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Estimation of Alterations of Serum Gamma Globulin by a Turbidimetric Technique.

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Increased attention has been devoted in recent years to the elevation of serum globulin encountered in patients with diseases of the liver. The wide use of the cephalin flocculation and the thymol turbidity reactions for the diagnosis of liver disease has been partially responsible for this renewed interest. These reactions have been shown to depend mainly on small changes in the globulin fraction of the serum.^{1,2} However, other factors are involved and the exact aberration of the serum that is measured has not been completely identified. In the case of the thymol turbidity test, for example, the concentration of the serum lipids has a definite effect on the intensity of the reaction.² It would seem important, therefore, to determine specifically and directly slight elevations of globulin that occur in acute liver disease.

The usual methods of globulin estimation which depend on nitrogen determinations following salting out procedures are subject to considerable error. The technique is time consuming and minor changes in the globulin level may not be apparent. Changes in the amount of serum globulin can best be detected at the present time by means of electrophoretic patterns. Such a technique, however, is not practical for routine application.

Dilution of serum with solutions of low ionic strength decreases the solubility of the proteins and under certain conditions the more insoluble globulins will precipitate. This technique is utilized in the thymol turbidity test³ and in the buffer dilution test of Wolff.⁴

Both of these reactions are positive in acute liver disease and appear to depend partly on increases in the gamma globulin fraction of the serum. In certain conditions, such as cirrhosis of the liver, large increases in gamma globulin may be present in the serum in the absence of increased values for these two tests. The intensity of these reactions, therefore, is not always proportional to the increase in amount of gamma globulin in the serum.

The present paper describes a turbidimetric technique which provides an index of the degree of elevation of the gamma globulin fraction of the serum of patients irrespective of their disease. Dilution of serum with solutions of the salts of heavy metals was found to precipitate various protein fractions according to the concentration of metal used. Fig. 1 illustrates the curve of protein precipitation from normal serum at various concentrations of CuSO_4 . Electrophoretic analyses showed that at the lower concentrations the precipitate consisted almost entirely of gamma globulin. By means of this curve it was possible to estimate a concentration of CuSO_4 that caused minimal precipitation of protein from normal serum. When hepatitis serum showing slight elevation of the gamma globulin fraction was diluted with CuSO_4 solution of this concentration, precipitation occurred, and the amount of precipitate was proportional to the increase in gamma globulin. The precipitated globulin could be accurately determined by turbidity measurements because the protein precipitated as a diffuse finely particulate suspension.

Zn, Hg, Pb, Cd, and Ur salts were found to produce an effect similar to that of CuSO_4 .

¹ Moore, D. B., Pierson, P. S., Hanger, F. M., and Moore, D. H., *J. Clin. Invest.*, 1945, **24**, 292.

² Kunkel, H. G., and Hoagland, C. L., *J. Clin. Invest.*, 1947, **26**, 1060.

³ MacLagan, N. F., *Brit. J. Exp. Path.*, 1944, **25**, 234.

⁴ Wolff, E. K., *Tr. Roy. Soc. Trop. Med. and Hyg.*, 1939, **32**, 707.

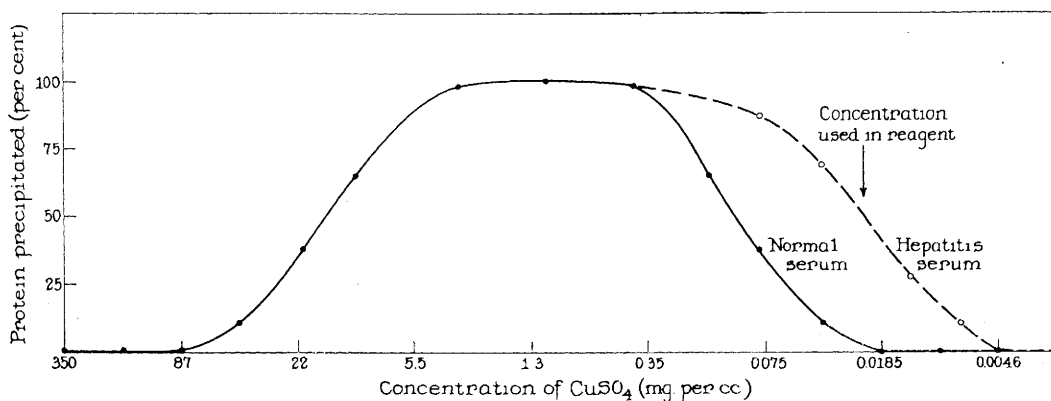


FIG. 1.

Per cent of the total protein of normal and hepatitis serum precipitated following a 60-fold dilution with various concentrations of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ in distilled water.

Curves illustrating the effects of various concentrations on the precipitation of protein from serum closely resembled the curve for CuSO_4 in Fig. 1. When buffered solutions were used, ZnSO_4 was found to have certain advantages over CuSO_4 .

The effect of the solutions of heavy metals in precipitating serum proteins was altered by small changes in pH and ionic strength. The results in Table I illustrate the comparative effect of the CuSO_4 solution usually used as reagent on normal and hepatitis serum under various conditions. The best differentiation between the two types of serum occurred at pH 6.5-7.5 in the presence of as low an ionic strength as it was practical to use.

Various concentrations of CuSO_4 reagent could be used for estimating changes in the globulin fraction. The concentration of 23 mg of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ per liter which causes minimal precipitation with normal serum was found to be the most useful. A zinc sulfate solution containing 24 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ per liter gives similar results, and has the advantage that it can be permanently buffered with barbiturate, whereas copper sulfate solution with barbiturate forms a precipitate. For routine use the solution eventually employed contained, per liter, 24 mg of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 280 mg of barbituric acid, and 210 mg of sodium barbiturate; the pH was 7.5.

Procedure. Measure one volume of serum (0.05 ml) into 60 volumes of either the CuSO_4

or the buffered ZnSO_4 reagent (3 ml). Allow to stand for 30 minutes, shake, and then read the turbidity in the spectrophotometer at 650 $\text{m}\mu$.

The turbidity produced was translated into units by applying a standard curve similar to the one used for the thymol turbidity test.^{5*} All values reported in this paper were determined by means of a Coleman Jr. spectrophotometer at 650 $\text{m}\mu$. A colorimeter can also be used. In the absence of these instruments, a rapid estimate of the globulin elevation can be made by direct visual observation of the turbidity produced. The time necessary for flocculation to occur was a simple index of the globulin elevation. Some of the sera showing very high globulin levels demonstrated flocculation in a few minutes. The buffered ZnSO_4 reagent produced flocculation somewhat more rapidly than did

⁵ Shank, R. E., and Hoagland, C. L., *J. Biol. Chem.*, 1946, **162**, 133.

* 3 cc of a BaCl_2 solution (containing 1.15 g $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ per 100 cc) is made to 100 cc in a volumetric flask with 0.2N H_2SO_4 . This BaSO_4 suspension gives a turbidity equivalent to 20 units. By assigning the value 20 units to the optical density reading obtained in a colorimeter or a spectrophotometer at 650 μ with the BaSO_4 suspension, a standard curve can be constructed by drawing a line through the point obtained in the above manner and the 0 point on ordinary graph paper. The type of cuvette is not important as long as the same type is used for constructing the standard curve as for routine readings.

TABLE I.

Turbidity Produced by Adding 3 cc of a 2.3 mg % Solution of CuSO_4 to 0.05 cc of Serum Under Different Conditions of pH and Ionic Strength. Turbidities Are Expressed in Units Described in Text.

pH	3.0	3.5	4.0	4.5	5.0	5.5	6.0	6.5	7.0	7.2	7.5	8.0	8.5	Ionic strength	
Turbidity															
Normal serum	0	0	0	2	4	13	19	14	9	6	2	0	0	}	.01
Hepatitis serum	0	2	3	4	6	19	31	31	31	24	19	0	0		
Normal serum	0	0	0	0	0	0	4	2	0	0	0	0	0	}	0.1
Hepatitis serum	0	0	0	0	0	4	9	6	5	3	0	0	0		

the CuSO_4 reagent. Normal serum did not flocculate for at least 12 hours with the ZnSO_4 reagent while serum from patients with very slight elevation of the gamma globulin fraction usually flocculated within 4 hours.

The protein precipitated in the reaction dissolved readily on dialysis against sodium cyanide and the latter could be removed by further dialysis against barbital buffer at pH 7.8. Electrophoretic analysis of the protein treated in this manner revealed that the chief component precipitating in the reaction was a gamma globulin plus small amounts of other fractions (Fig. 2). This was true of the precipitate from 4 different sera that were studied. It appeared as if small amounts of other protein fractions, which normally would not precipitate at such a concentration of metallic salt, were carried down with the gamma globulin of these abnormal sera. Numerous observations of electrophoretic patterns were carried out on whole sera from patients showing different values for the copper and zinc turbidity reactions. In every case the increase in the gamma globulin com-

ponent correlated well with the intensity of the reaction. Fig. 3 illustrates the close relationship between the turbidity as measured by dilution of serum with the zinc reagent and the gamma globulin concentration as calculated from electrophoretic patterns of various pathological sera showing approximately normal albumin levels.

Certain sera with elevated lipid levels showed a marked increase in the beta globulin fraction. This increase was not reflected in the copper turbidity reaction. It is well known that such beta globulin peaks will often disappear almost completely by extraction of the lipids from the serum⁶ and do not represent a true picture of the protein migrating in this fraction.

Addition of albumin to a positively reacting serum decreases the amount of globulin precipitated. Fig 4 demonstrates the *in vitro* change in the turbidity produced with the copper reagent upon the addition of increas-

⁶ Longworth, L. G., and MacInnes, D. A., *J. Exp. Med.*, 1940, **71**, 77.

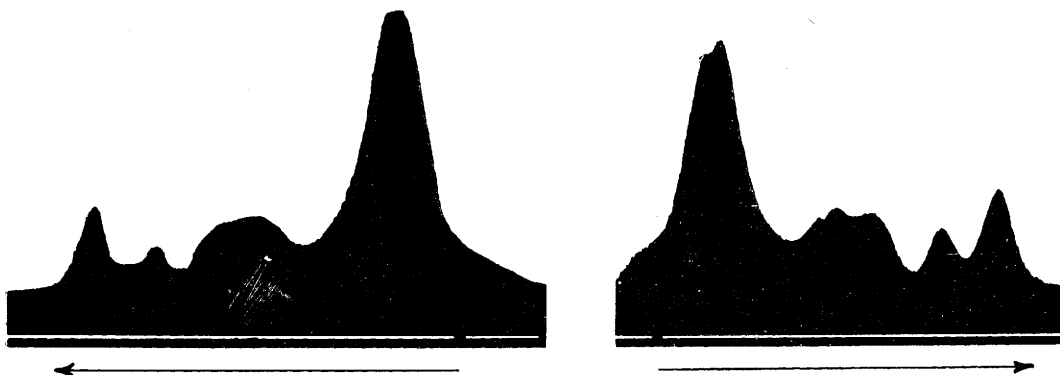


FIG. 2.

Ascending and descending electrophoretic patterns of the protein precipitated in the copper turbidity reaction. The major peak has the mobility of gamma globulin.

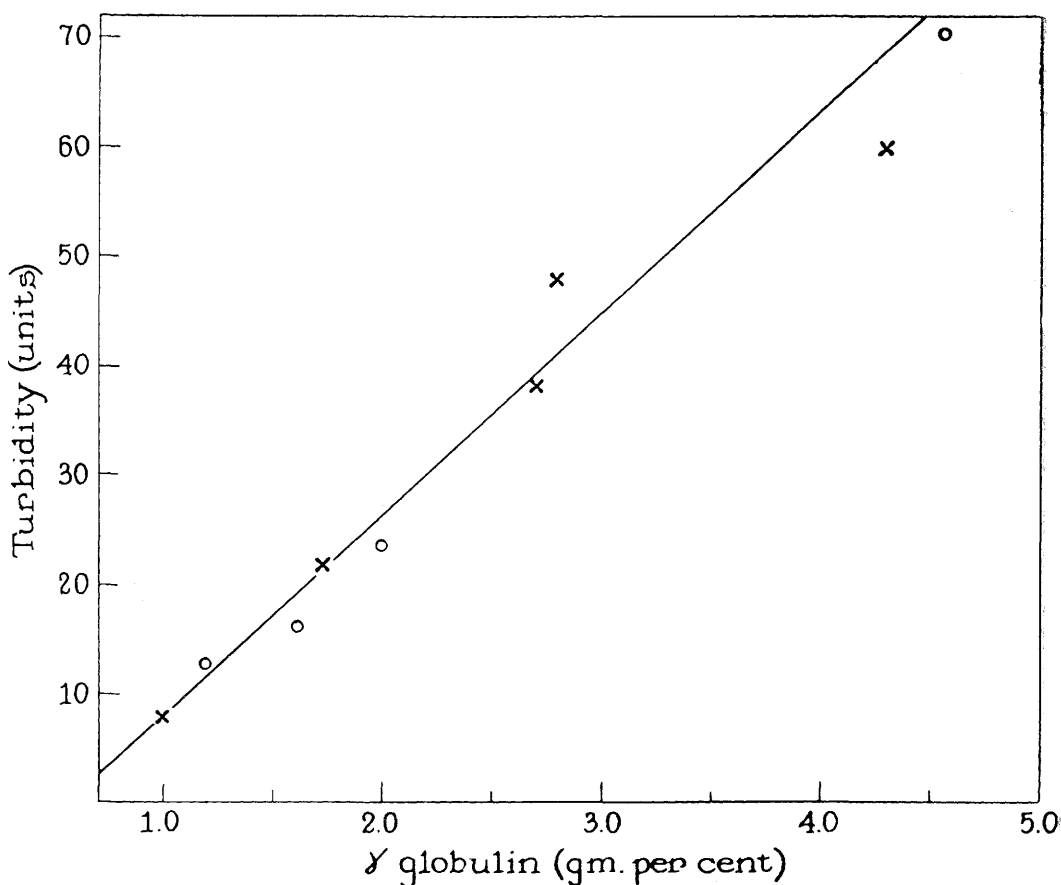


FIG. 3.

The correlation between the turbidity as measured in the zinc turbidity reaction and the gamma globulin concentration as calculated from electrophoretic patterns of various pathological sera. X = liver disease; O = other conditions.

ing amounts of human serum albumin. The intravenous administration of large amounts of concentrated human albumin to patients with liver disease demonstrated that a slight fall in the intensity of the reaction occurred and that it was similar to the fall expected from the *in vitro* experiments. The fact that an albumin deficit without hyperglobulinemia will not cause the copper turbidity reaction to become positive is clear, however, from observations on patients with nephrosis. The copper turbidity reaction was found normal despite the albumin deficit found in this disease.

Approximately 1000 determinations with the copper and zinc turbidity reactions have now been carried out on various sera. The normal range was found to lie between 2 and

8 units. Certain sera with globulin levels above 6 g % have shown values as high as 80 units. In these sera more than half of the protein was precipitated. Fig. 5 illustrates the close relationship between the turbidity in units and the globulin level of the serum. The reaction is not specific for liver disease but depends on the degree of elevation of gamma globulin. Serum from patients with multiple myeloma, for example, showed turbidity in proportion to the increase in gamma globulin. Forty-one patients with cirrhosis of the liver were tested and all were found to demonstrate a positive reaction. The test was of particular value when applied serially throughout the course of an acute illness. Fig. 6 shows the markedly delayed elevation in gamma globulin as determined by the zinc turbidity test

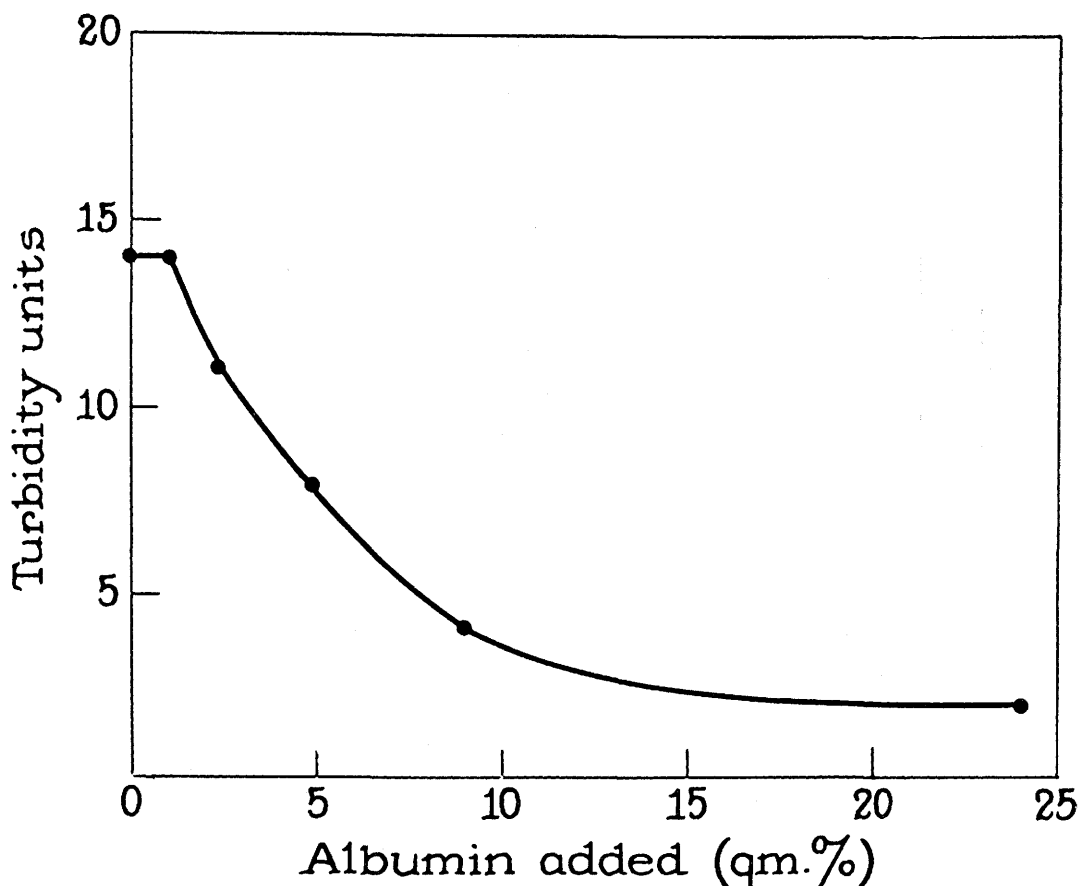


FIG. 4.

The effect of adding increasing amounts of concentrated human albumin to hepatitis serum on the intensity of the copper turbidity reaction.

during the course of a recurrence of acute infectious hepatitis. Similar changes with a delayed rise and prolonged elevation of the gamma globulin level have also been observed following an uncomplicated attack of acute infectious hepatitis.

Discussion. The Takata-Ara reaction and its many modifications, which have long been utilized for the diagnosis of liver disease, depend on the precipitation of protein from serum with a high globulin level. The mechanism of these reactions has always been obscure, but one of the substances added to serum in performing the tests is the metallic salt, HgCl_2 . The study of the effect of various concentrations of heavy metals on the precipitation of protein from serum has furnished an understanding of this reaction. Curves similar to the ones illustrated in Fig. 1 for

CuSO_4 may be constructed for various mercury salts and concentrations which will precipitate globulins from abnormal sera may be obtained. The Takata-Ara reaction does not employ the dilution technique and is only positive when marked elevation of the globulin level occurs.

The concentrations of zinc and copper sulfates used in the reagents described above were useful for detecting elevations in gamma globulin in liver disease. However, other concentrations may also be used for different purposes. For example, a slightly higher concentration will precipitate globulin from serum with a normal or an abnormally low globulin level. The procedure of determining protein aberrations by the turbidity developed in dilute, metallic salt solutions is so simple in use and consistent in results that it may find

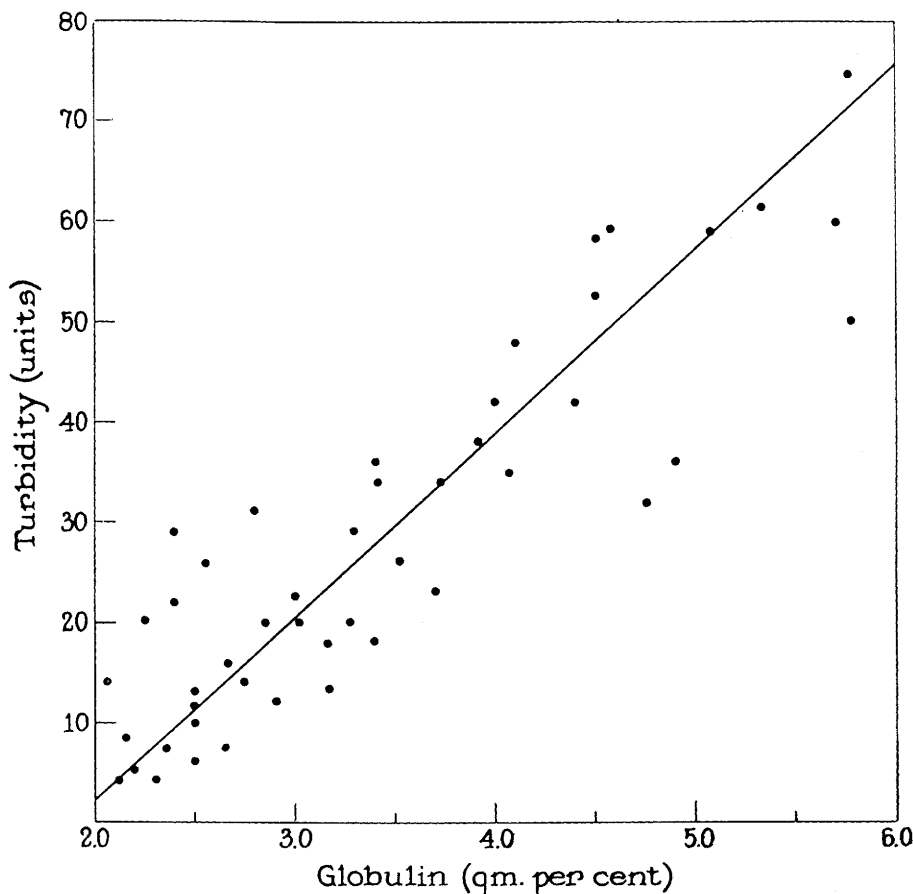


FIG. 5.

The correlation between the turbidity as measured in the zinc turbidity test and the total globulin level as determined by the Howe fractionation in various pathological sera.

application for a wide variety of purposes.

Measurable increases in the total globulin of serum in liver disease and in other conditions are almost always due to elevation of the gamma globulin fraction.^{2,7} The beta globulins may show significant changes electrophoretically but these depend mainly on the lipid level of the serum and do not represent true protein alterations. As a result, any technique which measures increases in gamma globulin in pathological sera is really a measure of the elevation in total globulin.

Significant curves of the globulin changes during the course of acute infectious hepatitis

were not obtained until the copper and zinc turbidity techniques were adopted. The delayed rise and prolonged elevation during the course of this disease is of considerable interest. It suggests the possibility that the globulin elevation may reflect the production of antibodies rather than a disturbance in liver function. The albumin level of the serum was often unaltered during the period of hyperglobulinemia. Serial determinations of the thymol turbidity test closely paralleled those of the globulins during the convalescent phase of infectious hepatitis, but marked differences were readily apparent in cases of cirrhosis of the liver. In this condition the thymol turbidity reaction was of considerably less value and showed little relation to the globu-

⁷ Gray, S. J., and Guzman Barron, E. S., *J. Clin. Invest.*, 1943, **22**, 191.

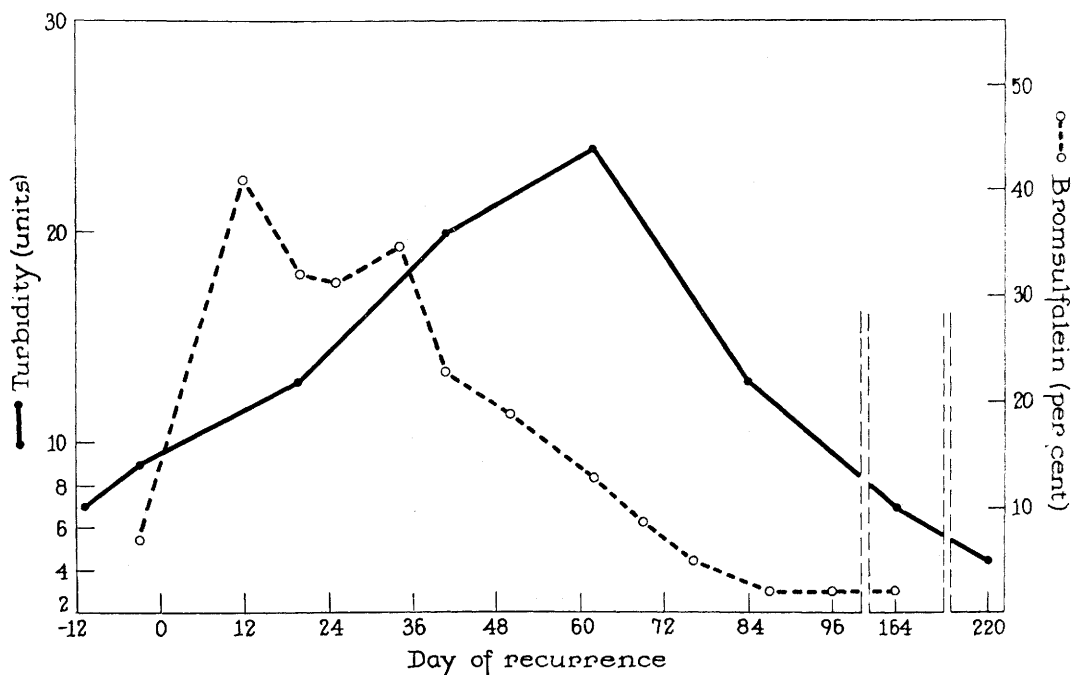


FIG. 6.

The delayed rise and prolonged elevation of the gamma globulin level in the serum following a recurrence of infectious hepatitis as determined by the zinc turbidity test. The curve for bromsulfalein retention is added for purposes of comparison.

lin aberration.⁸

In chronic infectious hepatitis alterations in the globulin components of the serum may be the only indication of persisting liver disease. In previous studies^{8,9} it was demonstrated that the thymol turbidity test was positive more often than any other single liver function test that could be applied to patients with persistent vague complaints more than 6 months after an attack of acute infectious hepatitis. Electrophoretic patterns showed that values for the thymol test paralleled the changes in the gamma globulin fraction closely in these patients. All patients with abnormalities in the thymol test also showed abnormalities in the copper and zinc turbidity reactions. In addition, the latter frequently remained positive after the thymol test had become negative. The zinc turbidity test, although not specific for liver disease, was found to be the most sensitive method of

detecting a lingering hepatitis. Five patients have been observed who developed cirrhosis of the liver following infectious hepatitis. A characteristic feature of this group was marked elevation of the gamma globulin fraction of the serum electrophoretically. In two of these patients the gamma globulin reached values above 6 g %. These aberrations were easily detected by the addition of the metal reagents to serum. The use of this test provided a rapid method of screening patients with persistent symptoms following infectious hepatitis in searching for the more severe complications of the disease.

The main advantage in the use of a test of this type instead of the cephalin flocculation or the thymol turbidity reaction is that a single known alteration in the serum is measured. This alteration is an elevation in the gamma globulin fraction. The other reactions depend partly on this alteration but also on other factors less well understood, such as the lipid level of the serum.

Another application of the zinc turbidity test has been in the study of the gamma globu-

⁸ Kunkel, H. G., and Hoagland, C. L., *Am. J. Med.*, in press.

⁹ Kunkel, H. G., and Hoagland, C. L., *Proc. Soc. Exp. Biol. and Med.*, 1946, **62**, 258.

lin changes associated with the development of antibodies following scarlet fever in patients with normal convalescence and patients developing rheumatic fever. Increased turbidity was found to closely parallel the rise in antistreptolysin and antistreptokinase titers. These results will be reported in a separate communication.

Summary. 1. When serum with abnormally high gamma globulin concentration is diluted with a solution containing a certain small amount of copper or zinc sulfate, a turbid precipitate forms and the optical density of the suspension is proportional to the concentration of gamma globulin.

2. Such an estimation of increase in gamma globulin really measures the total globulin elevation in pathological sera because hyperglobulinemia is almost always due to an alteration in the gamma globulin fraction of the serum.

3. The test has proved useful for determining alterations in gamma globulin during the course of an acute illness such as infectious hepatitis.

4. It was found to be of particular value in detecting persistent liver disease following infectious hepatitis. In a group of 41 patients with cirrhosis of the liver the reaction was positive in every case.

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Black Pigmentation in Feathers of Buff Orpington Chicks Caused by Vitamin D Deficiency.*

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Abnormal amounts of black pigment in feathers of New Hampshire chicks fed vitamin D deficient diets were observed by Glazener, Mattingly, and Briggs.¹ Domm² reported that feeding desiccated thyroid caused abnormal black coloring in Brown Leghorn males, females, and capons. Juhn and Barnes,³ working with capons of the same breed, produced similar results. Both the New Hampshires and the Brown Leghorns normally have some black pigment in the feathers. Buff Orpingtons, on the other hand, do not normally have black pigment in the feathers. It was of interest, therefore, to determine whether a deficiency of vitamin D would cause the

black-pigmented feathers in this breed.

Experimental Methods. The percentage composition of the vitamin D deficient basal diet fed in this experiment is as follows: ground yellow corn 30.0, ground wheat 25.5, ground barley 10.0, dehydrated alfalfa 5.0, B-Y riboflavin concentrate (250 μ g riboflavin/g) 0.5, ground limestone 2.0, dicalcium phosphate 1.5, soybean oil meal (expeller) 20.5, fish meal 4.0, salt (iodized) 0.5, and soybean oil 0.5. Manganese was added at a level of 75 p.p.m.

Chicks were examined and weighed at weekly intervals. At 6 weeks the feather color of each chick was given a numerical score, ranging from 0 to 5 depending upon the intensity of black pigment in the back feathers. A score of 0 indicated no black pigment whereas a score of 5 indicated very markedly blackened feathers. Since some of the chicks were extremely slow feathering, it was not possible to score all of them.

At the end of the 6-week period, a few chicks from each vitamin D deficient diet

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¹ Glazener, E. W., Mattingly, J. P., and Briggs, G. M., *Poultry Sci.*, 1946, **25**, 85.

² Domm, L. V., *Anat. Rec.*, 1929, **44**, 227.

³ Juhn, M., and Barnes, B. O., *Am. J. Physiol.*, 1931, **98**, 463.