procedure should be determined for other organs. If one wants to be sure that 100 % of the glycogen present in any organ is determined, the usual hydrolysis of the tissue with strong alkali followed by precipitation of the glycogen remains the method of choice. Using the iodine reagent only approximate figures are obtained for the glycogen content of a liver extract. Nevertheless, this very convenient method may be useful for investigations where some accuracy may be sacrificed in order to make possible experiments with a large series of rats. For instance this was the case in our studies of the glycogenetic properties of adrenal steroids by estimation of liver glycogen, in which the experimental animals are treated following a standard procedure, and the results are always mutually compared. The results of these tests were hardly influenced by the variable colour intensities of the glycogen from different livers. However, if approximate values cannot be accepted the use of the anthrone method is indicated. Providing the lower saccharides are previously destroyed with alkali, more correct results are obtained, but the method is more elaborate than the estimation with iodine.

SUMMARY

1. Two methods are described for the determination of glycogen. In both procedures the glycogen is extracted from the tissue with trichloroacetic acid.
2. In these extracts glycogen can be estimated approximately by means of an iodine reagent. The

colour intensity of the glycogen-iodine complex varies from one liver to another and is further influenced by the treatment of the animals.

3. More correct results are obtained if the glycogen in the extract is determined using an anthrone reagent, after treating the liver extracts with alkali which eliminates interfering glucose.

4. The application of the methods is briefly discussed.

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The Use of Substances Depressing Antithrombin Activity in the Assay of Prothrombin

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Determination of the prothrombin activity of body fluids has become of clinical interest in connexion with the diagnosis of haemorrhagic disorders, and during treatment with anticoagulants. Most techniques for the determination of prothrombin are based on one or other of two principles. In both, prothrombin is converted into thrombin in the presence of optimum amounts of the other clotting factors. In one, the time interval required for clot formation in the specimen is taken as a measure of prothrombin activity; this is a direct method, and is referred to in the literature as the one-stage technique (Quick, 1942). In the other, the thrombin potency developed is estimated with an added

substrate containing fibrinogen; this is an indirect method often called the two-stage technique (Warner, Brinkhous & Smith, 1936).

In the direct method a fibrin clot appears at a certain time corresponding to the formation of a certain concentration of thrombin. The rate of thrombin formation depends upon the concentration of prothrombin and the activity of accelerating and inhibiting factors. The direct method is useful in determining the haemostatic condition of a patient, but it does not indicate the actual prothrombin concentration of the specimen.

In the direct method, fibrin formation takes place at a very low thrombin concentration, which is

normally only a small fraction of the potential total thrombin yield. The bulk of thrombin production will therefore occur after coagulation has taken place. Most biological fluids which contain the clotting components can also inactivate thrombin. Therefore the yield of thrombin is determined by the balance between formation and inactivation. It is apparent that this will vary with the concentration of prothrombin and the antithrombic potency of the specimen.

On the other hand, in the indirect method the total yield of thrombin obtainable from the prothrombin-containing preparation is determined, and this is not dependent on the rate of thrombin formation. The indirect method is less suitable for the detection of a haemorrhagic condition, but gives an indication of the quantitative relation between prothrombin activity and its concentration. Because of the different principles involved the results obtained by the two methods do not always agree. In the most widely used indirect process for the determination of prothrombin devised by Warner *et al.* (1936), the activity of antithrombin is reduced by dilution of the specimen and by addition of gum acacia (Seegers & Smith, 1942). Sternberger (1947) has found that the presence of 25% ethanol ensures thrombin stability. The following experiments indicate that a number of phenols which accelerate the thrombin fibrinogen reaction have also the property of depressing antithrombin activity. A prothrombin assay including such substances is presented.

EXPERIMENTAL

Materials

Venous blood from humans of both sexes, aged 2–70 years, was obtained at least 2 hr. after a meal. Animals were anaesthetized with Nembutal and ether, and blood was obtained from guinea pigs by heart puncture, and from rabbits by cannulating the carotid artery. Blood (9 vol.) was mixed quickly with either 1 vol. of 0.1 M sodium oxalate or with 1 vol. of 0.13 M trisodium citrate. Cell-free plasma was obtained after centrifuging at 2300 *g* at 6° for 20 min. Both cell and plasma volume were measured. The plasma specimens were kept at 6° until the test was carried out, which was not more than 6 hr. after collection. Human serum was obtained from blood collected without any addition. It was separated from the clot after at least 24 hr. storage at 20°. Usually serum from two donors was pooled.

BaCO₃ was freshly prepared according to Fantl & Nance (1947); BaSO₄ was prepared from equivalent amounts of Ba(OH)₂ and dil. H₂SO₄, and washed with distilled water until neutral. The wet suspensions of the barium salts were used for not more than 1 week after preparation.

Prothrombin-free plasma, which will be called Ba-plasma, was obtained by mixing suspensions containing 10–20 mg. BaSO₄, or BaCO₃/ml. of oxalated plasma, and frequent stirring in ice water for 30 min.

Thromboplastin preparations were made from human or rabbit brain, which was thoroughly rinsed with 0.15 M-NaCl

and dehydrated with acetone according to Quick (1942). The dry powder was kept in evacuated ampoules in the refrigerator. On the day of use, a 6% suspension in 0.15 M-NaCl was prepared, kept at 36° with occasional stirring for 1 hr., and then on ice for 1–2 hr. The sediment-free fluid was used.

Isolation of the euglobulin fraction from plasma and its conversion into thrombin was carried out essentially according to Mellanby (1909). Bovine thrombin was a commercial product.

Dilutions were made from a 0.25 M-CaCl₂ stock solution, preserved with 1×10^{-4} part of merthiolate.

Human fibrinogen was prepared by the process of Kekwick, Mackay & Record (1946).

A commercial preparation of gum acacia was purified according to Seegers & Smith (1942). Phenol, *m*- and *p*-cresol, pyrocatechol, resorcinol and quinol were commercial products of analytical grade.

The veronal buffer of Michaelis (1931) was used.

Procedure

In the determination of the depression of antithrombic activity of human serum by phenols, the phenol to be tested was dissolved to 0.036 M in 1.5 ml. of a solution containing 0.01 M-CaCl₂, 0.06 M-NaCl; this solution contained 0.3 ml. of veronal buffer, pH 7.4. To this mixture were added 0.1 ml. human serum and finally 0.2 ml. of a human or bovine thrombin preparation containing 0.15 M-NaCl. In control experiments the phenols were replaced by NaCl. From the reaction mixtures, 0.2 ml. samples were removed at known intervals, and blown into a mixture of 0.2 ml. human Ba-plasma and 0.05 ml. of 0.18 M solution of the phenol to be tested. The reactions were carried out in a water bath maintained at $28 \pm 1^\circ$. The clotting times were measured and converted into thrombin units (see below).

RESULTS

Choice of phenol. The results in Fig. 1 show the quantitative differences among the phenols with regard to their ability to stabilize thrombin in the presence of human serum. *p*-Cresol and *m*-cresol were more effective than quinol. During an incubation time of 3 min., approximately 10% thrombin disappeared in the presence of pyrocatechol, phenol or resorcinol, whilst in the absence of the phenols, more than 40% thrombin activity disappeared. The antithrombic activity of human serum varied. In several other experiments the loss of thrombin could be prevented by phenols for a period of 5–10 min. A comparison between the activity of gum acacia, which is used in the prothrombin assay of Warner *et al.* (1936), and pyrocatechol was carried out, and the results are given in Fig. 2.

The results given in Fig. 2 show two facts. First, the clotting time in the presence of pyrocatechol corresponded to 1.2 units of thrombin, whilst 0.85 unit seemed to be present with gum acacia (see Fig. 3). Secondly, pyrocatechol showed greater antithrombin depressing activity than gum acacia. These two properties appeared to be of advantage

in an assay of prothrombin. Satisfactory results could be obtained in the presence of 0.03M *m*-cresol with plasma; however, isolated prothrombin preparations gave a reduced yield of thrombin. Pyrocatechol did not interfere with clotting reactions if used in concentrations between 0.03 and 0.04M.

Fig. 1. Depression of antithrombic activity of human serum by phenols. ○—○, 0.1 ml. serum plus thrombin; ●—●, 0.1 ml. serum plus quinol; □—□, 0.1 ml. serum plus resorcinol; +—+, 0.1 ml. serum plus pyrocatechol; △—△, 0.1 ml. serum plus phenol; ■—■, 0.1 ml. serum plus *m*- or *p*-cresol.

Fig. 2. Depression of antithrombic activity of human serum by pyrocatechol 0.03M or by gum acacia 2.5%. Pyrocatechol experiments: ○—○, serum plus thrombin; +—+, serum plus pyrocatechol. Gum acacia experiments: □—□, serum plus thrombin; ■—■, serum plus gum acacia. Experimental conditions similar to those given in Fig. 1.

Definition of prothrombin units. The relationship between clotting time and thrombin activity was established with varying concentrations of a thrombin preparation under similar conditions to the assay of prothrombin, except that the addition of the prothrombin-containing material was omitted. One unit (PTU) of prothrombin is here defined as that amount of prothrombin present in 0.2 ml. fluid containing 0.036M pyrocatechol which, when converted into thrombin and added to a mixture of 0.2 ml. human Ba-plasma and 0.05 ml. of 0.18M pyrocatechol in veronal buffer, pH 7.4, produces a clot in 15 sec. at 28°. One unit of prothrombin corresponds to 1 unit of thrombin. In the method of Warner *et al.* (1936) a different unit of thrombin is used. A definition of it is quoted by Ware & Seegers (1949) as follows: 'A thrombin unit is that amount of thrombin which will clot 1 ml. of standardized fibrinogen solution in 15 sec., under the conditions imposed for the two-stage analysis'. The test is carried out in the presence of 2.5% gum acacia. This unit will be referred to here as gum acacia thrombin unit (GTU). On a volume basis one PTU corresponds to 0.45 GTU. In both definitions 1 unit is based on a clotting time of 15 sec., but a given clotting time in the range of 9–15 sec. indicates approximately 1.4 times greater thrombin potency in the presence of 0.036M pyrocatechol than it does in the presence of 2.5% gum acacia. Thus, to convert PTU into GTU, it is necessary to divide by 1.4×2.22 .

It was thought advisable to introduce the PTU for the following reason. In the method of Warner *et al.* (1936), a 1% fibrinogen solution is diluted with the thrombin-containing material to 0.2%. Such concentrations cannot be obtained when normal human plasma is used as fibrinogen solution; but it is desirable to carry out the thrombin assay with

Fig. 3. Relationship between thrombin concentration and clotting time. ●—●, pyrocatechol; ○—○, gum acacia. Experimental conditions similar to those given in Fig. 1.

fibrinogen concentrations above 0.1% because a small variation of fibrinogen concentration in this region has little influence on the thrombin clotting time (Deutsch & Gerarde, 1946; Fantl & Nance, 1948). The relationship between the $\log_{10}$ of the thrombin concentration, and the $\log_{10}$ of the clotting time is given in Fig. 3. For practical purposes it is convenient to draw a line from one point corresponding to 1.9 units and 10 sec. clotting time to 0.5 unit and 25 sec. Using this graph, an error not greater than 10% from the calculated value will be found. This is sufficiently accurate because duplicates of clotting times cannot be reproduced with greater precision.

Assay of prothrombin. For the assay of prothrombin, the following reaction mixture was prepared: into Pyrex tubes (9–10 mm. diameter, 80 mm. length) were measured 1.5 ml. of a mixture consisting of: 0.15 ml. of the thromboplastin preparation, 0.6 ml. 0.025M-CaCl₂, 0.45 ml. 0.15M-NaCl, 0.3 ml. 0.18M pyrocatechol in veronal buffer pH 7.4; to this was added 0.3 ml. of the material to be tested.

At known periods beginning 1–2 min. after the last addition, 0.2 ml. was blown into a mixture of 0.2 ml. human Ba-plasma and 0.05 ml. 0.18M pyrocatechol, and the clotting times were determined. The reactions were carried out in a water bath at $28 \pm 1^\circ$. Three successive samples which gave identical clotting times indicated a maximum yield of thrombin.

To obtain reliable results it was necessary to use very active thromboplastin preparations, and the optimum concentration of brain extract to give the shortest clotting time had to be established in preliminary tests. The concentration of the material to be tested depended on its prothrombin activity.

Fig. 4. Prothrombin assays in human oxalated plasma with and without defibrination. □—□, plasma not defibrinated; ○—○ plasma defibrinated with 2 units of bovine thrombin; ●—●, plasma with 1 unit of bovine thrombin.

Samples of reaction mixtures containing 0.015 ml. of normal oxalated or citrated human plasma should clot in 15–20 sec. after 2–5 min. incubation. The assays were carried out with either human- or rabbit-brain extract. This did not seem to make any difference to the result. In the recommended assay of prothrombin, fibrinogen is not removed from the test fluid. Some workers using the technique of Warner *et al.* (1936) defibrinate the specimen, while others omit this step (Herbert, 1940). Prothrombin assays were carried out on specimens with and without defibrination. The results in Fig. 4 show that the presence of fibrinogen or fibrin had little influence on the rate of thrombin formation and thrombin yield. Therefore it seems unnecessary to remove fibrinogen prior to the assay of prothrombin.

A further simplification in the proposed assay is the use of human Ba-plasma instead of isolated fibrinogen. In order to test the validity of this substitution, prothrombin assays were carried out simultaneously with human Ba-plasma and human fibrinogen, and also with rabbit Ba-plasma.

It is apparent that the results (Table 1) with human Ba-plasma or isolated human fibrinogen are not significantly different, and justify the replacement of fibrinogen by human Ba-plasma in the prothrombin assay. The results also indicate that the sensitivity of thrombin to rabbit's Ba-plasma is somewhat less than to human Ba-plasma. Finally, with regard to the optimum Ca ion concentration, it was observed that the prothrombin results did not vary significantly when the assays were carried out in reaction mixtures containing between 0.017M- and 0.0085M-CaCl₂.

Variation of prothrombin activity in normal mammalian plasma. It appeared of interest to determine, by the assay method described, prothrombin activity in a number of human and animal plasma specimens. The 126 results recorded in the histogram (Fig. 5) were obtained with either pooled or single plasma specimens from 159 human donors, who on clinical grounds were expected to have a normal prothrombin level. 94% of the results are between 375 and 575 units (PTU) of prothrombin/ml. oxalated plasma. This corresponds to 470–720 (PTU)/ml. of native plasma. Results obtained by previous workers using the method of Warner *et al.* (1936) are quoted in

Table 1. Prothrombin assays of oxalated plasma with different sources of fibrinogen

Source of fibrinogen	Results are given in units PTU/ml. oxalated plasma							
	Plasma							
	Bu*	Bl	Ma	Ea	Ro	Gr	Be	Rabbits
Human Ba-plasma	600	400	380	460	470	430	450	380–450
Human fibrinogen	550	370	340	—	—	—	—	—
Rabbit Ba-plasma	—	—	—	410	380	380	330	310

* Bu...Be refer to the name of the donor.

Table 2. The apparently higher results obtained with the pyrocatechol technique than with the gum acacia technique are due to the difference in the definition of the thrombin units.

As an essential difference between the technique devised by Warner *et al.* (1936) and the one suggested here is that gum acacia is replaced by pyrocatechol; it was necessary to compare the results obtained from assays using these two substances. The results given in Table 3 indicate that prothrombin assays

carried out in the presence of pyrocatechol gave 20–60 % higher results than in the presence of gum acacia.

Occasionally prothrombin determinations have to be carried out in plasma, serum or isolated fractions, in which it may be necessary to add prothrombin accelerator to obtain the maximum thrombin yield. The addition of 0.1 ml. of a 20 % Ba-plasma dilution to the reaction mixture has been found adequate for this purpose.

Prothrombin has been assayed with venous and capillary blood. In the latter case, 0.027 ml. blood was taken from a finger or heel puncture. The results obtained from venous and capillary blood were identical.

DISCUSSION

The experimental results presented show that the inactivation of thrombin by serum can be delayed and partly depressed by several phenols. Provided that these substances have no influence on the formation of thrombin, they can be used in the assay of prothrombin. One of the phenols used, pyrocatechol, belongs to a series of compounds which accelerates the thrombin fibrinogen reaction. Gerber & Blanchard (1945) assume this to be due to a catalytic influence of phenols on thrombin, and so the addition of pyrocatechol to a clotting system would give erroneously high values of the thrombin content. However, it was shown recently (Fantl & Ebbels, 1953) that pyrocatechol affects the surface structure of fibrinogen, making it more sensitive to the action of thrombin.

It is generally agreed that the method of Warner *et al.* (1936) gives absolute prothrombin values. Modifications of that method using different buffers have been suggested by Rieben (1947), Hurn & Mann (1947), Mawson (1949) and Herbert (1940). Ware & Seegers (1949) add prothrombin accelerator to ensure complete conversion of prothrombin into thrombin. In these techniques, isolated fibrinogen is used as the indicator of thrombin activity. As the variable reactivity and limited stability of most fibrinogen preparations are difficulties in these techniques, Sternberger (1947) has used plasma instead of fibrinogen, and ethanol as antithrombin depressing agent. The disadvantage of this procedure is that in the presence of ethanol, thrombin produces a granular fibrin precipitate which is not easily detected.

The present experiments indicate that it is unnecessary to defibrinate plasma specimens prior to the test, and that fibrinogen can be replaced by human Ba-plasma. Rabbit Ba-plasma is less reactive towards thrombin. Pyrocatechol as an antithrombin depressing agent has its limitations. In the suggested assay, plasma with a normal prothrombin concentration is diluted 120 times. It has

Fig. 5. Prothrombin assays in 126 specimens of human oxalated plasma.

Table 2. Results of prothrombin assays on normal mammalian blood plasma

Figures are given in units/ml. of native plasma. Number of specimens in brackets.

Man	(45)	244–452 GTU	Mann <i>et al.</i> (1948)
	—	300–350 GTU	Ware & Seegers (1949)
	(126)	470–720 PTU	Present work
Rabbit	—	220–270 GTU	Murphy & Seegers (1948)
	(5)	450–670 PTU	Present work
Guinea pig	—	210–250 GTU	Murphy & Seegers (1948)
	(2)	310–330 PTU	Present work

Table 3. Prothrombin assays of citrated and oxalated human plasmas and a citrated euglobulin fraction of human plasma

The results are given as units/ml.

Source	Pyrocatechol (0.03M)	Gum acacia (2.5 %)
Hu. citrated	470	380
Hu. oxalated	440	290
Lo. citrated	450	380
Lo. oxalated	430	300
Go. citrated	300	180
No. oxalated	420	270
Euglobulin citrated	660	480

often been found that the thrombin formed in the presence of pyrocatechol was stable for periods up to 20 min., whilst in its absence, loss occurred after 4–5 min. On the other hand, in the case of a prothrombin concentration of approximately 20% of the normal value, or less, where the plasma is diluted 12 times, significant losses of thrombin occur even in the presence of pyrocatechol after 3 min. incubation. Despite this, a satisfactory assay can be carried out since complete conversion of prothrombin into thrombin occurs in that period. It is preferable to carry out the assay of prothrombin with citrated plasma, because calcium oxalate adsorbs prothrombin (Menghini, 1949). The assay both in a macro- and in a micro-form has been carried out in blood, plasma, and serum of man and other mammals and also in isolated plasma fractions.

SUMMARY

1. Phenols depress the antithrombic activity of serum.
2. This property, as well as the accelerating effect of phenols on the thrombin fibrinogen reaction, is utilized in a prothrombin assay.
3. Results of prothrombin assays in mammalian plasma in the presence of pyrocatechol are presented.

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Creatine Phosphokinase: Assay and Application for the Micro-determination of the Adenine Nucleotides

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The determination of creatine phosphokinase activity has been complicated by the fact that although creatine phosphate is extremely labile in acid solution, the assays of this enzyme which have been described in the literature (e.g. Banga, 1943; Askonas, 1951; Narayanaswami, 1952) have at some stage usually involved exposure to a pH well below 7. Creatine phosphate breakdown during deproteinization with trichloroacetic acid can be minimized by working at low temperature, but the subsequent determination of this substance by methods involving phosphate analysis presents

difficulties when the system contains other labile organophosphates or free phosphate. Usually creatine phosphate has been determined by phosphate analysis after fractionation (Meyerhof, 1930; LePage & Umbreit, 1945), or after hydrolysis with acid molybdate (Fiske & Subbarow, 1929). The latter method is not entirely satisfactory, however, as a number of workers, particularly Weil-Malherbe & Green (1951), have indicated that under the acid molybdate conditions, creatine phosphate may not be the only labile organophosphate. When creatine phosphate is estimated in systems containing