obtainable with any other single drug. S. L. W.

Noradrenaline and Adrenaline Content in Cat Organs, Effect of Increased Adrenergic Nerve Activity on. U. S. von Euler and S. Hellner-Björkman. (*Acta physiol. scand.*, 1955, **33**, Suppl. 118, 17.) Electric stimulation of the splenic nerves of the cat caused no significant change in the adrenaline and noradrenaline content of the stimulated portion of the spleen, when compared with the non-stimulated portion. Increased reflex adrenergic activity, elicited by occlusion of the carotid artery or by denervation of the carotid sinus, also caused no significant change in the noradrenaline content of the spleen, heart and liver. These facts suggest that the release of adrenergic transmitter is normally followed by rapid resynthesis, thus allowing the store of amine to be maintained at an approximately constant level. M. M.

***iso*Quinoline Unsymmetrical Bis-quaternary Hypotensive Agents, Pharmacology of.** T. B. O'Dell, C. Luna and M. D. Napoli. (*J. Pharmacol.*, 1955, **114**, 317.) A series of unsymmetric bis-quaternary derivatives of *iso*quinoline and related compounds have been studied for hypotensive activity. Many of them are potent hypotensive agents with primarily a central site of action, showing a biphasic fall in blood pressure in the anæsthetised dog. The bis-quaternary salt seemed essential for overall hypotensive activity. Increasing the methylene chain showed a chain length of three to provide maximum activity. Variations in the small cationic head resulted in very pronounced differences in activity, while substitution on the large cationic (*iso*quinoline) head caused considerable variations in hypotensive activity. Derivatives of the benz*iso*quinolines were generally more active and more toxic than the simple *iso*quinoline analogues. Most interesting were the tetrahydro derivatives (tetrahydro*iso*quinoline and benztetrahydro*iso*quinoline) all of which were more active than the analogous *iso*quinoline derivative. There was no correlation between hypotensive activity and sympathetic ganglion blockade. Toxic doses caused respiratory paralysis. *In vitro* the compounds showed varying degrees of blockade to acetylcholine but they did not block the vasodepressor action of acetylcholine in the dog. The vasopressor action of adrenaline was not blocked or depressed by any of the compounds. G. F. S.

Roter Tablets in the Treatment of Peptic Ulcer. R. R. Hamilton. (*Brit. med. J.*, 1955, **2**, 827). Seventy-nine cases of peptic ulcer and 19 of presumptive peptic ulcer were treated with Roter tablets. Immediate clinical response was satisfactory in 90 per cent. of patients (81 per cent. became symptom-free within 2 weeks), with a relapse rate of 57 per cent. in the first year. A reduction in the average number of relapses was recorded in 15 cases kept on a maintenance dose for 1 year. Roter tablets contain heavy magnesium carbonate 400 mg., sodium bicarbonate 200 mg., bismuth subnitrate 350 mg., calamus 25 mg., an aromatic bitter, and frangula. The patients (all of whom were ambulant) were instructed to take 2 tablets three times a day after food for 3 weeks; thereafter 1 tablet three times a day for 2 months. Fifteen patients with a long history of frequent relapses continued on a maintenance dose of 2 tablets daily for a year. The patients were advised to take an average diet but to avoid fried foods. In 75 per cent. of the cases the patients were of the opinion that the tablets were superior to alkaline powders, and they found they were able to take foods they had avoided for years. No serious toxic effects were observed in any of the patients treated. Though the author admits that a study of the individual drugs contained in these tablets yields no solution to their mode of action, he considers that the treatment is of value for general practice, where its simplicity appeals to both doctor and patient. S. L. W.

Serotonin Release as a Possible Mechanism of Reserpine Action. A. Pletscher, P. A. Shore and B. P. Brodie. (*Science*, 1955, **122**, 374.) Reserpine (5 mg./kg.), injected intraperitoneally in rabbits, effected the release of up to 85 per cent. of total serotonin in intestinal tissue. The serotonin content of the small intestine decreased progressively for about 16 hours after administration of the reserpine and, after remaining constant for a further 16 hours, regained normal levels after 5 days from injection. Serotonin depletion in the intestine was demonstrable with doses down to 0·25 mg./kg. Phenobarbitone and barbitone in doses inducing deep narcosis had no effect on intestinal serotonin content. The results were consistent with the view that reserpine owes some of central sedative actions to the release of serotonin.

G. P.

Steroid Anæsthesia in Surgery. F. J. Murphy, N. P. Guadagni and F. DeBon. (*J. Amer. med. Ass.*, 1955, **158**, 1412.) This is a report on the use of the steroid Viadril (21-hydroxypregnanedione sodium succinate) as a basal anæsthetic on 125 patients in a variety of surgical procedures. Viadril is a white, crystalline substance, soluble in water and having an alkaline reaction (pH 8·5 to 9·8). It is administered intravenously in a freshly prepared 2·5 per cent. solution in either distilled water or isotonic sodium chloride solution. The Viadril solution is introduced via the intravenous tubing into an intravenous drip of 5 per cent. glucose in distilled water, so that mixing and dilution of the two solutions takes place in the tubing before the needle is reached. Approximately 5 minutes is required to give 1500 mg. and an additional 5 minutes is allowed before the patient is considered ready for further manipulation. In this series pre-medication usually consisted of administration of 50 to 75 mg. of pethidine hydrochloride, and hyoscine or atropine in the appropriate dosage. In the lightly pre-medicated patient Viadril produces sleep in 5 to 10 minutes, without an excitement phase. The authors conclude that Viadril is a true anæsthetic agent, as evidenced by its ability to control pain, obtund reflexes, produce relaxation, and produce sleep, without depression of vital functions. Most satisfactory results have been obtained with administering the compound with nitrous oxide 75 per cent. and oxygen 25 per cent. In many cases it has been found necessary or advantageous to add pethidine hydrochloride, other agents, or relaxants, but in these cases the dosages required have been considerably smaller than would have been employed had the patient been receiving thiopentone sodium-nitrous oxide-oxygen. No complications were observed following the use of Viadril except for three cases of thrombophlebitis in the injected vein: in each of these cases the patient had widespread vascular disease and was receiving the anæsthetic for an aortogram. S. L. W.

Synthalin A as Selective Mitotic Poison Acting on Alpha-Cells of Islets of Langerhans. H. Ferner and W. Runge. (*Science*, 1955, **122**, 420.) Synthalin A (decamethylene diguanidine dichlorhydrate), decreased the mitotic rate of the alpha-cells of the islets of Langerhans (considered to be the producers of glucagon) in one- to five-day-old rats. The drug was given in a single subcutaneous injection of 10 mg./kg. and the rats were killed 12 to 18 hours later. In none of the animals were there any signs of lesion of the alpha-cells, although lesions and alpha-cell destruction often occur in adult rats after Synthalin A administration. Beta-cell mitosis was not affected by the drug.

G. P.

BACTERIOLOGY AND CLINICAL TESTS

Mumps Virus, Reproduction in the Chorio-allantoic Membrane. H. L. Wolff. (*Nature, Lond.*, 1955, **176**, 604.) The cultivation of mumps virus is described in the chorio-allantoic membrane of the incubated hen's egg. Using "Enders" mumps strain, compliment fixing antigen could not be detected until 18½ hours after inoculation, and hæmagglutin after 19½ hours. The infection titre decreased from 2½ hours after inoculation, reaching a minimum after 5 hours and remaining low until 14½ hours when it slowly rose again. Experiments were also carried out using the chorio-allantoic membrane of 9 day old chicks cut into small pieces and placed with the virus into a petri dish. It is concluded that mumps virus is reproduced in the living chorio-allantoic cell in 14 to 17 hours.

G. F. S.

Mustard Oils, Bacteriostatic Action of. P. Klesse and P. Lukoschek (*Arzneimitt.-Forsch.*, 1955, **5**, 505.) A strong bacteriostatic action was observed with a number of synthetic mustard oils: methyl, ethyl, allyl, phenyl, benzyl and phenylethyl mustard oil. Effective concentrations ranged between 1:60,000 and 1:2,000,000 against *Staph. aureus*, *E. coli* and *Sarcina lutea*. The action is also observed in the urine—after the administration of 10 mg. of benzyl mustard oil the urine was found to be bacteriostatic for more than 10 hours. In plate tests the action is decreased by a factor of from 3 to 10 by the presence of blood or serum. By subcutaneous injection into mice, the toxicity showed a variation between LD50 = 0·05 g. per kg. for methyl mustard oil, and LD50 = 0·25 g. per kg. for phenylethyl mustard oil. G. M.

Usnic Acid, Antibacterial Action of. J. R. Möse. (*Arzneimitt.-Forsch.*, 1955, **5**, 510.) Tests were carried out with pure usnic acid against a large variety or micro-organisms, using the plate method. With the exception of meningococci and *Neisseria flava*, only Gram-positive bacteria were affected. No action was observed on moulds or viruses. The minimum concentration required (for *Staph. aureus* and *N. flava*) was 1:64,000. Thirty different strains of *Clostridium tetani* showed particularly fine bacteriostatic zones of 10 to 12 mm., which is considerably greater than with penicillin and streptomycin. Although there were differences between the many different strains of *Staph. aureus* tested, only one proved resistant. The effect differed according to the solvent used and the pH value of the solution, and was also modified by various additions such as powder and ointment bases. Usnic acid is not affected by the action of heat or of ultra-violet light. Its toxicity appears to be low. The oral administration of 2 g. of the pure acid to a guine-pig of 4·08 kg. weight showed no permanent ill effects. In this case the bacterostatic action of the urine was nil. G. M.

Water-soluble Filter for Trapping Airborne Micro-organisms. M. Richards. (*Nature, Lond.*, 1955, **176**, 559.) The preparation of a water soluble "wool" of sodium alginate to trap spores is described. Calcium alginate yarn is cut into short lengths and stirred into a large volume of water to untwist the filaments. After draining on a Buchner funnel the mass of calcium alginate wool is soaked in N hydrochloric acid to leech out the calcium, drained, suspended in 50 per cent. ethanol and neutralised with sodium hydroxide. The sodium alginate yarn produced is soluble in water. It is washed with 50 per cent., then absolute ethanol and dried. The wool is used to trap micro-organisms when packed as a plug in a special brass tube. It is very useful for sampling airborne fungi and bacteria for culture. G. F. S.

BOOK REVIEWS

THE QUANTITATIVE ANALYSIS OF DRUGS, by D. C. Garratt. Second edition. Pp. xv + 670. Chapman and Hall, London, 1955. 70s.

The first edition of this book was published in 1937 under the title of *Drugs and Galenicals: Their Quantitative Analysis*. Since that date many changes have occurred in pharmacy and some of these changes are reflected, in the work under review, by the inclusion of new sections dealing with synthetic organic chemicals and by the deletion of the word "Galenicals" from the original title. It was stated, in the first edition, that the author had relied mainly on his own collection of notes and references collected during his analytical experience; it was, in fact, this which gave the work much of its practical value. The new edition has been awaited for some time and it is of interest to see how far, after a period of 18 years, the original practical approach to the subject could be maintained in the much wider field of present day pharmaceutical analysis.

The general arrangement of the first edition has been retained, a substance, its salts, similar compounds, and preparations containing the substance, being grouped together. Thus barbiturates are listed and described as a group, as are the sulphonamides, sugars, soaps, paraffins, flavines, and compounds containing mercury. New sections have been added where necessary and those describing the penicillins and soapless detergents are particularly informative. The appendices to the original volume have been enlarged and new additions include an account of titration in non-aqueous solvents and a review of physical methods of analysis.

In describing the various methods of assay available for a substance or group of substances, the author clearly states the alternative methods available and gives reasons for his final choice. For many official B.P. or B.P.C. assays, points of practical analytical detail from the author's experience are given which will greatly assist any newcomer to the method.

Few criticisms can be made and a preliminary reading did not show any typographical errors. If one wished to be hypercritical the choice and exclusion of certain substances might be queried. Thus a monograph on solapsone is included but not one on cyanocobalamin; mephenesin and chloramphenicol have also been omitted and future editions might well include a separate section on the antihistamines. These points however, do not detract from the value of the book but serve to emphasize the rapid changes which now occur in the contents of official volumes.

The book is attractively produced and bound, and well indexed. It can be stated that the author has, in fact, maintained his original standard, resulting in a practical handbook and reference work which will be essential for any laboratory engaged in pharmaceutical analysis.

R. E. STUCKEY.

METHODEN DER ORGANISCHEN CHEMIE (Houben-Weyl). Volume III, Part 2. Physikalische Forschungsmethoden. Fourth Edition. Edited by Eugen Müller. Pp. xxviii + 1078 (including 507 illustrations and Index). Georg Thieme Verlag, Stuttgart, 1955. Moleskin Dm.186.00.

This latest volume of the new fourth edition of Houben-Weyl is the second of two which are to be devoted to physical methods, and deals solely with electrical, optical, magnetic and acoustic analytical methods. The layout of this book

follows the now usual pattern with detailed monographs each by a specialist on the major subdivisions of the subject. Included are chapters on the measurement of pH; potentiometric and conductimetric titrations; redox potentials; polarography; dielectric constants; refractometry; polarimetry; fluorescence; phosphorescence; electron microscopy; X-ray crystallography; visible, ultraviolet, infra-red and Raman spectroscopy; microwave spectroscopy; magnetic methods and ultrasonics. The theory of pH is presented, as was to be expected, from the standpoint of the Lowry Brønsted theory of acids and bases, so that such sections as those on the measurement of pH in non-aqueous media, Hammet acidity functions and indicator theory, fall naturally into place. On the experimental side, the description of apparatus and methods for the measurement of pH and redox potentials, and for conductimetric and potentiometric titration is given, as in all these volumes, in a wealth of detail. These descriptions are often quite sufficient in themselves for the average worker to establish the necessary technique without further reference, but, as always, in addition full reference to original literature is also included. By comparison the single chapter on the polarography of organic compounds is a little disappointing. Much of it has been devoted to general theory and the description of apparatus which, although excellently presented in itself, is general to all polarographic work, whilst what appears to the reviewer as the real purpose of the chapter has been relegated to the last few pages. As may be expected, a large section of this volume has been allocated to the theory and practice of light absorption measurements. Especially welcome here is the chapter on Raman spectra which are, of course, complementary to the infra-red and for which few volumes of this type provide adequate treatment. The tabulation of solvents suitable for infra-red measurements in various ranges of the spectrum provides a useful collection of information, whilst the collection and diagramatic representation of absorption data for the various functional groups is a valuable addition. The inclusion of examples of the use of infra-red spectra in the determination of organic structures greatly enhances the value of this section. The newer techniques of micro-wave spectroscopy and ultrasonics are also briefly described. The volume is remarkably free from misprints, beautifully finished, and although part of a large series can be seriously entertained as a work, complete in itself, which will be of value both to the specialist and the routine analyst.

J. B. STENLAKE.

LETTER TO THE EDITOR

Thiambutene and Barbiturate Anæsthesia in the Dog

SIR,—I regret and apologise for certain errors in doses which occur in the article "Thiambutene and Barbiturate Anæsthesia in the Dog," published in this Journal 1955, 7, pp. 533–540. In all cases subcutaneous doses of thiambutene were 22 mg./kg. and intravenous doses 4·4 mg./kg. Intravenous doses of nalorphine were 2·2 mg./kg.

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