THE INTRINSIC VISCOSITY OF MIXED PROTEIN SYSTEMS, INCLUDING STUDIES OF PLASMA AND SERUM*

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INTRODUCTION

In recent studies of plasma viscosity in pulmonary tuberculosis, rheumatic diseases, and certain diseases of an infectious nature Houston, Harkness, and Whittington (1, 36) have shown what appears to be a general phenomenon, that plasma viscosities are in all cases greater than normal. The values of the viscosity of plasma and serum measured at normal concentrations, although having physiological significance, are subject to the criticism that the apparent viscosity usually measured does not really represent the true viscosity of the system. Houston, Harkness, and Whittington were aware of this fact and attempted to eliminate it in correlating their data.

The use of the concept of intrinsic viscosity[1] introduced by Kraemer (2) reduces some of the disturbing factors of the so called "colloidal effects" in protein viscosity measurements and also allows us to describe on a simple physical basis what appears to be the mechanism of enhanced viscosity in certain pathological plasmas.

We have studied the intrinsic viscosity relationships of purified bovine serum protein fractions in order to test a theoretical relationship. Thence we have studied the intrinsic viscosity of human plasma and serum, of clinically "normal," and of clinically pathological subjects. Our results indicate that plasma viscosities greater than normal reflect primarily a higher weight fraction of

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[1] The intrinsic viscosity is defined as $H = \lim_{c \to 0} \left[\frac{1}{c} \ln \frac{\eta}{\eta_o} \right]$ where c is the concentration of solute macromolecules in grams per 100 ml., η is the measured viscosity of the solution, and η_o the viscosity of the solvent system. H has the dimensions of cubic centimeters per gram. While this definition is a departure from Kraemer's original definition and is based upon an earlier equation introduced by Arrhenius (3) it gives the same value of H on extrapolation to infinite dilution and has the advantage of increased accuracy in plotting. Kraemer defined $H = \lim_{c \to 0} \frac{\eta_{sp}}{c}$ where $\eta_{sp} = \eta_r - 1 = \frac{\eta - \eta_o}{\eta_o}$ is called the specific viscosity increment.

fibrinogen and those globulins (α_2 and γ) which have higher intrinsic viscosities. This conclusion, although anticipated by many, has not been demonstrated previously in a quantitative fashion.

THEORY

The viscosity coefficient of solutions of macromolecules is both a colligative property of the solution and a constitutive property of the macromolecule. The measured viscosity coefficient of a protein solution is, in general, a function of many variables. The more important of these variables are:

(*a*) the viscosity coefficient of the solvent,
(*b*) the concentration of the solute,
(*c*) the temperature of the solution,
(*d*) the pH of the solution (4),
(*e*) the ionic strength of the solution (5–7),
(*f*) the electric charge on the molecule (6, 7),
(*g*) the velocity gradient under which the viscosity coefficient was measured (8–12),
(*h*) the shape of the macromolecule (13),
(*i*) the intramolecular flexibility of the macromolecule (14),
(*j*) the solvation of the macromolecule,
(*k*) physical interactions between macromolecules,
(*l*) the "chemical" interactions between macromolecules.

Intrinsic viscosity measurements eliminate the effects of (*a*), (*b*) and to a considerable extent of (*g*) and (*k*), and, if performed with (*c*), (*d*), (*e*), and (*f*) as parameters allow one to measure a function depending primarily upon (*h*) (*i*), (*j*), and (*l*). The "electroviscous" effect (*f*) is associated with the effects of pH and ionic strength, and as Briggs has shown (7) it can be reduced to insignificance by working at ionic strength values greater than 0.02.

Factors (*h*), (*i*), and (*j*) are all constitutive properties for a particular molecular species. In the absence of actual chemical combination of molecules, the intrinsic viscosity (H_T) of a solution containing several molecular species is an additive property of the system (15).

$H_T = \sum_1^n x_i H_i$ where H_T is the intrinsic viscosity of the mixture and x_i and H_i represent the weight fraction and the intrinsic viscosity respectively of a particular molecular species.

One must distinguish between two types of molecular interactions. One type is the purely physical effect of the presence of one molecule upon the behavior of another molecule and the effect on the hydrodynamic behavior of the solvent. This effect is enhanced in more concentrated solutions and leads to a failure of the solution to conform to Newtonian flow; *i.e.*, the rate of shear is not proportional to the applied stress. In addition, there is the possibility of a second type of interaction in which two molecules actually react to form a new mole-

cule. Except under unusual circumstances, a solution containing the new species of molecule will not have the same intrinsic viscosity as a physical mixture of two molecular species. The departure of the measured value of H_T from the calculated value of H_T would serve as a criterion for detecting such interaction.

It is well established that highly asymmetric macromolecules form unusually viscous solutions and measured values of the intrinsic viscosity of these solutions can be used to calculate approximate molecular dimensions and the intramolecular flexibility of a molecule (13, 14). To what extent, if any, intramolecular flexibility operates in the viscosity of protein solutions is as yet unknown. On the other hand, the departure from a linear relationship between concentration and the viscosity coefficient occurs at lower concentrations as molecules become more asymmetric (16), indicating hydrodynamic characteristics in the system that are not easily interpreted, as mentioned above.

A disturbing effect encountered when measuring the viscosity of more concentrated solutions of macromolecules is the inability to reproduce data from one viscometer to another. This is due, in part at least, to the orientation of the macromolecule in the direction of flow and is proportional to the velocity gradient (10), and in part to the physical interactions mentioned above, the effects of which increase with concentration. The higher the velocity gradient the greater is the orientation of the major axis of the asymmetric molecule along the direction of flow and the lower is the measured apparent viscosity. Increased molecular asymmetry enhances the effect. When only the orientation effect is involved, extrapolation to zero velocity gradient, corresponding to completely random orientation, should lead to measured viscosities independent of the viscometer used (11). As Edsall and Mehl (9) and Lyons (12) have shown, the effect of the velocity gradient becomes less pronounced at low concentrations. The effect due to physical interactions can be reduced by working with dilute solutions and extrapolation to infinite dilution.

In measuring the viscosity coefficient of solutions of macromolecules, using a capillary viscometer, the mean velocity gradient $\bar{\beta} = \frac{8V}{3\pi r^3 t}$ (8) should be stated as an essential parameter of the experiment. In Kroeplin's equation V is the volume of flow of the apparatus, t represents the time of efflux, and r the radius of the capillary.

The intrinsic viscosity may be visualized as the specific volume occupied by the protein molecules while flowing in a viscometer; *e.g.*, 4.2 cc. per gm. for serum albumin and 25.0 cc. per gm. for human fibrinogen. Since the partial specific volume is usually about 0.75 cc. per gm. for most proteins, the difference between the intrinsic viscosity and the partial specific volume represents the increased volume (neglecting solvation effects) necessary for a gram of molecules to have available for random orientation in free flow. This increased volume is a function of the axial ratio of the molecule (17).

The above theoretical considerations indicate that *intrinsic* viscosity data are simpler to interpret than are viscosity data. An intrinsic viscosity measurement is often a powerful method of elucidating the physical nature of a solution containing several molecular species.

Experimental Methods

All viscosities were measured at 37.00 ± 0.05°C. in a water bath with a Fisher-Irany viscometer.[2] This is an Ostwald type viscometer in which the capillary spirals through four turns in a vertical distance of 5 cm. The capillary is approximatly 75 cm. long allowing the use of a small sample (1.1 ml.). The mean velocity gradient $\bar{\beta} = \frac{8V}{3\pi r^3 t}$ varied from 96 to 112 sec.$^{-1}$ in the concentration ranges in which we worked. With this viscometer we were able to obtain a precision of one part in one thousand in measured time of flow. The effect of the velocity gradient on the viscosity coefficient of plasma and serum diluted to concentrations of 1 per cent with 0.1 M sodium citrate solution was determined to be less than 0.1 per cent and well within the limit of error of our determined values of the intrinsic viscosity. Because of the long flow time and the small volume of sample, the kinetic energy correction is negligible and was neglected.

Careful cleaning was found to be a critical factor in obtaining reproducible viscosity values. Before each determination the viscometer was cleaned successively with 10 per cent sodium hydroxide followed by copious rinsing with distilled water and finally dried from an acetone rinse. All solutions used for cleaning, all air passed through the viscometer, and the protein solutions were filtered through fine sintered glass filters in order to remove particulate matter.

The viscometer was aligned vertically with a plummet, the vertical alignment points being the orifice into and the egress from the capillary. Failure to reproduce vertical alignment introduces rather large variations in efflux times. The direction of error is in general towards longer flow times.

Plasma and serum samples were secured as outlined below. Freshly drawn oxalated blood was immediately centrifuged and dialyzed 48 hours at 5°C. against 0.1 M sodium citrate at pH 7.4. A sample of 5 ml. was dialyzed against 250 ml. of dialysate with three changes of the dialysate in 48 hours. Serum samples were obtained by clotting an aliquot of plasma by the addition of calcium chloride. The clot was held for an hour at 37°C., then squeezed to remove as much serum as feasible, and weighed wet; after which it was dried in a vacuum oven at 105°C. for 12 hours. The dry weight, corrected for salt content of the moisture removed, and for the precipitated calcium oxalate, furnished the fibrinogen values used throughout the work. Although the determination of fibrinogen in this manner has been shown to be of questionable accuracy (18), nevertheless, due to the limited amount of each plasma sample available to us, it was the only feasible method. The error due to loss in calcium oxalate in squeezing the clot is hardly significant. However, errors due to occlusion in the clot of other proteins in plasma, particularly lipoprotein, will cause our fibrinogen determinations to be

[2] Purchased from Eimer and Amend, 635 Greenwich St., New York.

high (18). This will be discussed further in a subsequent part of the report. The serum was dialyzed against 0.1 M sodium citrate at pH 7.4 as with plasma above.

In general, the protein concentrations were determined on the basis of colorimetric nitrogen analysis according to the method of Johnson (19), using the factors 6.73 for plasma and serum, 5.9 for fibrinogen, and 6.2 for albumin and globulin (20). The determination of the concentration of protein represents the chief source of error in our measurements of the intrinsic viscosity. The reported values for the intrinsic viscosity (H) of plasmas and sera are accurate to 3 per cent. In the case of the preliminary studies using purified serum components, concentrations were determined from dry weight analyses, in which cases an accuracy of 1 per cent was obtained. In the case of dry weight analysis an aliquot of the solution was dried overnight in a vacuum oven at 105°C. and corrected for the salt content.

DATA AND RESULTS

The intrinsic viscosity for crystalline bovine albumin,[3] bovine fibrinogen,[3] and for bovine gamma globulin[4] is shown in Table I. The values of (H) based upon extrapolation to infinite dilution are as follows: albumin $H = 0.042$ ml./gm., fibrinogen $H = 0.204$ ml./gm., and for gamma globulin $H = 0.070$ ml./gm. The intrinsic viscosity data for a mixture of 54.3 per cent albumin and 45.7 per cent fibrinogen and for a mixture 51.2 per cent albumin and 48.8 per cent gamma globulin are also shown in Table I. In all the above solutions the protein was dissolved in 0.1 M sodium citrate at pH 7.4 and dialyzed 48 hours at 5°C. in order to remove inorganic ions other than ions from sodium citrate. In Fig. 1 we have plotted certain of the experimental data in order to indicate the form of the experimental curves.

In another experiment (the data are also given in Table I) the intrinsic viscosity of a mixture of 44.0 per cent globulin and 56.0 per cent albumin was determined in 0.1 M sodium citrate at pH 6.4. An interaction to produce an albumin-globulin salt should occur at a pH between the "average isoelectric points" of these two components. The close agreement between the calculated and experimental data at pH 6.4, given in Table I, indicates such an interaction did not occur. The results of the studies on mixtures of bovine serum fractions indicate the validity of the equation $H_T = \sum_1^n x_i H_i$ and demonstrate that intrinsic viscosity data for mixed protein systems are additive. From the published data of Oncley, Scatchard, and Brown (21) giving the intrinsic viscosity data for various human plasma fractions and from the data on the composition

[3] Obtained from Armour Laboratories, 1425 W. 42nd St., Chicago.

[4] A preparation made by one of the authors according to methods given in *J. Am. Chem. Soc.*, 1948, **70**, 84.

TABLE I

The Intrinsic Viscosity of Bovine Serum, Protein Fractions and "Normal" Human Plasmas and Sera

Solute*	H experimental	H calculated $= \Sigma x_i H_i$	Fibrinogen	Protein content plasma	Fibrinogen
	ml./gm.		*gm./100 ml.*	*gm./100 ml.*	*per cent*
Bovine serum albumin	0.042				
Bovine fibrinogen	0.204				
Bovine gamma globulin	0.070				
54.3 per cent albumin 45.7 per cent fibrinogen	0.118	0.116			
51.2 per cent albumin 48.8 per cent globulin	0.056	0.057			
56.0 per cent albumin‡ 44.0 per cent globulin	0.055	0.054			
"Normal" plasma E. L. H.	0.059				
"Normal" serum E. L. H.	0.052	0.052§			
"Normal" plasma A. C.	0.062	0.063‖	0.424	7.48	5.60
"Normal" serum A. C.	0.052	0.052§			
"Normal" plasma A. W.	0.062	0.061‖	0.395	8.35	4.70
"Normal" serum A. W.	0.052	0.052§			
"Normal" plasma G. T.	0.059	0.061‖	0.373	7.30	5.10
"Normal" serum G. T.	0.051	0.052§			
"Normal" plasma J. D.¶	0.063	0.063‖	0.455	7.42	6.16
"Normal" serum J. D.	0.053	0.052§			
"Normal" plasma J. N.	0.060	0.061‖	0.355	6.92	4.85
"Normal" serum J. N.	0.052	0.052§			
"Normal" plasma R. M.¶	0.060	0.061‖	0.334	6.60	5.00
"Normal" serum R. M.	0.052	0.052§			

* Solvent 0.1 M sodium citrate pH 7.4 except where indicated otherwise.

‡ pH 6.4.

§ Calculated from data given in reference 21–23.

‖ $\Sigma\ x_s H_s + x_f H_f$. s = serum, f = fibrinogen.

¶ Children (J. D., 9 year old female; R. M., 12 year old male).

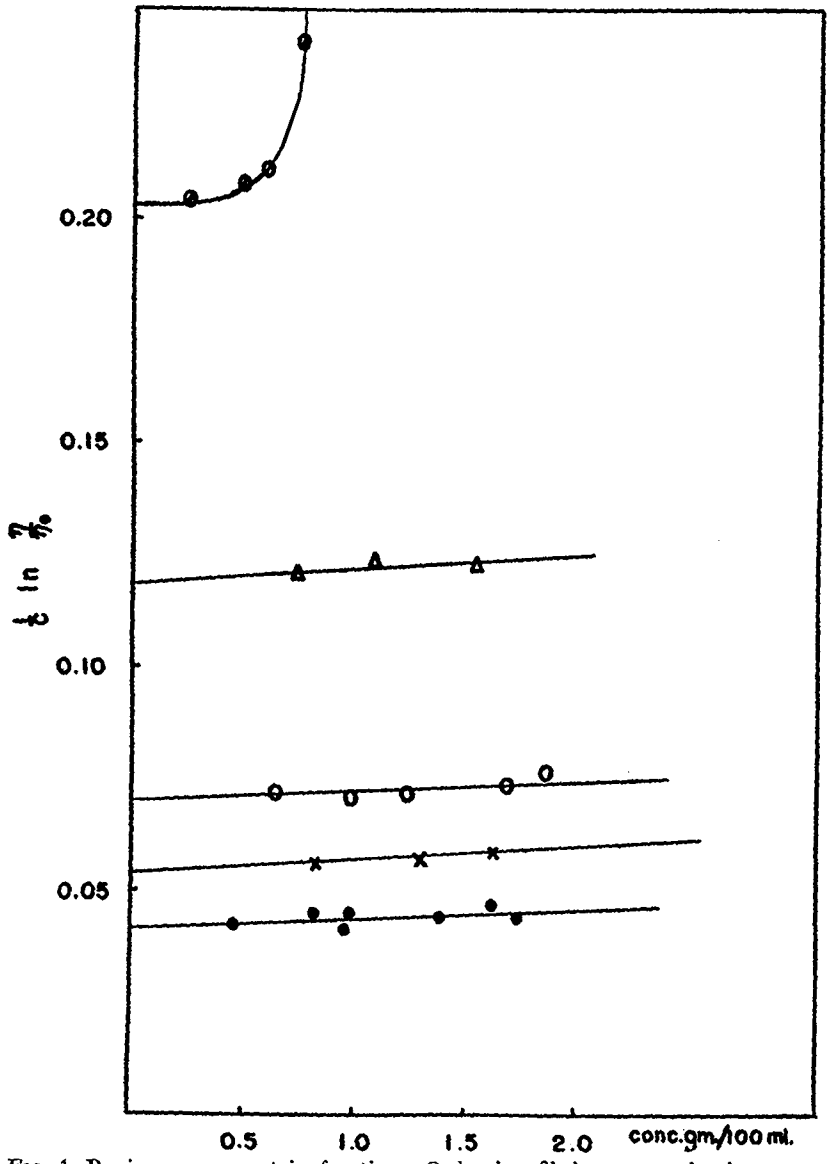

FIG. 1. Bovine serum protein fractions. ⊖, bovine fibrinogen. ○, bovine gamma globulin. ●, bovine serum albumin. △, 54.3 per cent albumin 45.7 per cent fibrinogen. ×, 51.2 per cent albumin 48.8 per cent globulin. All proteins dissolved in 0.1 M sodium citrate pH 7.4.

of plasma by Armstrong, Budka, and Morrison (22) and by Perlmann and Kaufman (23) we have calculated the intrinsic viscosity of normal plasma according

to the equation $H_{\text{plasma}} = \sum_{1}^{n} x_i H_i = 0.063$ ml./gm. Data used for the calculation are shown in Table II. The intrinsic viscosity of normal serum is calculated to be $H_{\text{serum}} = 0.052$ ml./gm.

It is obvious that the intrinsic viscosity of plasma is most sensitive to the variations in the fibrinogen content. In order to correlate the measured intrinsic viscosity of individual plasma samples with fibrinogen concentration we calculated $x_s H_s$, where $1.00 - x_f = x_s$ and H_s is the experimental value. From the fibrinogen concentration and the total plasma protein concentration for each

TABLE II

Weight Fractions and Intrinsic Viscosities of "Normal" Human Plasma and Serum Proteins

	x_i (22, 23)	H_i (21)	$x_i H_i$
		ml./gm.	
Albumins	0.54	0.042	0.0227
α-globulins	0.14	0.079	0.0110
β-lipoproteins	0.060	0.041	0.0025
β-globulins	0.080	0.055	0.0044
γ-globulins	0.120	0.071	0.0085
Fibrinogen	0.060	0.228	0.0137
Plasma	1.000		$H_p = 0.0628$ ml./gm.
	x_i	H_i	$x_i H_i$
Albumins	0.575	0.042	0.0242
α-globulins	0.149	0.079	0.0118
β-lipoproteins	0.064	0.041	0.0026
β-globulins	0.085	0.055	0.0046
γ-globulins	0.128	0.071	0.0092
Serum	0.999		$H_s = 0.0523$ ml./gm.

individual plasma sample we calculated $x_f H_f$. This experimentally determined value added to $x_s H_s$ gave us the calculated value of H_{plasma}. The close correlation between the calculated and experimental values can be seen in Table I. Where the fibrinogen level is high the experimental value for the intrinsic viscosity of plasma is likewise high. We have measured the intrinsic viscosity of the plasma and serum of seven clinically normal individuals (2 adult females, 3 adult males, and 2 children: a 9 year old female and a 12 year old male). In all cases the experimental values agree with the calculated values within 3 per cent.

Abundant experimental evidence exists (24–26) indicating that in most diseases involving infection with fever, general variations in the plasma protein levels occur. Fibrinogen, alpha globulin and gamma globulin levels increase

and the albumin level decreases somewhat. Intrinsic viscosity data reflect these changes. In Table III are listed data from several types of pathological plasmas and sera. The marked elevation of intrinsic viscosity of the plasma reflects the

TABLE III

Pathological Plasmas and Sera

	H experimental	H calculated	Fibrinogen	Protein content plasma	Fibrinogen	Diagnosed
	ml./gm.	*ml./gm.*	*gm./100 ml.*	*gm./100 ml.*	*per cent*	
Plasma P. P. T.	0.117	0.113	0.970	4.51	21.0	Tuberculosis, advanced, with cardiac failure and edema terminating in death—39 yr. old male
Serum P. P. T.	0.080					
Plasma P. C.	0.085	0.082	0.731	6.30	11.8	Nephritis—9 yr. old male
Serum P. C.	0.063					
Plasma P. J. L.	0.065	0.065	0.388	7.85	4.9	Mild rheumatic fever on salicylate therapy—12 yr. old male
Serum P. J. L.	0.056					
Plasma A. R. J.	0.082	—	0.850	8.05	10.5	Acute rheumatic fever—25 yr. old male. Without therapy
Serum A. R. J.	0.016*	0.063*				
Plasma A. R. X.	0.079	0.078	0.714	7.10	9.95	Acute rheumatic fever—7 yr. old male. Without therapy
Serum A. R. X.	0.058					
Plasma C. A. C.	0.074	0.076	0.394	8.71	4.52	Duodenal carcinoma—37 yr. old male. Metastasis to jaw
Serum C. A. C.	0.068					
Plasma C. A. D.	0.080	0.077	0.700	7.02	10.0	Squamous cell carcinoma—54 yr. old male
Serum C. A. D.	0.057					

* The measured value of H for A. R. J. serum was unusually low indicating proteolysis had occurred during dialysis. N. P. N. determination gave additional confirmation for this conclusion.

higher levels of fibrinogen as well as the elevated intrinsic viscosity of the serum. The agreement between experimental and the calculated values for the intrinsic viscosity of plasma is within 4 per cent. The data of Morrison (18) would suggest that the calculated value for H_p should be higher than the experimental value of H_p due to the presence of alpha and beta lipoproteins occluded in the fibrin clot. Thus our calculated value of $x_f H_f$ for fibrinogen added to the intrin-

sic viscosity increment due to serum ($x_s H_s$) would give us a calculated value for H_p greater than the experimental value. In most cases, our calculated value for the intrinsic viscosity of plasma is less than our experimental value.

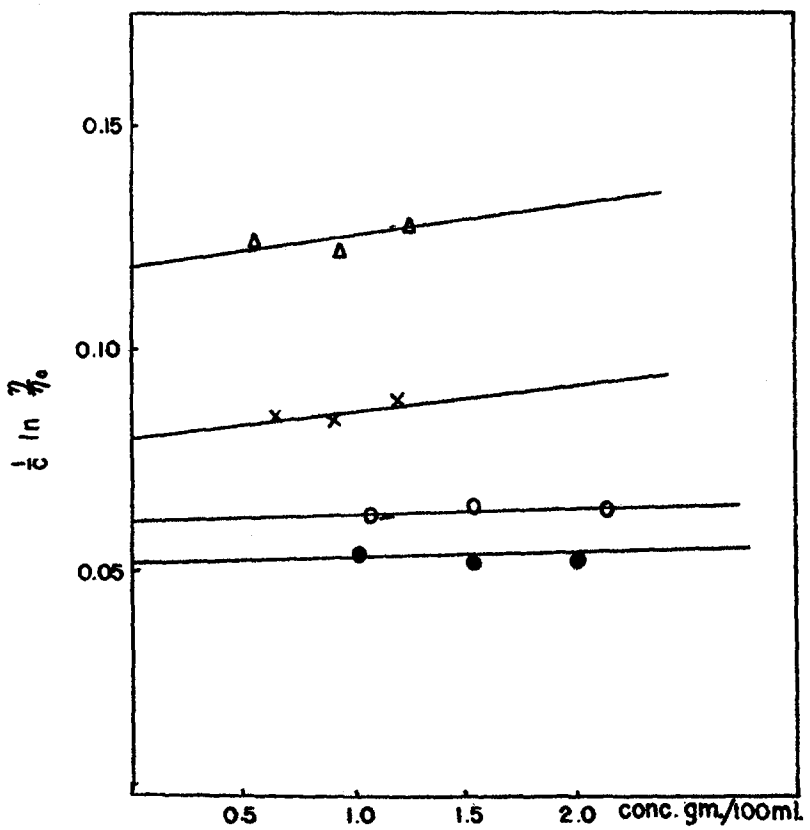

FIG. 2. Human plasma and serum. △, pathological plasma P.P.T. ×, pathological serum P.P.T. ○, "normal" plasma G.T. ●, "normal" serum G.T. in 0.1 M sodium citrate pH 7.4.

The intrinsic viscosity of the pathological sera reflects the elevated levels of those proteins possessing higher intrinsic viscosities (H_i); namely, α_2-globulins and γ-globulins, and the relative decrease of albumin with its low H_i value. In Fig. 2 are plotted experimental curves for a "normal" and a pathological plasma and serum.

DISCUSSION

The excellent agreement between the measured and the calculated values for the intrinsic viscosity of "normal" sera serves as additional confirmation that

the ethanol fractionation procedures developed by Cohn and his associates (27) yield products essentially unaltered with regard to the shape of the molecule.

Recently several investigators have postulated the combination of serum and plasma components into larger aggregates (28, 29). Our results do not support such a hypothesis. On the contrary, the intrinsic viscosity data appear to be strictly additive, which is the expected behavior of a mixture of molecules in the absence of interaction. It seems likely that these measurements are sufficiently sensitive to detect such interactions should they occur.

The viscosity measurements of Harkness and Whittington (1, 16, 29, 36) were made with undiluted plasma and serum. The departure from Newtonian flow behavior of such systems makes quantitative interpretation difficult. The fact that such systems exhibit non-Newtonian flow characteristics is evidence of physical interactions but does not warrant any conclusions as to stoichiometric chemical interactions such as Harkness and Whittington have attempted (29).

The measured intrinsic viscosity of the pathological serum (PPT) was 0.080. This is an unusually high value and is difficult to reconcile with the known protein composition of serum. Only one protein component in normal serum, the α_2-globulin ($H_i = 0.092$), possesses an intrinsic viscosity greater than this value. It is necessary to assume that 25 per cent of the serum protein is α_2-globulin and a corresponding decrease in albumin to 30 per cent of the serum protein to calculate an intrinsic viscosity for serum of 0.064. Since the α_2-globulin value reported by Oncley, Scatchard, and Brown (21) was based upon a fraction known to be a complex mixture of several molecular species, it seems probable that one of these species is a very asymmetric molecule which imparts a large viscosity increment. Seibert (30, 31) has reported a large increase in serum polysaccharide in the case of advanced tuberculosis and carcinoma, and lesser increases in other diseases. Recently Surgenor (32) has separated a mucoprotein from the α_2-globulin fraction of normal serum. He states that this protein forms rather viscous solutions. It seems probable that it is this protein augmented in pathological conditions which produces the unusually high serum intrinsic viscosities.

The data based upon the plasma and serum C.A.C. from the patient with duodenal carcinoma are interesting since the fibrinogen level is normal. The enhanced value of H_p is entirely due to the high value of H_s. The large value of H_s in C.A.C. again demonstrates the presence in the serum of a component with marked molecular asymmetry. This is significant since Seibert finds that serum polysaccharide levels in carcinoma sera may be 300 per cent above normal (30).

The data on patient (P.J.L.), a mild case of rheumatic fever, perhaps reflect the effects of salicylate therapy, since there is evidence that salicylate therapy

reduces the level of fibrinogen (37). This patient, a 12 year old boy, had been on salicylate and penicillin therapy prior to hospitalization. The elevation in his serum intrinsic viscosity, although slight, is significant and accounts for the elevation in the plasma intrinsic viscosity.

Although the evidence is circumstantial, it is interesting and reasonable to speculate that the "C-reactive protein" reported by Tillett and Francis (33) is the component in serum possessing the high intrinsic viscosity. Tillett and Francis found this protein, although normally not present in serum, to occur in the serum of patients with acute rheumatic fever, bacterial endocarditis, pneumonia, and staphylococcal osteomyelitis. Ash (34) confirmed the work of Tillett and Francis and found the "C-reactive protein" present in the serum of patients infected with colon-typhoid group bacilli. Dole and Rothbard (35) found the "C-reactive protein" to occur in the α-globulin components of serum.

Intrinsic viscosity studies of mixed protein systems are useful in characterizing these systems and in serving to distinguish subfractions of the system, particularly if one or more of the individual components are well characterized. The technique can easily be adapted to routine clinical procedures. One ml. of each plasma and serum diluted to approximately 1 per cent protein concentration can be used to determine $\frac{1}{c} \ln \eta/\eta_o$, which reflects the departure of plasma and serum protein composition from the normal levels.

SUMMARY

Experimental evidence is presented that the intrinsic viscosity of solutions of mixed proteins obeys the additive equation $H_T = \sum_1^n x_i H_i$. The datum serves to characterize the system, and combined with other analytical techniques and fractionation procedures, enables one to analyze and characterize subfractions. The plasmas and sera of clinically "normal" individuals give intrinsic viscosity values agreeing with calculated values. The intrinsic viscosity values for pathological plasmas and sera in all cases are greater than normal and reflect the augmented levels of those proteins fibrinogen, α_2-globulins, and γ-globulins occurring in the pathological state. The method is readily adaptable to routine clinical use and furnishes a measure of the departure from normal of protein levels in serum and plasma.

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