Immunochemical Determination of Orosomucoid.* (22121)

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A low molecular weight, acidic glycoprotein having the mobility of an alpha-1-globulin at pH 8.6 has been isolated from human serum and has been extensively characterized(1-5). This protein, designated orosomucoid(5,6), is a major component of the "seromucoid"(7) or "mucoprotein"(8) fractions of serum, fractions which increase significantly in a number of diseases(6).

Studies on the concentration of orosomucoid in serum and other body fluids, as well as in various tissues, may be expected to yield information relating orosomucoid to disease and bearing on the origin of this glycoprotein. An immunochemical determination seemed best suited to the determination of orosomucoid in solutions containing proteins having similar stability and solubility characteristics. Previous attempts to prepare precipitating antibodies to this protein have been only moderately successful(9,10), due to the low antigenicity of orosomucoid. In the present paper it is shown that precipitating antibodies against orosomucoid can be prepared in chickens, and that these can be used to quantitate orosomucoid in serum and in tissue extracts.

Materials and methods. Orosomucoid was prepared from fraction VI of pooled normal human serum‡ by the method of Kefalides and Winzler(5). Antiserum was produced in healthy hens about one year old. One hundred mg of orosomucoid dissolved in 3 ml of water and mixed with an equal volume of aluminum hydroxide adjuvant was injected into the breast muscle. Two weeks later the injection was repeated, and 7 days later the chickens were bled out by cardiac puncture. The blood was citrated to prevent coagulation and to obtain maximum yields, and the cells separated by centrifugation. One hundred fifty mg of solid sodium chloride was added for each ml of plasma. The suspension was centrifuged and the fibrinogen which was salted out was removed as a gelatinous mat at the surface. The serum was then diluted with an equal volume of water to make the final sodium chloride concentration about 8%. The procedures developed by Goodman *et al.*(11-13) were adapted for the present immunochemical studies. Qualitative tests were carried out by adding a constant amount of antiserum (0.2 ml of a 1:2 dilution of antiserum) to 0.2 ml portions of serial dilutions of antigen in 8% NaCl, and examining for the presence of a precipitate after 1 hour at 38°C and again after 18 hours at 4°C. Quantitative determinations were carried out in 8% NaCl by adding 0.5 ml of the antiserum preparation to 0.5 ml of the antigen dilutions. The reactants were incubated at 38°C for 1 hour and then placed in a refrigerator for 18 hours. The tubes were then centrifuged at 2500 rpm for 30 minutes. Supernatants were decanted and saved for tests for antigen or antibody excess. Precipitates were resuspended in 5 ml of 8% saline and centrifuged again. A control containing antigen and normal chicken serum, as well as one containing 8% saline and the antiserum were carried out with each series of determinations. In no case did significant precipitation occur in the controls. The amount of protein in the precipitates was determined either from nitrogen determination by the method of Lanni *et al.*(14), using a factor of 6.25 to convert nitrogen values to

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TABLE I. Quantitative Orosomucoid-Antiorosomucoid Interactions.

Orosomucoid, μg	Total protein in precipitate, μg	Orosomucoid precipitated, μg	Antibody precipitated (by difference), μg	AB/AG	Supernatant tests, excess
0	0	—	—	—	AB
.7	13.8	.7	13.1	19.2	"
1.4	26.6	1.4	25.2	18.0	"
2.8	50.0	2.8	47.2	16.9	"
5.5	75.0	5.5	69.5	12.6	"
11	100.6	11.0	89.6	8.2	"
21	126.2	—	—	—	AG
42	158.2	—	—	—	"
84	113.4	—	—	—	"
167	94.2	—	—	—	"

0.5 ml antiserum PC-7 (diluted 1:2) plus 0.5 ml orosomucoid solutions in 8% NaCl. (Incubation 1 hr, 38°C, 18 hr 4°C. Precipitated nitrogen determined and converted to protein by multiplying by 6.25.)

protein, or by the method of Lowry *et al.* (15) using serum albumin as the standard. The serum seromucoid fraction was determined by the method of Weimer and Moshin(16).

Results. The antigenicity of orosomucoid is rather low in comparison with other plasma proteins. Preparation of antiserum from different chickens varied markedly, containing from 100 to 400 μg of antibody protein per ml.§ The preparation used to obtain the data shown in Table I contained between 90 and 100 μg of antiorosomucoid in 0.5 ml of the 1:2 dilution of the antiserum.

Table I shows a typical set of results when pure orosomucoid is mixed with antiserum prepared against it. As with other chicken precipitin systems(9) in which the reaction is carried out in concentrated NaCl, a relatively large excess of antigen is needed before inhibition of precipitation begins. The ratio of antibody to antigen in the precipitate formed in the antibody excess zone ranged from 19.2 to 8.2. Amounts of precipitate in this antibody excess zone, when plotted, serve as a calibration curve for determining the orosomucoid content of unknown mixtures. Since as little as 0.7 μg of the antigen yields a precipitate 19 times its own weight, it is unlikely that the precipitate results from a reaction with a minor component of the antigen.

To ascertain the homogeneity of the orosomucoid-antiorosomucoid system, the precipitation reaction was studied in a gel medium by the technic developed by Oudin(17). Two types of antisera were employed in these studies, antiorosomucoid and antibodies against fraction VI from which orosomucoid was prepared. This fraction contains albumin as well as several α-1 and α-2 proteins in addition to orosomucoid. The results of the studies using the Oudin technic are summarized in Table II. Since single bands were observed in series 1, 2 and 3, it appears that the orosomucoid-antiorosomucoid system is a homogeneous one. This band had a rate of migration which was proportional to the square root of the time, as shown by Munoz and Becker(18).

TABLE II. Orosomucoid-Antiorosomucoid Interactions in a Gel System.

	In gel	Overlying solution	Results
	1:2 dilution of antiserum in 8% NaCl, 0.15 M pH 7.4 phosphate & 0.6% agar	1% sol. of antigen in 8% NaCl	
1.	Antiorosomucoid	Orosomucoid	Single strong band
2.	"	Fraction VI	*Idem*
3.	Antifraction VI	Orosomucoid	Single weak band
4.	"	Fraction VI	Four distinct bands

§ It has been subsequently found that 4 to 8-fold higher antibody titers can be obtained by a single intramuscular injection of 200 mg of orosomucoid to chickens and collection of the blood in 10 days. It is likely that other injection schedules may result in still more effective antisera.

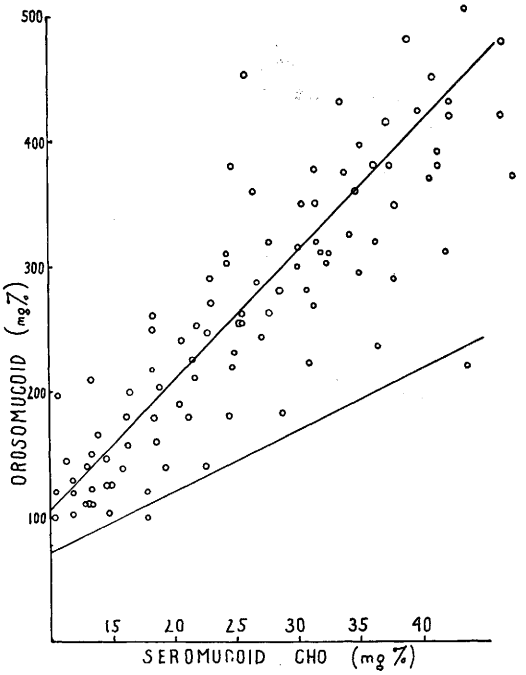

FIG. 1. Comparison of seromucoid fraction and orosomucoid levels in normal and abnormal sera.

The orosomucoid content of serum can be estimated by the immunochemical method described. Two-tenths ml of serum is diluted to 40 ml with 8% NaCl. Five-tenths ml of the diluted serum is added to 0.5 ml of 1:2 antiserum, and the incubation and protein determination carried out as previously described. The amount of orosomucoid is then determined from calibration curves of the type shown in Table I. Fifty normal sera examined in this manner showed a mean value and standard deviation of 103 $\pm$ 26 mg%. A single sample of serum was assayed repeatedly over the course of several months and gave consistent results. Thus, serum orosomucoid appears to be stable to storage by refrigeration.

In many clinical conditions including cancer, tuberculosis, etc., the orosomucoid levels are significantly elevated. Detailed data on serum orosomucoid levels in disease will be considered in subsequent reports. Values for orosomucoid are slightly higher than those for the seromucoid fraction determined in perchloric acid filtrates of serum(6). This is due primarily to the fact that there is considerable (30-50%) coprecipitation of the seromucoid fraction with the proteins precipitated by perchloric acid. Since it has been shown that orosomucoid is a major component of the seromucoid fraction(19), studies on seromucoid and orosomucoid levels of a series of 100 normal individuals and patients with various diseases associated with high serum glycoprotein levels were carried out. The results, shown in Fig. 1, demonstrate a rough correlation between the serum levels of orosomucoid and of the seromucoid fraction. The lower line in Fig. 1 is that expected from orosomucoid values corrected to a carbohydrate basis by multiplying by .16 (the fraction of hexose in orosomucoid)(3). Since the values of the seromucoid fraction are low by 30 to 50% due to coprecipitation with other proteins during perchloric acid precipitation(8), a further correction of the seromucoid values has been made by multiplying by 0.6. This second correction, which depends in part upon the total protein value, results in the upper line shown in Fig. 1. The fact that most of the experimental points lie along this line suggests that orosomucoid is the major protein in the seromucoid fraction. This substantiates the observation to be reported elsewhere(19) in which the seromucoid fraction has been shown by immunological methods to contain orosomucoid.

Much of the deviation of individual points from the best straight line is undoubtedly due to differences in coprecipitation or to combined experimental errors of the two methods. Points far to the left of this line, however, may be due to a cross reaction of the antibody with proteins precipitated by perchloric acid.

The present results demonstrate that an increase in the levels of orosomucoid in the serum of patients with tuberculosis, cancer, and a variety of other diseases can be determined with a specific immunological method.

The specificity and ease with which this determination can be carried out should make it a useful adjunct for the study of the source and significance of the serum glycoproteins. A possible extension of this work involves the coupling of fluorescein isocyanate with the antibodies to orosomucoid and the investiga-

tion of the deposition of the labeled antibodies in freeze-dried sections of various tissues suspected of being sources of orosomucoid.

Summary. A homogeneous glycoprotein, orosomucoid, isolated from normal human serum was found to be weakly antigenic in chickens. The antibody-antigen system appeared to consist of a single antigen and its specific antibody. A procedure for determining immunochemically the orosomucoid content of serum has been described. The results show a reasonably good correlation of orosomucoid with the levels of the seromucoid fraction (mucoprotein) of normal and pathological serum.

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Inhibition Studies with *Streptococcus faecalis*. Quinolines. (22122)

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Farber and his associates(1) observed that the anti-folics may be used to treat leukemia. This observation gave impetus to the synthesis and examination of metabolite blocking compounds. One outgrowth of this was the observation by Hitchings and his co-workers (2,3) that some anti-folics may have antimalarial properties. In view of these findings we have examined compounds initially prepared for testing against malaria(4-9) for their ability to interfere with the utilization of folic acid.

In this communication the results of tests on 93 quinolines for anti-folic properties are given. It may be indicated at the outset that some of the most useful anti-malarials are without effect against folic acid. While some specificity of activity was suggested by the study, the compounds do not compare favorably with some of the known anti-folics. Accordingly, the results appear to be only of theoretical interest at this time.

Experimental. The method of test was based on the use of *Streptococcus faecalis* (ATCC No. 8043) with a slightly modified version of the medium of Teply and Elvehjem (10). The modifications consisted of omitting the xanthine from the medium and of substituting "vitamin-free" casein hydrolysate (Nutritional Biochemicals) for the charcoal treated preparation suggested by the above authors. Under our conditions of test, with increasing amounts of folic acid in the medium up to 1 mμg per ml of medium, the