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Semi-Quantitative Immunoelectrophoresis and Its Application to the Study of Serum α-Globulins.* (28888)

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Immunoelectrophoresis has been used extensively for identification of subfractions of serum proteins and other biological materials. Quantitative analysis using immunoelectrophoresis has also been reported. Ouchterlony (1) reported a method of quantitative immunoelectrophoresis in which antigens, after electrophoretic separation, were allowed to diffuse into an agar-antiserum mixture, and the distance from the interface to the leading edge of the precipitin arc was measured. In a method reported by West *et al*(2), the antiserum was absorbed with increasing amounts of antigen, and the volume of antigen solution which resulted in nearly complete consumption of antibody was determined. Neither of these, however, is fully satisfactory for routine clinical use, owing to their relatively complicated procedures and also to their requirements for large amounts of antisera. In the present study, a simple system of criteria for a semi-quantitative interpretation of immunoelectrophoretic patterns was devised, and was used for analysis of serum α-globulins in various diseases.

Materials and methods. Serum specimens. Serum was obtained from 3 groups of normal healthy adults, each group representing a specific haptoglobin type. A total of 162 sera was tested from hospital patients of whom 30 had non-neoplastic inflammatory diseases, 37 malignant neoplastic diseases, 38 liver diseases, 22 renal diseases, and 25 other miscellaneous diseases.

Immunoelectrophoresis. Immunoelectrophoresis on microscopic slides was carried out using the method of Hirschfeld(3) who employed the discontinuous Laurell buffer system. Electrophoretic separation was continued for 120 minutes at 10 v/cm.

The identification of the orosomucoid precipitin arc was made with a rabbit anti-human orosomucoid serum. Ceruloplasmin and haptoglobin were identified with the *p*-phenylendiamine reaction of Uriel(4), and the anisidine stain of Javid(5), respectively.

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The precipitin arc due to α_1-lipoprotein was identified with the Sudan black B stain. Identifications of the other subfractions were made according to the technique of Hirschfeld(3).

Antisera. (a) Rabbit anti-human whole serum protein sera were prepared from normal human sera pooled in accordance with haptoglobin types. (b) Rabbit anti-human α_2-globulin serum was prepared from normal human α_2-globulin separated by means of starch block electrophoresis(6). (c) Horse anti-human whole serum protein serum was obtained from Hyland Laboratories, Los Angeles, Calif. (d) Rabbit anti-human orosomucoid serum was likewise obtained from Hyland Laborotories. Antiserum (a) was mixed with an equal volume of antiserum (b).

Paper electrophoresis. Sera were subjected to paper electrophoresis using veronal buffer, pH 8.6, $\mu = 0.05$, for 4 hours at 0.5 ma per strip and at 250 v. The strips were stained with amido black 10B for protein fractions and with PAS for glycoproteins and were scanned with a densitometer. The SB unit method(7) was employed for lipoprotein fractions using Sudan black B stain.

Biochemical determinations. Total serum protein was determined with cupric sulfate gravimetry. Total serum protein-bound hexose was determined by the orcinol method(8). Determinations of seromucoid, ceruloplasmin activity, and of haptoglobin were carried out according to the methods of Weimer and Moshin(9), Ravin(10), and of Javid(5), respectively.

Results. When undiluted serum was allowed to react with the undiluted anti-human whole serum, a total of from 14 to 16 precipitin arcs were formed consisting of 1 in the albumin zone, 3 in the α_1-globulin zone, 4 or 5 in the α_2-globulin zone, 3 or 4 in the β_1-globulin zone, and 1 each in the β_2- and γ-globulin zones.

Precipitin arcs in the α_1-globulin zone were identified, from anode to cathode, as α_1-lipoprotein, α_1-glycoprotein, and orosomucoid. Precipitin arcs in the α_2-globulin region were haptoglobin, ceruloplasmin, α_2-macroglobulin, β-lipoprotein and either 1 or 2 unidentified subfractions. Differentiation between haptoglobin types Hp 2-1 and Hp 2-2 was readily achieved in normal sera, but often difficult in pathologic sera.

Effect of dilution of antigen and of antisera on precipitin reaction. To find an optimal condition for development of immunoelectrophoretic precipitin arcs, effects of dilution of either the antigen or the antiserum were studied. Dilution of the antigen resulted in clearer demonstration of precipitin arcs in the α_1-globulin region, whereas clearer demonstration of precipitin arcs in the α_2-globulin region was achieved by dilution of the antiserum. These findings were common to both normal and most pathologic sera, except that the sera with decreased α-globulins sometimes had an optimal dilution ratio different from that of normal serum. Results of a reproducibility study and of a comparison between a large number of sera indicated that under the experimental conditions, semi-quantitative analysis was possible in a ratio of antigen and antiserum dilutions of 3.8 $\times$/undiluted for α_1-globulin subfractions, and undiluted/2.25 $\times$ for α_2-globulin subfractions (Fig. 1).

Immunoelectrophoretic quantitation of subfractions. Each of the precipitin arcs formed in the α-globulin regions was classified into 7 grades, ranging from a negative (grade 0) to the strongest positive (grade 6) reaction, according to the distance from the axis of electrophoretic migration ($a/a+\beta$, where a represented the distance from the convex summit of the precipitin arc to the line drawn

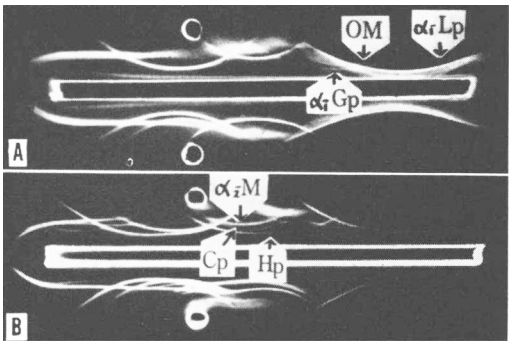

FIG. 1. Identification of precipitin arcs in α-globulin regions. Anode to the right. (a) Precipitin pattern of 3.8$\times$ diluted serum reacted with undiluted antiserum. (b) Precipitin pattern of undiluted serum reacted with 2.25$\times$ diluted antiserum.

TABLE I. Values of $\alpha/\alpha+\beta$ for Semi-Quantitation of Serum α-Globulin Subfractions.

Subfraction	Grade 1	Grade 2	Grade 3	Grade 4	Grade 5	Grade 6
Orosomucoid	<.57	.57–.62	.62–.67	.67–.72	>.72	
α_1-Glycoprotein	<.63	.63–.69	.69–.74	.74–.79	>.79	
α_1-Lipoprotein	<.62	.62–.65	.65–.69	.69–.72	>.72	
Haptoglobin	<.55	.55–.58	.58–.62	.62–.66	>.66	
Ceruloplasmin		<.35	.35–.42	.42–.53	>.53	
α_2-Macroglobulin		<.34	.34–.39	.39–.43	.43–.45	>.45

parallel to the length of the agar plate from the central brim of the specimen well, and β represented the distance from the former to the nearest brim of the antiserum trough) (Table I). In cases where the above objective criterion was inapplicable for a clear-cut grading, due to the insufficient sharpness of the precipitin arcs in certain pathologic sera, the following subjective criteria were also taken into consideration in determining the grades: (a) radius, (b) density, and (c) width of the precipitin arc (Fig. 2). The intensity of the reaction of normal sera corresponded to grade 3. To minimize possible subjective errors, reactions between the undiluted serum and the undiluted antiserum were referred to as controls. The reader had no knowledge of the patient's illness.

Correlations between immunoelectrophoretically determined values and values determined by the biochemical methods for the same subfractions were studied. Haptoglobin had a highly significant coefficient of correlation of $r = 0.90$ ($P<0.01$) between immunoelectrophoretically determined values and values determined by the method of Javid (Fig. 3-a). Ceruloplasmin also had a highly significant coefficient of correlation of $r = 0.90$ ($P<0.01$) between immunoelectrophoretically determined values and activities determined by the method of Ravin (Fig. 3-b). Similarly, the coefficient of correlation between values of orosomucoid levels determined by immunoelectrophoresis and the seromucoid levels determined by the method of Weimer and Moshin was $r = 0.91$ ($P<0.01$) (Fig. 3-c). The coefficient of correlation between the immunoelectrophoretically determined values of α_1-lipoprotein and the values of SB units was $r = 0.61$ ($P<0.05$) (Fig. 3-d). These findings support the possibility of simultaneous quantitation of the serum protein subfractions with the immunoelectrophoretic method.

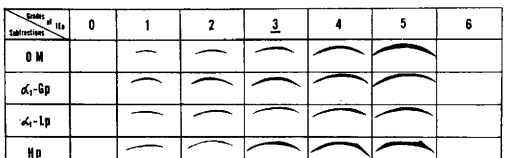

FIG. 2. Auxiliary criteria for immunoelectrophoretic semi-quantitation of α-globulin subfractions.

Quantitative changes of subfractions accompanied by the changes of α_1- and α_2-globulin fractions. The semi-quantitative immunoelectrophoresis was applied to a study of factors responsible for the quantitative changes of α-globulins in pathologic states. Protein and glycoprotein concentrations of α_1- and α_2-globulins as determined by paper electrophoresis were compared with the values of α-globulin subfractions determined by the semi-quantitative immunoelectrophoretic method. Orosomucoid and α_1-glycoprotein paralleled the protein concentration of α_1-globulin fraction, and haptoglobin paralleled the protein concentration of α_2-globulin fraction (Fig. 4-a and b). The correlation also applied to the concentration of protein-bound hexose in α_1- and α_2-globulins.

Precipitin arcs of the unidentified subfractions of α_2-globulin were detected only in a few of the neoplastic and inflammatory cases with increased α_2-globulin concentrations. Such subfractions were barely detectable in the serum from patients with nephrotic syndrome. These components are possibly glycoproteins, inasmuch as they are stained by amido black 10B and have a positive PAS reaction. Sudan black did not stain these components.

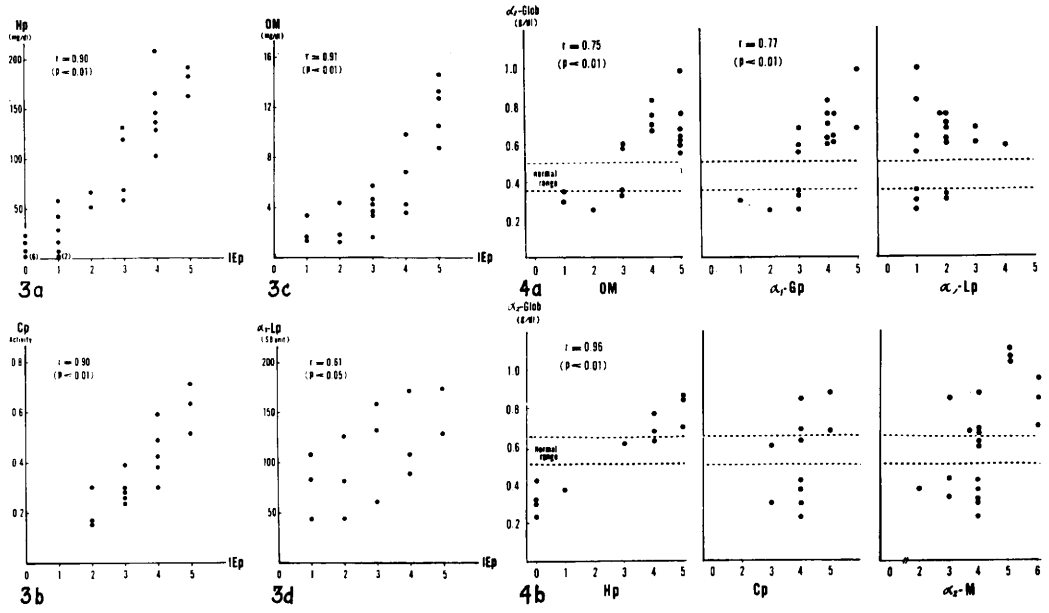

FIG. 3. Correlations between biochemically determined levels and immunoelectrophoretically determined values of subfractions of α-globulins. (a) Haptoglobin (Javid's method); (b) ceruloplasmin (Ravin's method); (c) orosomucoid (seromucoid, Weimer and Moshim method); (d) α_1-lipoprotein (modified Swahn's method).

FIG. 4. Correlations between total α_1- and α_2-globulin protein values and immunoelectrophoretic values of subfractions. (a) α_1-globulin; (b) α_2-globulin.

Discussion. The technique of immunoelectrophoresis of serum protein components has been greatly improved recently, and as many as 30 precipitin arcs have been reported(11). Of these, however, only a small number of precipitin arcs have been identified, leaving the physiologic significance of most of the components undetermined. Hirschfeld(3) reported the largest number of precipitin arcs. He detected 4 subfractions in the α_1-globulin region and 12 in the α_2-globulin region. Although the technique of Hirschfeld was essentially followed in the present study, fewer precipitin arcs were observed than were reported by him. In this study, 3 precipitin arcs in the α_1-globulin region and 4 or 5 in the α_2-globulin region, including 1 or 2 unidentified components in pathologic sera with augmented α_2-globulin, were detected. The difference was probably due to the antibody titers of the antisera and to minor details of the techniques. The present study was especially designed for quantitative analysis of a few specified subfractions of α-globulins in pathologic sera.

Each of the precipitin arcs had a fairly constant relative mobility. They were identified to be orosomucoid, α_1-glycoprotein, and α_1-lipoprotein in the α_1-globulin region, and α_2-macroglobulin, ceruloplasmin, haptoglobin and β-lipoprotein in the α_2-globulin region.

Under these standardized conditions, the immunoelectrophoretic method for the quantitative analysis was successful, and a close correlation was demonstrated between immunoelectrophoretically and chemically determined values. The principle utilized for the quantitation was essentially similar to that used in the immunologic quantitation of C-reactive protein(12). The possibility of direct and simultaneous measurement of several subfractions of α-globulin by the immunoelectrophoretic method was thus established.

By the use of this method, it was demonstrated that orosomucoid and α_1-glycoprotein played the dominant roles in the quantitative changes of total α_1-globulin, and that haptoglobin played the dominant role in the changes of α_2-globulin concentration. It is already known that the increase of α_1-globu-

lin in various diseases is due to increase of mucoprotein, or seromucoid(13), the heterogeneity of which is well established(14). An increase of α_1-glycoprotein, however, has been frequently encountered in the absence of increased total α_1-globulin concentration, especially in advanced neoplastic diseases(15). Thus the present method which enables simultaneous quantitations of both orosomucoid and α_1-glycoprotein may have an advantage over the conventional method of quantitation of total α_1-globulin in estimating the clinical severity of the disease. Similarly, the present method may have an advantage over the conventional method in cases with decreased haptoglobin and increased α_2-macroglobulin, not infrequently encountered in cases of nephrotic syndrome and of hepatic diseases(16), with an apparently unchanged or increased over-all α_2-globulin concentration. The above information indicates the promising applicability of semi-quantitative immunoelectrophoresis to the study of pathologic physiology of the subfractions of serum proteins.

Summary. A method is described for making direct and simultaneous quantitation of the subfractions of serum proteins by a modification of the immunoelectrophoretic method. By applying the method to the study of serum α-globulin subfractions, highly significant correlations were observed between biochemically determined values and intensities of their immunoelectrophoretic reactions. The method is simple, yet satisfactorily reproducible, and has a promising applicability to the study of pathologic physiology of the subfractions of serum proteins.

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