of the oestrogens administered to human subjects as a sulphate ester. The absence of any detectable change in the excretion of ethereal sulphate after giving the synthetic oestrogens to rabbits (Dodgson *et al.* 1947) and the occurrence of only small changes of doubtful significance in the ethereal S : inorganic S ratio in the cow (Malpress & Owen, 1947) suggest, however, that these substances are normally excreted either in the free form or combined as glucuronide. If this is so the methods described in this paper should be capable of yielding comprehensive data on the quantitative excretion of the oestrogens, and afford some insight into the measure of any metabolic breakdown.

SUMMARY

1. Methods are described for the estimation of the synthetic oestrogens stilboestrol and hexoestrol and their glucuronides in cow's or human urine.

2. After addition to human urine 80 % or more of these substances were recovered, except with stilboestrol glucuronide where decomposition during hydrolysis reduces the figure to 63 %. Recoveries from cow's urine were slightly lower in all cases.

I wish to express my thanks to Dr R. T. Williams for samples of stilboestrol glucuronide and glucurone, and to Prof. D. C. Harrison and Dr S. J. Folley for their interest in the progress of this work.

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The Fifth Coagulation Factor ('Factor V'). Preparation and Properties

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In 1943 a previously unknown coagulation factor was discovered and termed the fifth coagulation factor, or 'Factor V' (Owren, 1944). Factor V is necessary for the conversion of prothrombin to thrombin, and the rate of this reaction increases with increasing amounts of Factor V up to a certain limit. Lack of Factor V produces a haemorrhagic diathesis, termed *parahaemophilia* (Owren, 1946, 1947*a*). The isolation of this factor and its function in the coagulation process has been discussed in detail elsewhere (Owren, 1944, 1945, 1946, 1947*b*).

In the present paper experiments are described on the basis of which a new method for the preparation of Factor V has been developed, providing a more highly purified product.

METHODS

Ox plasma

Ox blood (9 vol.) was mixed with 1 vol. of 4% (w/v) potassium oxalate monohydrate solution, the mixture cooled to 1° and the plasma obtained by centrifuging at this temperature.

Prothrombin-free plasma

In order to obtain Factor V absolutely free from prothrombin or thrombin, prothrombin-free plasma was prepared. Prothrombin is far more readily adsorbed than Factor V by adsorbents such as $Mg(OH)_2$, $Al(OH)_3$ and $Ca_3(PO_4)_2$, but it is difficult to obtain plasma absolutely free from prothrombin with these agents. On the other hand, 10–40% of Factor V in the plasma is adsorbed simultaneously with the prothrombin depending on the agent used and the relative quantities of adsorbent and plasma (Owren, 1947*b*, p. 78). Asbestos differs from these agents in adsorbing prothrombin selectively. Using asbestos-paper filter pads containing 30–40% asbestos, prothrombin-free plasma with an almost unchanged content of Factor V can be obtained. Filters of higher asbestos content remove some of the Factor V.

The following procedure was adopted. The plasma was passed through a clarifying filter containing about 20% asbestos, and then twice through pads containing 40% asbestos (Hodgkinson, Wookey Hole, Somerset). Before filtration the pads were washed with citrate-saline (0·4% (w/v) trisodium citrate, 0·9% (w/v) NaCl).

Preparation of Factor V

Principle

Human fibrinogen prepared by the ether-fractionation method of Kekwick, Mackay & Record (1946) appeared to be free from Factor V, and this encouraged an examination of the applicability of the procedure to bovine plasma.

The crude fibrinogen precipitate obtained by addition of 0·1 vol. ether to plasma at unadjusted pH and 0° was found to contain 10 % of the plasma Factor V. This was removed by repeated washing with distilled water and the fibrinogen was further purified by reprecipitation.

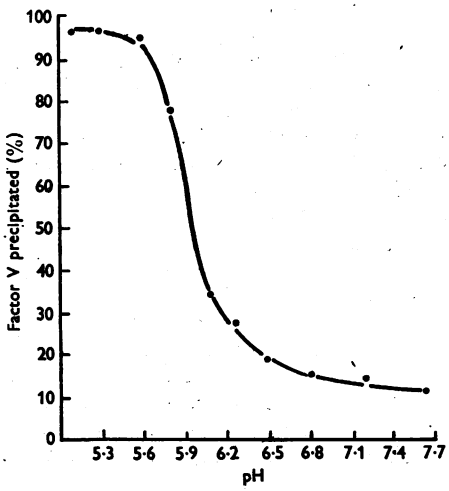

Fig. 1. The precipitation of Factor V from diluted plasma with 0·1 vol. ether at various pH values ($\mu = 0{\cdot}05$, and $-1°$. Protein conc. approx. 2%).

After the removal of the fibrinogen, about 50 % of the plasma globulins were precipitated by adjusting the pH to 6·5 and lowering the ionic strength to $\mu = 0{\cdot}05$ by dilution with distilled water, the ether being maintained at 10 % (v/v). The supernatant fluid from this precipitation contained substantially all the plasma Factor V.

The pH of samples of this fluid was further lowered and the precipitates which formed were assayed for Factor V. The precipitation of Factor V as a function of pH is shown in Fig. 1. It can be seen that by lowering the pH to 5·3, the Factor V is almost completely precipitated. The amount of protein and Factor V extracted from this precipitate by acetate buffers of pH 5·23 and varying ionic strength is shown in Fig. 2. From this it is obvious that by extracting the precipitate with acetate buffer, pH 5·23, $\mu = 0{\cdot}04$, about 30 % of the protein can be removed with only a minimal loss of Factor V.

The precipitate formed at pH 5·3 contains a sticky yellow substance which like Factor V is soluble in water at pH 6·5 and above, but unlike Factor V is insoluble in acetate buffer, pH 5·23, $\mu = 0{\cdot}10$. Consequently it can be removed by dissolving the precipitate and reprecipitating under these conditions. In the presence of a certain amount of sodium chloride the solubility of Factor V is increased, and by this means the loss of Factor V in the precipitate is reduced to about 10 %.

On the basis of these findings, the following preparative procedure was developed.

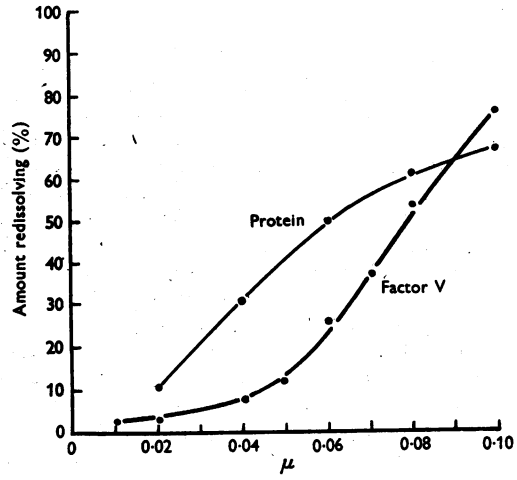

Fig. 2. The solubilities of Factor V and protein (precipitated from plasma at pH 5·3 with ether) in acetate buffer at pH 5·23 and various ionic strengths.

Procedure

(1) Prothrombin-free ox plasma was diluted with 2 vol. of distilled water, cooled to $-1°$, and 10 % (v/v) ether was added slowly through a capillary jet, the temperature being kept at -1 to $-2°$. The precipitate was allowed to settle for 3 hr. and was then removed and used for the preparation of fibrinogen.

(2) The supernatant fluid was adjusted to pH 6·5 by the addition of 0·1 N-acetic acid and left for 2 hr. at $-1°$. The precipitate was removed.

(3) The supernatant fluid from (2) was adjusted to pH 5·3 by further addition of 0·1 N-acetic acid. After 1 hr. at $-1°$ the precipitate was collected by centrifuging at $-2°$. This precipitate contains about 80 % of Factor V in the plasma.

(4) The precipitate was washed at 0° with acetate buffer, pH 5·23, $\mu = 0{\cdot}04$, by suspending and centrifuging. It was then suspended in a volume of distilled water equal to 20 % of the original plasma volume, and dissolved by adjusting the pH to 6·5 with 0·1 N-NaOH. Factor V dissolved completely, and a small amount of undissolved material was removed by centrifuging.

(5) To the solution NaCl was added to give an ionic strength of $\mu = 0{\cdot}10$, and the pH was then lowered to 5·3 by the addition of $\frac{1}{3}$ vol. of acetate buffer, pH 5·23, $\mu = 0{\cdot}10$. A precipitate of yellow protein was formed and removed after 1 hr. by centrifuging in the cold.

(6) Factor V was precipitated from the supernatant fluid by lowering the ionic strength to 0·04 by the addition of 150 ml. distilled water to each 100 ml. solution. After 2 hr. at 0°, the precipitate was collected on the centrifuge, washed with distilled water and dissolved by suspending it in distilled water and adding 0·1 N-NaOH to pH 7·0.

(7) The solution of Factor V was dried from the frozen state.

The yield obtained was about 60% and the purification 100–150 times in terms of activity/mg. of nitrogen. It is essential that the whole procedure should be carried out at low temperature as Factor V is inactivated by ether above 0°, especially at pH 5·3.

Properties of Factor V

The dried preparation of Factor V is a white, amorphous water-soluble protein material.

Stability. The dried product, in sealed bottles, is stable indefinitely. When stored in solution at 0° the activity decreases about 50% in the 1st week. Higher temperatures increase the rate of inactivation. The stability on storage of Factor V in human plasma is of the same order, with a reduction to about 30% of the original activity after 8 days at 0°, and to below 10% in the same time at 10°. The rate of inactivation varies considerably. In ox plasma the stability as a rule is greater. The inactivation by heat, acid and alkali was reported upon previously (Owren, 1947*b*, pp. 85, 90).

Adsorption. As stated above, Factor V is partly adsorbable from plasma with $Mg(OH)_2$, $Al(OH)_3$ and $Ca_3(PO_4)_2$. From purified solutions Factor V may be removed almost completely by these agents, and Seitz-filtration reduces the activity considerably.

Function. The influence of Factor V on thrombin formation has been described previously (Owren, 1944, 1945, 1947*b*, p. 182). Without Factor V no thrombin is formed.

The dried preparation of Factor V described above (0·2 mg.) produced the following effects: (1) The time for complete conversion of prothrombin to thrombin in 1 ml. of a mixture containing prothrombin (10 P.U.), thrombokinase (human brain, optimal amount) and Ca (2·5 mM) was shortened from ∞ to 75 sec. at 37°. (2) The coagulation time for 1 ml. of a mixture containing prothrombin (100 P.U.) thrombokinase (optimal amount), Ca (2·5 mM) and fibrinogen (0·10%) was shortened from ∞ to 16 sec. at 37°. (3) The 'prothrombin time' by Quick's method in a case of parahaemophilia was shortened from 68 to 15 sec. The 'prothrombin time' of human oxalated plasma stored 1 week at 5° was shortened from 38 to 16 sec.

DISCUSSION

Quick (1943, 1946) has postulated the existence of two components of prothrombin, designated prothrombins A and B. Prothrombin A is labile and disappears when plasma is stored, prothrombin B is more stable, is completely adsorbed by $Al(OH)_3$ (which does not remove prothrombin A) and its content is lowered in dicumarol poisoning.

The hypothesis is chiefly based on the fact that the prolonged 'prothrombin time' (Quick, 1943) of stored plasma is shortened by the addition of plasma, previously treated with $Al(OH)_3$ to remove adsorbable material, or plasma from animals poisoned with dicumarol.

Quick's theory has been supported by others (Oneal & Lam, 1945; Zondek & Finkelstein, 1945; Munro, Hart, Munro & Walking, 1945; Munro & Munro, 1947), but questioned by Link (1945), Banfi, Bay & Tanturi (1945); Loomis & Seegers (1947). Loomis & Seegers (1947) believe that the labile component partially inactivated during storage is fibrinogen. They hold the opinion that plasma treated with $Al(OH)_3$ acts by supplying reactive fibrinogen, since the same effect could be produced with stored plasma by the addition of a fibrinogen preparation.

It has been shown previously that Factor V is the most unstable coagulation component in plasma during storage; prothrombin usually remains unchanged for 6 days in plasma stored at 0°, and the reactivity of fibrinogen keeps unaltered for at least 10 days at 0° (Owren, 1947*b*, pp. 84, 143, 271). The prolonged 'prothrombin time' by Quick's method in stored plasma is, therefore, first and foremost due to the inactivation of Factor V. After storage for a long time, reduced prothrombin concentration and decreased activity of fibrinogen may exert some influence. This is confirmed by the following experimental evidence.

Oxalated human plasma stored at 5° for 8 days showed a decrease of Factor V to 10% of the original value, and of prothrombin to 75%. The prothrombin time was reduced from 34 to 15 sec. on addition of Factor V, whilst the addition of prothrombin (20 P.U. free of Factor V) and fibrinogen (0·10%, free of Factor V and profibrin) gave prothrombin times of 30 and 32 sec. respectively.

The prothrombin time of plasma from a patient with parahaemophilia after storage for 3 weeks at 0° was reduced from 110 to 17 sec. on addition of Factor V, whilst fibrinogen and prothrombin, both free from Factor V, were without influence.

The effect of fibrinogen in the experiment of Loomis & Seegers (1947) can be explained by the fact that fibrinogen precipitated from bovine plasma by the ethanol method used can be shown to contain about 10% of the plasma Factor V. Further, it should be mentioned that fibrinogen which contains profibrin will also shorten the clotting time when added to plasma.

Fantl & Nance (1946) have found that prothrombin-free plasma contains a factor which accelerates

the conversion of prothrombin to thrombin, and Ware, Guest & Seegers (1947*a*) have isolated such a factor by fractionation of prothrombin prepared by $Mg(OH)_2$ adsorption. They suggest that this factor is not identical with any of Quick's prothrombins.

There seems to be no doubt that Quick's prothrombin A and Factor V are identical. The factor is concerned with the conversion of prothrombin to thrombin, however, and is not itself a component of prothrombin. The factor is partly adsorbed by $Mg(OH)_2$, and prothrombin prepared by this method always contains some Factor V. (Prothrombin which is absolutely free of Factor V is not converted to thrombin by thrombokinase and Ca alone (Owren, 1944, 1947*b*, p. 186).) Factor V is precipitated by the ammonium sulphate fractionation used by Ware *et al.* (1947*a*) for isolating the accelerator factor from prothrombin preparations. The relatively slight activity of the material isolated (shortening of the conversion time to 3, 7 and 10 min.) indicates that the amount of Factor V is low.

The mode of action described by Ware *et al.* (1947*b*) for their accelerator factor, however, conforms with the previously described action of Factor V (Owren, 1944, 1945, 1947*b*), and there is no reason to believe that they are different substances.

The recorded properties of Factor V and experiments on a quantitative basis point to the fact that prothrombin A, Seegers's accelerator factor and Fantl & Nance's factor are identical with Factor V.

SUMMARY

1. A new method for the preparation of 'Factor V' is described, and its properties are outlined.

2. Experiments are submitted indicating that the prothrombin A of Quick (1943, 1946) and the accelerator factor of Fantl & Nance (1946) and of Ware *et al.* (1947*a*, *b*) are identical with Factor V.

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The Detection of Creatine and Creatinine by Partition Chromatography

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Methods commonly used for the identification and quantitative estimation of creatinine, based on the Jaffé reaction, suffer from the lack of specificity of this reaction. Hunter (1928) has listed 40 compounds which give a positive creatinine reaction with alkaline sodium picrate. The use of 3:5-dinitrobenzoic acid (Benedict & Behre, 1936), although it provides a slightly more specific reaction, introduces the serious complications of colour fading and sensitivity of the coloured product to the presence of other substances. The picric acid method of estimation is reliable, however, if precautions are taken to exclude the interference of other chromogenic substances, or when combined with specific enzymic destruction of creatinine by *Corynebacterium ureafaciens* (Miller & Dubos, 1937; Krebs & Eggleston, 1939).

The estimation of creatine in the presence of creatinine by the picric acid or 3:5-dinitrobenzoic acid methods has the added disadvantage of being