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Sodium Citrate Salting-out of the Human Blood Serum Proteins

by

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Salting-out methods have proved of value for blood serum proteins fractioning. A wide bibliography has been published on this matter, being known the solubility curves for total serum and its several fractions, when increasing the concentrations of various salts (1). However, data regarding the precipitating effects of sodium citrate upon human serum protein has not been published, although the equation constants of the solubility curve for carboxihemoglobin in sodium citrate solutions are known (5). It appears that sodium citrate could be a good protein precipitating agent, as it is very water soluble, being possible to obtain very concentrated solutions of great ionic strength. It is also a polivalent anion, and we know that the alkaline salts of polivalent anions are more effective for protein precipitation than monovalent anions are (1).

The evergrowing progress and multiplicity of fractioning techniques, improved in recent years, have not vanished the classic salting-out methods, having even enhanced their use. Therefore we were interested in improving our knowledge about the action of non-used salts, especially sodium citrate, being an organic metabolite of suspected little denaturating action. The object of this work has been to study the serum protein solubility curves with different sodium citrate concentrations, controlling the nature of the soluble protein fractions by electrophoresis.

Material and Methods

The following techniques were used: a) Obtainment of human blood serum. b) Preparation and control of sodium citrate solutions at known concentrations. c) Precipitation of serum proteins adding sodium citrate solutions. The precipitate was separated from the soluble fraction by filtration. d) Cuantitative estimation of the soluble proteins in the filtrate. e) Electrophoresis of the soluble fraction.

All the results were statistically treated.

1. Blood serum. Blood was taken from twelve healthy young people in fast-

ing conditions. This was done to avoid the presence of chylomicrons which could opacify the serum.

2. SODIUM CITRATE SOLUTIONS. They were prepared from a Merck p. a. sodium citrate concentrated solution by dilution in bidestilated water. The saline concentrations were estimated measuring the refraction indices, because saline concentration and refraction index are related by an exponential equation (7). Four readings were made from every solution, using an ABBE CARL ZEISS refractometer, keeping a 20° C temperature with a Colora thermostat type K, and taking the mean values. With this method it is possible to measure sodium citrate concentrations in grammes per cent, with an approximation up to the 2nd decimal figure. Table I represents the relation between refraction indices and concentrations of the used sodium citrate solutions.

3. PROTEIN PRECIPITATION. The blood and the sodium citrate solutions were mixed at a ratio of 1:25, leaving 0.5 ml of serum and 12 ml of citrate solution in each test-tube. The mixture with the serum implies a sodium citrate dilution of 96 %. Table I contains the real concentrations of citrate in the used samples. Three and a half hours later filtration was carried out with an Albet 242 filter paper, refiltering several times until the filtrate became completely transparent (3).

4. MEASUREMENT OF PROTEIN CONCENTRATION IN THE SOLUBLE FRACTION. This was measured by the biuret reaction, using the GORNALL *et al.* reactive (2).

A pattern curve was plotted containing values double of those of the maximun protein concentrations used by us in order to know how the method fulfilled the Beer's law. Four series of ten protein solutions were prepared using as serum, Lab-trol from Dade Reagents, containing 7.35 gm/100 ml. Two ml of serum diluted in isotonic sodium chloride, were mixed with eight ml of biuret reagent. Half an hour later, the optical densities were measured with a Spectronic 20 colorimeter of Bausch and Lomb, at 540 mμ, using the biuret reactive mixed with an isotonic sodium chloride solution for blank. Table II gives the mean optical densities related to every protein solution used. The slope of the straight line was calculated with all the obtained values using the square least method, resulting m=37.969, being 0.0219 the mean square error. With

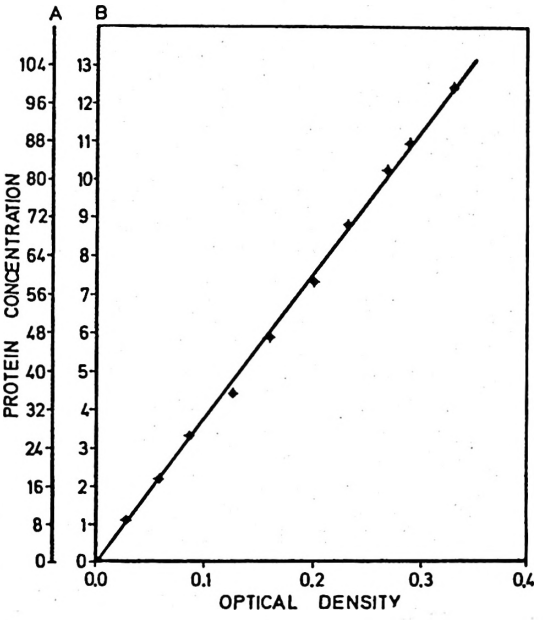

FIG. 1. Calibration curve obtained with the least squares method, correlating protein concentrations and optical densities after the biuret reaction. The points are the mean arithmethic values of optical densities for each concentration. Absolute values expressed in mg/100 ml of dissolved protein concentration in the reactive, appear in A in ordinates. B represents the protein concentrations in gm/100 ml in the samples, which, according to the type of dilution used, are 125 times greater than the absolute values.

all this data, a straight line was plotted placing the protein concentration values in ordinates and optical densities in abscises (fig. 1). In the diagram, the absolute values for protein concentration appear in ordinates, as well as the equivalence to protein concentration in g/100 ml of serum, according to the used diluting type. The precision of the method is reflected by the narrow limits of confidence shown in table II, as well as by the nearness of mean values on the straight line, according to the equation values. It is evident that the biuret reaction, using the GORNALL *et al.* reactive, was a precise enough method for our determinations.

To evaluate the protein concentration of the filtrate, 2 ml of it were mixed with 8 ml of the biuret reactive. A mixture containig 10% sodium citrate solution and biuret reactive was used for blank. The total protein concentration was measured using the same dilution.

5. PAPER ELECTROPHORESIS OF THE FILTRATES. Control and identification of the soluble protein fractions at different sodium citrate concentrations, were made by electrophoresis of the filtrates. The filtrates were dialyzed against water at 4° C temperature, during 24 hours to eliminate the excess of salt. They were then concentrated in a Büchi Rotavapor R at 30° C and 380 mm Hg vacuum, until the volume of the sample was only 0.5 ml, usually seven to eight hours later. One of the serum samples was subjected to complete series of precipitations, and two electrophoretic fractionings were carried out of every filtrate with a Dosimat Elphor electrophoretic machine. Samples of 15 μl were put on Whatman No. 1 paper strips, 4 cm width and 32 cm length. Sodium veronal buffer pH 8.6 and $\Gamma/2 = 0.1$ was used (8). The paper strips were left during 20 hours at 100 V and 7 mA. Finally they were dyed with bromophenol blue, using a technique which allows a quite reliable cuantification (11). With the results read in a EEL photodensitometer the pattern of the curves was drawn.

6. STATISTICAL TREATMENT OF THE RESULTS. The results were statistically treated finding the mean values, parameters and confidence limits (9). The electrophoretic results were not cuantificated. The equations which correlate logarithms of solubility to ionic strength were calculated using a 1620 IBM computer with a least squares method program, giving also mean square errors.

Results and discussion

1. CURVE OF RESIDUAL DISSOLVED PROTEIN. Increases in sodium citrate concentration, origin a progressive decrease in the residual dissolved protein. The salt concentrations used appear in table I, and the solubility curve in fig. 2. The points over the curve are the mean values, and

TABLE I

Relation between refraction index and concentration of sodium citrate solutions.

R I	C	0.96 C	R I	C	0.96 C
1.3587	15.95	15.35	1.3790	29.97	28.77
1.3600	16.81	16.13	1.3803	30.92	29.68
1.3615	17.80	17.09	1.3817	31.94	30.67
1.3629	18.74	17.99	1.3833	33.13	31.80
1.3658	20.69	19.87	1.3859	35.07	33.67
1.3672	21.65	20.78	1.3860	35.14	33.74
1.3704	23.86	22.90	1.3875	36.28	34.83
1.3717	24.76	23.77	1.3898	38.04	36.52
1.3730	25.68	24.65	1.3909	38.89	37.33
1.3743	26.60	25.53	1.3922	39.90	38.30
1.3756	27.52	26.42	1.3950	42.10	40.42
1.3776	28.96	27.80	1.3958	42.74	41.03
1.3789	29.90	28.70	1.3967	43.45	41.72

R I: Refraction index of sodium citrate solutions. C: Sodium citrate concentration in water solution (g/100 ml). 0.96 C: Sodium citrate concentration in final serum-salt mixtures.

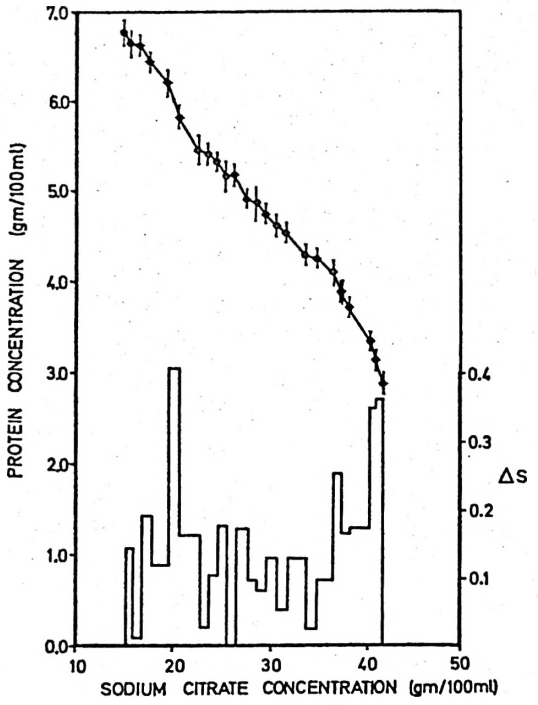

FIG. 2. Serum proteins solubility curve at progressively increasing sodium citrate concentrations. In the lower zone, differentiation diagram, where ΔS corresponds to a decrease in dissolved residual protein for each concentration increase. The scale of the latter graph appears on the right.

the vertical lines are the confidence limits for $P = 0.05$ (9). The lower curve is a differentiation diagram, where the ΔS values correspond to the relation between residual protein decrease and increase in salt concentration (10). The residual solubility curve falls progressively and evenly without important directional changes. We can observe an inflexion point between concentrations of 25.53 and 26.42 g/100 ml. In both, the mean concentration value of the soluble protein fraction was 5.15 g/100 ml, with a $\pm$ t.$s_m = 0.19$ for the first point and 0.11 for the second ($P = 0.05$). The differentiation diagram reveals clearly a $\Delta S = 0$ between the two concentrations. The inflexions on the solubility curves are taken as limits which mark the end of the precipitation of a protein fraction, a precipitation of a new fraction starting after them. The γ-globulins precipitation ended at this inflexion, as it was proved by electrophoresis control of the proteins contained in the soluble fraction.

From a sodium citrate concentration of 26.42 g/100 ml, the curve keeps without apreciable change in the slope. At a concentration of 40.42 g/100 ml there is a sudden fall of protein solubility. The differentiation diagram shows clearly this change in the curve, with wider increases in the ΔS value. The electrophoretic results showed the meaning of the sharp fall of protein solubility starting from that salt concentration.

2. ELECTROPHORETIC CONTROL OF THE SOLUBLE FRACTION. The electrophoretic controls showed that between 15.31 and 25.53 g/100 ml sodium citrate concentrations, the γ-globulins decreased progressively in the filtrates till disappearing completely in the last concentration. The

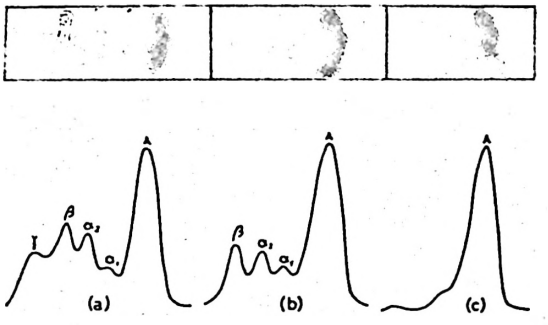

FIG. 3. Electrophoretic fractions contained in the filtrates.

a) A non-precipitated serum pattern.

b) Protein fractions of the filtrate at 25.53 g/100 ml sodium citrate concentration. The γ-globulins have completely disappeared.

c) Only albumins remain in the filtrate at a 40.42 g/100 ml concentration.

other serum protein fractions remained unchanged in the electrophoretic strips, so that we must admit that the γ-globulins had a very selective precipitation between those concentrations. At 40.42 g/100 ml concentration, the globulins disappeared completely, but albumin did not, suggesting that their precipitation had not occurred or did in a very small proportion (fig. 3c). Sodium citrate concentrations above 40.42 g/100 ml caused albumin precipitation.

There is a parallelism between fractions precipitated by sodium citrate and those obtained by electrophoresis. This concordance has been noticed by other authors when investigating the solubility curves of serum proteins in the presence of salts. SVENSSON (12) pointed out a similar parallelism when analysing the precipitation of serum proteins from several animals using ammonium sulfate, MAJOOR (10) arrived to similar conclusions with sodium sulfate, and GRAS and SALAZAR (4) with sodium thiosulfate. Using sodium citrate, three fairly pure fractions were separated: γ-globulins, α- + β-globulins and albumin. While the γ-globulin had its own solubility curve, the α- and β-globulin curves overlapped. The electrophoretic patterns showed that β-globulins precipitated at lower saline concentrations than the α_2-globulins, and the latter did so with respect to α_1-globulins, but similarly than Svensson's observations, all these globulins had very large areas of precipitation which overlapped, precipitating as if they were a single protein.

TABLE II

Relationship between optical density and protein concentration after biuret reaction. Values are corrected to express grammes per 100 ml of serum. The real concentration of diluted proteins in the reactive is 1/125 of the values expressed in the table.

O D	$\pm t.s_m$	P C
0.0292	0.00292	1.1025
0.0587	0.00321	2.2050
0.0875	0.00963	3.3075
0.1263	0.08251	4.4100
0.1590	0.00759	5,8800
0.1987	0.00969	7.3500
0.2300	0.00408	8.8200
0.2680	0.00969	10.2900
0.2867	0.01109	11.0250
0.3275	0.01226	12.4950

OD: Mean optical desinty. $\pm t.s_m$: Limits of confidence for P = 0.01. P C: Equivalence of protein concentration in the serum samples.

3. SALTING-OUT EQUATIONS. The fact that before the first inflexion of the solubility curve only the γ-globulins disappeared from the filtrates, and that from 40.42 g/100 ml sodium citrate concentration only the albumin remained, moved us to verify how the solubility experimental data of these two fractions would adjust with the salting-out equations of isolated proteins (6), yet knowing that the γ-globulins are not a pure fraction. On the other hand, it was interesting to know with what mean square error would fit the α- + β-globulins values to a salting-out equation.

In concentrated saline solutions the graph of the logarithm of protein solubility related to ionic strength is a straight line. Expressed in mathematical terms:

$$\log S = \beta - K_s \Gamma/2$$

where S is the solubility of the protein in grammes per litre of solution, $\Gamma/2$ is the ionic strength in moles/litre, and β and K_s are constants representing respectively the intercept and slope of the straight line. From that, it emerges trat protein solubility in concentrated saline solutions, can be expressed in terms of two constants: β and K_s.

To find the values of these two constants, which correspond to the three serum protein fractions precipitated with sodium citrate, the least square method was used, computing the experimental data on residual dissolved protein at different salt concentrations, the latter expressed in terms of ionic strength. The values corresponding to the first two points on the γ-globulin precipitation curve were excluded, because the salting-in effect masked the salting-out effect, and the decrease of solubility was less than at higher concentrations (1). Also the two values corresponding to the inflexion between γ- and β-globulins were excluded. The values of β and K_s in the three equations and the mean square errors, are consigned in table III.

TABLE III

Values of the slope and intercept of blood serum proteins solubility curves in sodium citrate concentrated solutions.

	K_s	β	M S E
γ-Globulins	- 0.0549	2.0347	0.02287
α + β-Globulins	- 0.0456	1.9894	0.02767
Albumins	- 0.2098	3.4969	0.03587

K_s: Slope of the straight line. β: Intercept. M S E: Mean squares error.

The mean square error values are not high, and for albumins are greater than for α- + β- and γ-globulins. This fact apparently abnormal, being albumin a single protein, occurs because we analysed only 72 points for the albumin compared with 288 for α- + β-globulins, and 168 for γ-globulins. It should be pointed out the great precision with which the experimental values of solubility for α- + β-globulins fit to the salting-out equations of single proteins, suggesting that α_1-, α_2- and β-globulins, with all their pure protein fractions, will have similar β and K_s values. The same can be said for those contained in the γ-globulins.

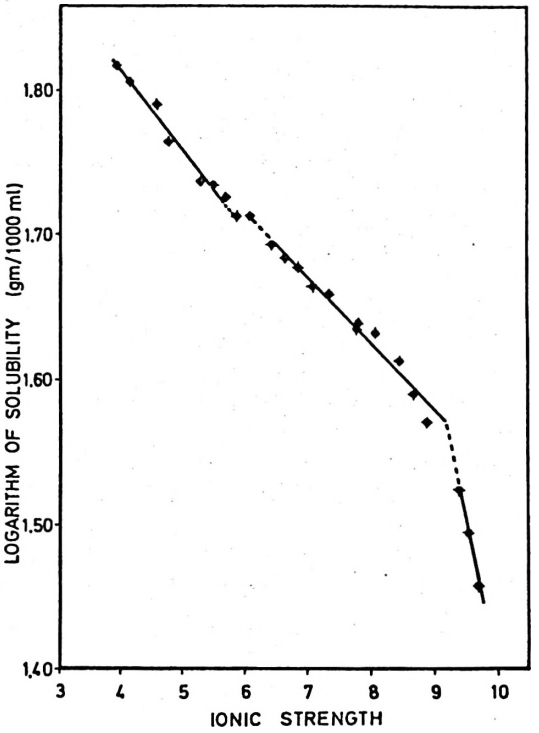

FIG. 4. Representation of the resulting curves after aplying salting-out equations to the three separated fractions. The upper segment corresponds to γ-globulins, the central part to α- +β- globulins, and the lower segment to albumins. Each point is the mean arithmethic value out of 24 values.

Drawing the corresponding curves of the three fractions, according to their equation values (fig. 4), results a slope for albumin with a greater value than that for α- + β-globulins, hence, the intersection point marks with precision the ionic strength where ends the precipitation of globulins and starts albumin precipitation. Expressed in numbers, the intersection point between these two lines was $\Gamma/2 = 9.180$, corresponding to a sodium citrate concentration of 39.48 g/100 ml, where the remaining dissolved albumin had a mean concentration of 3.72 g/100 ml

in total serum, with an albumin/globulin ratio of 3.72/2.98.

The salting-out protein mixtures has been criticized, assuming that high salt concentrations could produce protein denaturation. The protein molecule is so fragile and its spontaneous tendency to denaturation so great, that none of the known fractioning methods can escape from similar criticism. Nevertheless salting-out procedures are still used in the laboratory, and they are good enough to separate proteins as fragile as enzymes are. These experiences carried out with sodium citrate, do not allow to state whether or not protein denaturation has ocurred. However, we can assume that the citrate anion, being a Krebs cycle metabolite, will not have a denaturating action greater than that of the sulfate anion. On the other hand, if this salt would modify the chemical structure of the protein molecule, these changes could not be relevant because they do not alter electrophoretic migrations. The electrophoretic patterns of the proteins of the filtrates show clear fractions after dying, similar to those obtained with fresh samples of the same sera (fig. 3a). Therefore no important changes in the molecules of the soluble fractions occur, and if they did, would regress after dialysis.

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Summary

Human serum protein solubility curves in concentrated sodium citrate solutions, show an inflexion point between 25.53 and 26.42 g/100 ml concentrations, and a definite change in the slope starting at the concentration of 40.42 g/100 ml. With the electrophoretic control of the soluble fractions, γ-globulins can be seen to precipitate before the inflexion point. Between the inflexion and the change in the slope, α- + β-globulins precipitate. Albumin precipitation starts at a concentration of 40.42 g/100 ml. There is a high degree of correlation between both fractioning techniques, as has been proved with the solubility curves obtained with different salts by others.

The obtained values correlating logarithms of solubility and ionic strength, have mean square errors comparatively low for each of the three precipitated fractions, suggesting that pure proteins contained in every fraction, have similar straight lines equations constants.

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