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Flocculation Tests with Electrophoretically Separated Serum Proteins

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There are now a number of flocculation tests carried out on blood serum which have proved useful indicators of hepatic dysfunction, and the mechanism involved in these tests is becoming much clearer as a result of American work with electrophoretically separated proteins. Thus the γ-globulin is very active in precipitating the gold and cephalin-cholesterol reagents (Gray, 1942; Kabat, Moore & Landow, 1942) and this fraction is also known to be increased in the serum from patients with various types of hepatitis (Gray & Barron, 1943). In both these tests albumin has an inhibitory effect and, moreover, albumin from hepatitis serum has less inhibitory power than albumin from normal serum (Kabat, Hanger, Moore & Landow, 1943; Moore, Pierson, Hanger & Moore, 1945). Very little information is available as to the effect of the α- and β-globulin fractions.

The thymol turbidity test (Maclagan, 1944*a*) appeared to be closely related to the above two tests both on clinical and chemical grounds, and the precipitate formed in the reaction was shown to be a globulin-thymol-phospholipid complex. It therefore seemed probable that a similar mechanism was operating in this test. The experiments reported here were undertaken to elucidate this point, and to investigate the α and β fractions more fully. In the meantime, Recant, Chargaff & Hanger (1945) and McCord (1945) have failed to produce a turbidity when the thymol reagent was added to pure γ-globulin solutions. A preliminary report of our experiments has already appeared (Maclagan & Bunn, 1946).

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MATERIAL

The results reported here were obtained with two collections of human serum: *N*, 40 ml. from a normal subject; *H*, 35 ml. pooled from two patients with typical infective hepatitis. Serum *N* was negative and *H* was strongly positive to all the tests used.

Preliminary experiments were also conducted on two other pooled sera with very similar results, but these will not be reported here as in these earlier separations the standard electrophoretic technique was combined with either precipitation or concentration by evaporation from the frozen state, such procedures being suspect in that they may cause modifications of the serum proteins or their complexes.

METHODS

Electrophoresis

The yields, particularly of α- and β-globulins, obtainable by the discontinuous electrophoretic technique of Tiselius (1938) (see, for example, Blix, Tiselius & Svensson, 1941) are so limited as to make a semi-continuous technique greatly preferable. Svensson (1942, 1946) has described a preparative apparatus which allows sampling and refilling to be carried out at will during the separation. For our purposes, Svensson's elaborate arrangement did not seem necessary, and a very simple modification of the standard Tiselius assembly employing the same principles was found adequate (Fig. 1).

The standard long cell (Longsworth, Cannan & MacInnes, 1940) (vol. of one limb + bottom section approx. 10 ml.) was connected to open electrode vessels (vol. 1700 ml.) by special junction pieces, J, allowing two withdrawal capillaries, C_U, C_L, to be permanently supported in each limb of the U-tube by rubber bungs, B. It was found to be a considerable advantage if the glass tubes (internal diam. 2·5 mm.) from which the capillaries were drawn, were gripped by the rubber bungs sufficiently loosely to allow the height of the capillary endings in the U-tube limbs to be

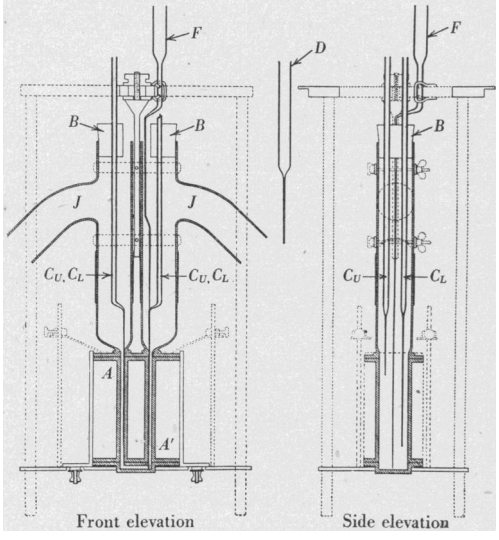

Fig. 1. Electrophoresis U-tube arranged for preparative separations; F, filling tube; D, compensator; B, rubber bungs; J, junction pieces; C_U, upper sampling capillaries; C_L, lower sampling capillaries; A, A', boundary starting positions. Electrode vessels not shown.

adjusted during the progress of the separation. A wider glass tube, F, also drawn out into a capillary, and supported by a clip on the frame so that its tip touched the floor of the cell, served as a filling tube. The apparatus was filled with buffer solution, and brought to 0–1° in a water thermostat, before cooled saturated KCl solution was carefully led into each electrode vessel to more than cover the Ag-AgCl electrodes.

About 35 ml. of serum were dialyzed in the cold, over a period of 3 days or more, against several changes of potassium phosphate buffer, ionic strength 0·2, pH 8·0. (The pH of this buffer lies within the stability range of the serum proteins (Svedberg & Sjögren, 1930); cf. the finding of Moore *et al.* (1945) that γ-globulin separated in diethylbarbiturate buffer at pH 8·5 does not flocculate cephalin-cholesterol emulsions. With the lower conductivity, however, it permits a more rapid separation than does the pH 7·4 phosphate-NaCl buffer used by the above authors, and is also preferable in that it involves virtually no losses by spontaneous flocculation of either sera or fractions.) The dialyzed sample was clarified by centrifugation and about 12 ml. introduced into the cell very slowly through the filling tube F. (The optical density of the icteric serum necessitated some prior dilution.) The lower withdrawal capillary, C_L, of each limb was used to sharpen the protein boundary as it approached the capillary tip in the U-tube. Very sharp boundaries were obtained in this way, and were brought to positions A and A' for the start of their migration by a slow feed of buffer solution into the appropriate electrode vessel from a removable glass tube drawn out into a capillary D. The current density in the U-tube was kept sufficiently low to avoid convectional disturbance of the boundaries, but was so chosen as to give about the maximum extent of migration allowed by the dimensions of the U-tube at convenient sampling times (9–19 ma./cm.²; sampling twice daily).

Sampling was controlled by observation of the boundaries by the optical method of Philpot (1938). Albumin and albumin + α-globulin were withdrawn in turn from the centre of their respective boundaries by the upper capillary C_U of the ascending limb, γ- and β + γ-globulins being recovered from the descending side. Sampling was accompanied and controlled by compensation with D and by the introduction of further material into the bottom of the U-tube, the lower boundaries in each limb being at the same time fused by removal, via the lower capillaries, of the material composing them. These operations were so ordered as to leave the system of boundaries in a position suitable for further migration. The multicomponent mixtures removed by the lower capillaries were combined, reconcentrated by pressure dialysis, centrifuged, and used to refill the U-tube when the first stock of filling solution had become exhausted. Advantage was taken of several such refillings during the course of the separation to reverse the polarity of the electrodes, which involved no great inconvenience, since refilling with a fresh stock solution in itself entailed the formation of a new set of boundaries. Only two or three reversals were required over a period of 10 days or more continuous running. Recovery of unwanted fractions and their return to the U-tube was continued until sufficient quantities of albumin + α-globulin, and β + γ-globulin fractions had been obtained. These were then separated in the same way.

Figs. 2 and 3 are the electrophoretic diagrams at equivalent migration times of the original sera and the fractions albumin and γ-globulin obtained from them, indicating the degree of purity of the latter. Insufficient α- and β-globulin was obtained to submit them to electrophoretic analysis, but there is no reason to doubt that they were of a similar degree of homogeneity, except that in the case of the hepatitis serum the α-globulin was replaced by a boundary migrating so close to the β-globulin that it was only possible to isolate them together as one fraction, designated arbitrarily as (α + β)-globulin in the text. The overall yield of albumin and γ-globulin from serum H amounted to 21 and 27% respectively of the quantities estimated from the diagrams to be present in the original serum.

Throughout the processing the material was maintained at 0–4°, except for short operations such as centrifugation, and the sera and fractions were sterilized by filtration through 0·7 μ pore Gradocol membranes (Elford, 1931) at various stages of the separation.

For the flocculation tests, the fractions were concentrated by pressure dialysis, and the buffer salt concentration reduced by dialysis against the volume of saline required to give at equilibration an ionic strength 0·09 in

NaCl, 0·01 in potassium phosphates. The final protein concentration was determined by the micro-Kjeldahl method, using a factor of 6·25 to convert nitrogen to protein.

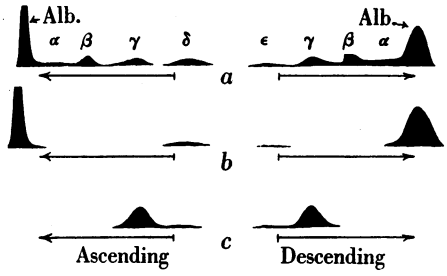

Fig. 2. Electrophoretic patterns of (*a*) normal serum *N* (by average of the two patterns 61·5% albumin, 9·7% α-globulin, 13·9% β-globulin, 14·9% γ-globulin); (*b*) isolated albumin fraction; (*c*) isolated γ-globulin fraction. K_2HPO_4 buffer, μ 0·2, pH 8·0. Refractive increment due to protein = 0·0030 for the serum, proportionately less for the fractions. Potential gradient = 3·21 V./cm., time 28,000 sec., electrode vessels open.

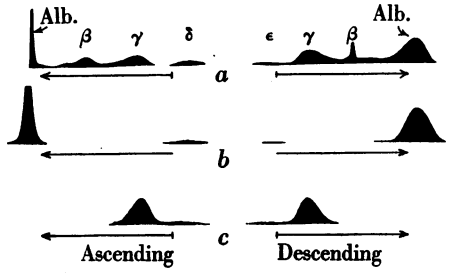

Fig. 3. Electrophoretic patterns of (*a*) hepatitis serum *H* (by average of the two patterns 54·0% albumin, 19·5% $\beta_1 + \beta$-globulin, 26·5% γ-globulin); (*b*) isolated albumin fraction; (*c*) isolated γ-globulin fraction. K_2HPO_4 buffer, μ 0·2, pH 8·0. Refractive increment due to protein = 0·0030 for the serum, proportionately less for the fractions. Potential gradient = 3·21 V./cm., time 28,000 sec., electrode vessels open.

Flocculation tests

The methods employed were:

(1) Cephalin-cholesterol test. Hanger (1939) 'Difco' antigen was used (Baird and Tatlock).

(2) Serum colloidal gold reaction. Earlier experiments were done with an oxalate sol at pH 7·8 (Maclagan, 1944*b*) and later ones with a citrate sol at pH 7·7 (Maclagan, 1946). These two methods are equivalent in sensitivity.

(3) Cerebro-spinal fluid colloidal gold reaction at pH 6·6 (Maclagan, 1946).

(4) Thymol turbidity test (Maclagan, 1944*a*). The photoelectric method of Shank & Hoagland (1946) was also tried for reading the turbidities, but the results appeared to be somewhat lower and this method was not pursued.

(5) Thymol flocculation test. This addition to the thymol turbidity test consists merely in leaving the mixture to stand overnight, after the turbidity has been read, as proposed by Neefe (1946). The amount of flocculation is graded as negative, 1, 2, 3 or 4+.

(6) Thymol-cephalin test. This was identical with the thymol turbidity test except that one drop of 1% cephalin in ether was added to the protein solution before the thymol reagent. The cephalin was prepared from sheep brain by the method of Hanger (1939). As explained below, this addition is not recommended for tests on whole serum.

(7) Takata-Ara reaction. Crane's modification as described by Chasnoff & Solomon (1938).

In all cases the actual volume of protein solution had to be varied up to a maximum of 0·5 ml.

RESULTS

The results obtained with single fractions are shown in Table 1, the only positive findings being that both γ fractions reacted with all three reagents, and the hepatitis (α + β) fraction reacted quite strongly with the cephalin-cholesterol reagent. Hepatitis γ-globulin was ten times as effective as normal γ-globulin against cephalin-cholesterol, and three times as effective against the gold reagent. The turbidity produced by the γ-globulin with the thymol reagent was however very small, being only about 1 unit/mg. in a volume of 3 ml. This result suggested the possibility that the serum phospholipids, which were known to be present in the precipitate obtained when the thymol test is done on whole serum, were also essential in the reaction and these might well be absent or diminished in pure γ fractions, being lost either during electrophoresis or during the preliminary dialysis.

Table 1. *Effect of single fractions*

(The weights given in the case of positive reactors are the amounts required to produce 1 unit of turbidity in the case of the thymol tests, and the least amounts required for complete precipitation in the other tests.)

Fraction	Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
Normal albumin up to 5 mg.	0	0	0	0
Normal α-globulin up to 0·4 mg.	0	0	0	0
Normal β-globulin up to 0·4 mg.	0	0	0	0
Normal γ-globulin	+ (1 mg.)	+ (0·1 mg.)	+ (0·075 mg.)	+ (0·5 mg.)
Hepatitis albumin up to 5 mg.	0	0	0	0
Hepatitis (α + β)-globulin up to 1·2 mg.	0	0	0	+ (0·06 mg.)
Hepatitis γ-globulin	+ (0·8 mg.)	+ (0·1 mg.)	+ (0·025 mg.)	+ (0·05 mg.)

The addition of various lipids to the fractions before the thymol reagent was therefore tried, and it was found that either lecithin or cephalin had the effect of giving a much increased turbidity in the case of γ fractions, amounting to 10 units/mg. for normal and 12 units/mg. for hepatitis γ-globulin. This addition had no effect on the negative results with albumin and α and β fractions. The technique finally adopted was that given under 'thymol cephalin test' above. It would appear from this that the serum phospholipids are an essential ingredient of the thymol reaction, and that results with electrophoretic fractions may be misleading unless the phospholipids are replaced.

The results with pairs of fractions from normal serum are shown in Tables 2 and 3. The principal points shown are:

(1) Normal albumin inhibits thymol-cephalin, gold and cephalin-cholesterol tests with some quantitative differences.

Table 2. *Effect of normal albumin on action of normal γ-globulin*

Note to Tables 2–6

The ratios given show the smallest amounts required to produce complete inhibition of one part of γ-globulin, the amounts of γ-globulin used being 0·1 mg. for the gold and cephalin-cholesterol tests and 0·6 mg. for thymol-cephalin tests. The experiments were done by a serial dilution technique with a dilution factor of $\frac{1}{2}$, so that an inhibition ratio of e.g. 10 : 1 may be taken to indicate that a 5 : 1 ratio failed to produce complete inhibition.

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
0	Inhibited 4 : 1	Inhibited 10 : 1	Inhibited 100 : 1

Table 3. *Effect of normal α- and β-globulin on action of normal γ-globulin*

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
0	10% increase of sensitivity	Inhibited $0{\cdot}5\alpha : 1\gamma$ $1\beta : 1\gamma$	Sensitivity unaltered but flocculation more rapid

(2) Normal α- and β-globulins are about ten times more effective than albumin in inhibiting the gold reaction but they do not inhibit the other two at all, producing instead a slight increase of sensitivity with thymol-cephalin and more rapid flocculation with cephalin-cholesterol.

Tables 4, 5 and 6 give the results obtained with pairs of fractions from hepatitis serum. They show:

(1) Hepatitis albumin still inhibits the gold and cephalin-cholesterol tests but fails to inhibit thymol-cephalin and slightly augments thymol alone.

Table 4. *Effect of hepatitis albumin on action of hepatitis γ-globulin*

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
Sensitivity increased threefold	0	Inhibition 40 : 1	Inhibition 40 : 1

Table 5. *Effect of hepatitis $(\alpha+\beta)$-globulin on action of hepatitis γ-globulin*

Thymol alone	Thymol-cephalin	Gold at pH 7·8	Cephalin-cholesterol
Sensitivity increased tenfold	10% increase of sensitivity	Inhibition 1·5 : 1	Sensitivity increased (active alone)

Table 6. *Effect of hepatitis albumin on action of hepatitis $(\alpha+\beta)$-globulin*

Cephalin-cholesterol: Inhibition 40 : 1

(2) Hepatitis $(\alpha+\beta)$-globulin behaves like normal α and β against thymol-cephalin and gold, but markedly increases the sensitivity of thymol alone and cephalin-cholesterol. This fraction is of course active by itself against cephalin-cholesterol and possibly influences thymol alone by virtue of its lipid content.

(3) With cephalin-cholesterol, hepatitis albumin inhibits $(\alpha+\beta)$ fractions in about the same ratio as that required for inhibition of γ-globulin.

Gold reaction at pH 6·6. Some experiments were also conducted with colloidal gold in the buffer of pH 6·6, ionic strength 0·067, recommended for cerebrospinal fluid tests (Maclagan, 1946). The principal differences noted were a much increased sensitivity, 2 μg. of normal γ-globulin being sufficient for complete precipitation. At this pH there was little difference between normal and hepatitis γ-globulin, the latter requiring 2·5 μg. for complete precipitation. Normal and hepatitis albumin were both inhibitory in the proportion of 40:1, and hepatitis $(\alpha+\beta)$-globulin was inhibitory at 3:1. In these inhibitory experiments both 5 and 50 μg. of γ-globulin were used, the ratios of inhibitor required being the same with the two amounts.

Takata-Ara reaction. Only the hepatitis fractions were available for this test. γ-Globulin and $(\alpha+\beta)$ fractions were active and both completely flocculated the reagent down to 0·25 mg. Albumin inhibited the γ-globulin in the ratio of 2·5:1, and also inhibited the $(\alpha+\beta)$-globulin in the ratio of 0·67:1 (0·3 mg. of γ-globulin used).

Cephalin and thymol turbidity test on serum. In view of the effects shown above it was obviously important to try the effect of added cephalin on the thymol turbidity test as carried out on serum. A number of normal and pathological sera were therefore tested with and without added cephalin, but no

appreciable increase of turbidity was noted. It would appear therefore that in whole serum the native phospholipids are usually sufficient in amount to give a maximal turbidity with the thymol reagent, and nothing is to be gained by the addition of cephalin.

Thymol flocculation test. It was originally noted by one of us (Maclagan, 1944*b*) that in the case of sera giving positive thymol turbidity tests the mixture usually flocculated on standing overnight, but it did not at that time appear that a test based on this observation would present any advantage over the quicker measurement of turbidity. However, Neefe (1946) and Neefe & Rheinhold (1946) have since made extensive observations on this 'thymol flocculation test' and regard it as an important addition to the thymol turbidity test. Particular note was therefore made in this study as to the occurrence of flocculation, with the rather startling result that the hepatitis γ fractions either alone or in combination always produced flocculation whereas the normal γ fractions did not. This was true with thymol alone as well as with the thymol-cephalin test. It appears therefore that the observation of flocculation does give extra information and shows a qualitative difference between normal and hepatitis γ-globulin fractions. The thymol flocculation test was not inhibited by any of the other hepatitis fractions, but it was not possible to test normal fractions for inhibitory power as they had all been used up before the hepatitis γ fraction became available.

DISCUSSION

Although the results presented are based principally on only two sera, we feel that they are sufficiently detailed to serve as a basis for the interpretation of the tests. The principal reason for undertaking this work was to elucidate the mechanism of the thymol turbidity test, and the results show that this test is in the first place dependent upon the presence of native serum phospholipids, as might have been anticipated from the character of the precipitate, which had previously been shown to contain an average of 0·33 % P, corresponding to 8·0 % of phospholipid (Maclagan, 1944*a*). Results with electrophoretic fractions are therefore liable to be misleading since the lipids are associated in high proportion only with the α- and β-globulins, and are particularly deficient in isolated γ-globulin fractions (Blix, Tiselius & Svensson, 1941). If, however, cephalin is added to the fractions results of the type anticipated from analogies with the related tests are obtained. These are given above under thymol-cephalin reactions, the principal features being:

(*a*) γ-Globulin is the only fraction active alone (activity 10–12 units/mg. in a volume of 3 ml.).

(*b*) Normal albumin inhibits the reaction in the ratio of 4:1, thus accounting for the negative reactions of normal sera.

(*c*) Hepatitis albumin and both normal and hepatitis α- and β-globulin have little effect.

(*d*) Only the turbidity developed by hepatitis γ-globulin proceeded to flocculation on standing.

Since the addition of cephalin to whole serum does not alter the results obtained, we consider that the test as originally described depends upon the mechanism outlined above. In any case there is no advantage in adding cephalin in the case of whole serum.

These results appear to contradict the work of Recant *et al.* (1945) who were unable to demonstrate any thymol turbidity with γ fractions separated electrophoretically at pH 7·4, either with or without added lipoids. These authors, however, do not give the details of their experiments.

The results obtained with colloidal gold show two important differences from those of previous workers: first, the considerably greater flocculating power of hepatitis γ-globulin as compared with normal γ at pH 7·8, and secondly, the powerful inhibitory effect of both normal and hepatitis α and β fractions. On the basis of the figures given above it is possible to formulate two equations expressing the excess flocculating activity (FA), in terms of γ-globulin, at pH 7·8 of the normal and hepatitis serum respectively. These are:

(1) $FA = \gamma - 2{\cdot}0\,\alpha - \beta - 0{\cdot}1$ Alb. for normal serum N,

(2) $FA = \gamma - 0{\cdot}67\,(\alpha + \beta) - 0{\cdot}025$ Alb. for hepatitis serum H.

The terms Alb., α, β and γ refer to the relative concentrations of the respective components expressed as percentages of the total serum protein. Although based on our nitrogen estimations, the approximate nature of these equations allow the use of uncorrected refractometric (electrophoretic) percentages. When applied to our whole sera they give the following values:

For the normal serum, $FA = -24{\cdot}5$,
For the hepatitis serum, $FA = +12$,

in qualitative accordance with the observed behaviour. Similarly all the normal sera analyzed by Gray & Barron (1943) give negative FA values by equation (1) and apart from one case of post-arsphenamine jaundice, a disease in which negative flocculation tests are particularly frequent (Maclagan, 1944*a*), four out of five of their hepatitis sera would be expected to have gold sol flocculating activity according to equation (2). It is of interest that most normal sera give negative values in (2) as well as in (1).

Our results with the cephalin-cholesterol test also differ from those of, for example, Moore *et al.* (1945) in showing a greater flocculating power of hepatitis as opposed to normal γ-globulin, and in the marked flocculating activity of hepatitis $(\alpha + \beta)$-globulin.

The latter has not previously been observed. The inhibitory ratio of 100:1 for normal albumin and γ-globulin does not account for the negative reaction of normal serum.

In comparing the various flocculation tests investigated (Table 7) it will be seen that there is a distinct family resemblance between them as γ-globulin is active in all of them and, so far as they have been tested, they are all inhibited by normal albumin. It is therefore not surprising that the tests have all proved useful as liver function tests and that the results on the whole run roughly parallel with each other. However, the failure of hepatitis albumin to inhibit the thymol reaction, and particularly the different functions of α- and β-globulin in the various tests, are sufficient to account for some individual sera reacting differently in different tests, as for example reported by Watson & Rappaport (1945). The thymol turbidity test appears to be the simplest in mechanism as it depends only upon the two fractions albumin and γ-globulin. It may therefore be described as a test of relative γ-globulin excess. The thymol flocculation test is a more qualitative procedure which is nevertheless of importance as it shows striking differences between normal and hepatitis γ-globulins. It is therefore evidently desirable to combine this test with the thymol turbidity test as proposed by Neefe (1946).

While the mechanism of these tests is not completely understood it is evident that they depend upon differences in chemical structure and not simply upon the net electric charge on the protein. This is shown by the fact that all the reagents are negatively charged colloids and are yet flocculated at reactions well above the isoelectric points of any of the serum proteins, so that the proteins must also be negatively charged under the conditions of the tests. The adsorption experiments of Elkes, Frazer, Schulman & Stewart (1945) suggest that this property, even if not peculiar to γ-globulin, is unusual in soluble proteins. The marked differences between the behaviour of normal and hepatitis fractions are not accompanied by gross electrophoretic differences. (Exact measurements of mobility were not however made during the course of this work.) The suggestion is that these flocculation tests can reveal differences in protein structure not shown by other physical methods, and may well lend themselves to wider application in protein chemistry. They have also the advantage of technical simplicity compared with, for example, serological tests.

Table 7. *Comparison of flocculation tests*

Test	Flocculator	Inhibitor	Normal-hepatitis differences
Thymol (cephalin) turbidity test	γ-Globulins	*N* albumin	*H* γ slightly more effective than *N*; *H* albumin not inhibitory
Thymol (cephalin) flocculation test	*H* γ-globulin only	None demonstrated (*N* fractions not tested)	*N* γ-globulin not effective
Gold at pH 7·8	γ-Globulins	Albumins, α- and β-globulins	*H* γ-globulin more effective than *N*
Gold at pH 6·6	γ-Globulins	Albumins, α- and β-globulins	None demonstrated
Cephalin-cholesterol	γ-Globulins; *H* (α + β)-globulin	Albumins	*H* γ much more effective than *N*; *N* α and β not effective
Takata-Ara	*H* γ-globulin; *H* (α + β)-globulin	*H* albumin	*N* fractions not tested

SUMMARY

1. A modified technique is described, suitable for the preparation of electrophoretically pure proteins from relatively small volumes of serum. Two human sera were separated, normal (*N*) and hepatitis (*H*).

2. The thymol turbidity test as carried out on serum depends partly upon the presence of native phospholipids. In tests on protein fractions with added cephalin γ-globulin acted as a precipitating agent; *N* albumin inhibited the reaction and *H* albumin did not; α and β fractions were without significant effect.

3. The thymol flocculation test, both with and without added cephalin, was positive with *H* γ-globulin, but was negative with *N* γ-globulin and with all other fractions. It was not inhibited by other *H* fractions (*N* fractions were not tested for inhibition).

4. Corresponding data are presented for the colloidal gold reaction, the cephalin-cholesterol test, and the Takata-Ara reaction. Two formulae have been obtained which qualitatively account for the behaviour of whole sera with the gold reagent.

5. The effect of α- and β-globulin was different in each test. Thus *H* (α + β)-globulin flocculated the cephalin-cholesterol and Takata-Ara reagents while both *N* and *H* α- and β-globulins were powerful inhibitors of the gold reaction.

6. The possible value of these tests as indicators of differences in protein structure is discussed.

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POSTSCRIPT

While this paper was in the press there have come to our notice two further references to similar work. Cohen & Thompson (1947) describe experiments on the electrophoresis of sera before and after treatment with the thymol reagent, which they interpret as indicating that the β-globulin fraction is the active agent in the thymol test. They report only slightly higher concentrations of β-globulin in positive sera and therefore suggest the possibility of a qualitative change in this fraction. There are two important differences in technique which may account for the discrepancy between their conclusions and ours. First, Cohen & Thompson's (1947) electrophoresis was conducted at pH 8·6 and ours at pH 8·0. The analytical classification is thus not necessarily identical, for as has been noted above γ-globulin separated at pH 8·6 is devoid of the flocculating activity associated with γ-globulin separated at a lower pH (Moore *et al.* 1945). Secondly, the conditions of precipitation were not identical, as both the initial ionic strength and the final protein concentration would be much higher in Cohen & Thompson's (1947) experiments. They added only 2 vol. of thymol reagent to one of serum, and then dialyzed against a further 20–25 vol. of reagent, whereas the thymol test is performed by adding 60 vol. of reagent to one of serum. Their results would thus not necessarily apply to the thymol turbidity test as actually performed on serum.

Hanger (1946) has made a brief report on work which appears to be entirely in agreement with our own, in that he finds γ-globulin to be the active precipitating agent in the thymol test with phospholipids as an essential adjuvant.

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A Method for the Estimation of Creatinine

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It has always been realized that the alkaline picrate method (Folin, 1905) for the estimation of creatinine suffers from the defect of being non-specific (Hunter, 1928). A wide variety of substances react, among them glucose, formaldehyde, acetaldehyde and many creatinine-like nitrogenous compounds, some of which are likely to be present in biological fluids. 3:5-Dinitrobenzoic acid (Langley & Evans, 1936) appears to offer greater specificity but only at the expense of sensitivity. Barrett (1936) described a qualitative test for creatinine using a reagent based on Nessler's solution. Further study has shown that such a reagent could be made the basis of a quantitative method, the estimation being carried out nephelometrically.