DETERMINATION OF PROTEINS IN THE PRESENCE OF PHENOL, SUCROSE, MANNITOL, GLUCOSE, FRUCTOSE, AND TRIS (TRIHYDROXY METHYL AMINO METHANE), BY LOWRY'S METHOD

by

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SUMMARY

1. It has been confirmed that the very strong interference of phenolic compounds in the protein determination by Lowry's method can be corrected according to Potty's procedure. This procedure is applicable if the phenol level does not exceed 30 µg. At higher phenol levels it is necessary either to eliminate the excess of phenol by absorption on ion exchange resins, or to precipitate the protein.

The content in phenols can be calculated with precision according to — Cu values.

2. The importance of sucrose interferences depends on the amount of proteins in the sample. For protein content of about 50 µg the determination can be made directly, whatever the sucrose concentration may be. For larger protein amounts the interferences become more and more important with increasing sucrose levels. So it becomes indispensable to dilute the solution in order to lower the sucrose concentration below 10 %.

3. A direct determination of proteins in the presence of a known and constant amount of mannitol encounters no obstacles if the same mannitol amount is included in standards.

4. The presence of Tris in the reaction medium leads to very important interferences and makes a direct determination of the protein impossible.

5. For a material rich in reducing sugars notable interferences in the determinations should be expected.

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RESUME

1. Il a été confirmé que la procédure proposée par Potty permet de corriger les interférences des composés phénoliques dans le dosage des protéines par la méthode de Lowry. Toutefois, la procédure n'est applicable que lorsque la teneur en phénol ne dépasse pas 30 µg. Pour des teneurs plus élevées il est nécessaire soit d'éliminer l'excès de phénol par absorption par des résines échangeuses d'ions, soit de précipiter les protéines.

La teneur en phénol peut être calculée avec précision suivant les valeurs — Cu.

2. L'importance des interférences du saccharose dépend de la quantité des protéines contenues dans l'échantillon. Pour une quantité de l'ordre de 50 µg le dosage peut être effectué directement quelque soit la concentration en saccharose. Pour des quantités plus élevées des protéines les interférences augmentent avec l'augmentation du saccharose. Dans ce cas il est indispensable de diluer la solution de manière à diminuer la concentration en saccharose en dessous de 10 %.

3. Il est possible d'effectuer le dosage directement en présence d'une quantité connue et constante de mannitol à condition d'introduire la même quantité de mannitol dans la gamme d'étalon.

4. La présence du Tris dans le milieu réactionnel entraine des interférences très importantes et rend impossible un dosage direct.

5. Dans un matériel riche en sucres réducteurs on peut s'attendre à des interférences très importantes.

∴

It is well known that the Folin reagent (1) gives a color reaction with phenol OH groups. This property has been used by Wu (2) for the determination of proteins on the basis of their tyrosine and tryptophane OH groups.

The modification introduced by Lowry et al. (3) consits of an alkali pretreatment of the protein solution with copper ions. This increases considerably the sensitivity of the reaction. The increase in color intensity is due to the formation of a Cuprotein complex and not to the presence of OH groups in the protein. So Lowry's method makes use of both the phenolic and peptidic groups reactivity.

Nowadays this technique is widely applied in biochemistry. Its real advantages are great simplicity, rapidity and at the same time sensitivity.

So, as it is not specific for proteins, it is subject to the interferences of various substances, endogenic as well as exogenic ones, the latter introduced with the extraction medium, the density gradients, etc.

This is why in spit of its advantages is should be used with caution.

When they published their method, Lowry et al. themselves made some restrictions regarding its application (differences in color development obtained with various proteins ; serious interferences of substances such as uric acid, guanine, hydrazine, glycine, ammonium sulphate).

Nevertheless a direct determination of protein in biological materials is of widespread use. This may give highly erroneous values.

Such is the case of protein estimation in enzyme preparations rich in phenolic compounds and pectins, as reported by Potty (4). We could notice it when estimating the proteins in subcellular fractions of cotton plant leaves rich in polyphenol compounds.

The same happens when proteins are determined in fractions obtained from sucrose gradients as noted by Gerhard and Beevers (5).

Sulfhydryl reagents (dithioerythritol, 2 mercaptoethanol, cysteine, glutathione and potassium ions) can also produce important interferences (6).

At last, some buffers in current use such as Hepes, Tricine, Taps (7) (8) are going to give incorrect results either because those compounds themselves give a color reaction in Lowry's procedure, or because they prevent the normal color produced by proteins developing.

From what is said above it appears that applying Lowry's technique directly to a complex medium containing, in addition to proteins, some other substances, meets with many difficulties.

The study being reported was undertaken in order to point out the errors which it is possible to make, to overcome the difficulties encountered and to specify some limits to the method.

Attemps are made to extend to otheir interfering substances Pottys' procedure of protein determination in the presence of phenolics.

METHOD

In all the experiments, two parallel determinations have been made: one according to Wu's method (2) using the Folin reagent without copper, named — Cu (phenolic groups) and the other one according to Lowry's method (3) symbolised by + Cu (phenolic and peptidic groups). The difference between the optical densitites + Cu and — Cu named Δ should represent according to Potty (4) the value corresponding to the protein.

Reagents

A-2 % Na_2CO_3 in O.1N NaOH solution.

B-Alkaline copper reagent prepared by adding 1 ml of a solution containing 0.5 % copper sulfate in 1 % sodium tartrate to 50 ml of reagent A.

C-Folin reagent diluted to a final acidity of 1 N.

Procedure

Two 1 ml samples of tested solution were made. One was vigourously mixed with 5 ml of reagent A (— Cu), the other with the same volume of reagent B (+ Cu). After 10 minutes, 1 ml of Folin reagent was added to each. Optical densities were measured after 30 minutes at 500 mμ in a Beckman DB spectrophotometer. Respective reagent blanks were run in parallel.

RESULTS

Phenol interferences

The standard curves of albumin (Bovine Albumin Fraction V, B. grade, lot 72129 CA Biochem. Los Angeles) and of phenol (Merck, Darmstadt), purified according to Draper and Pollard's technique (9) are shown in Figures 1 and 2.

The data in Table I illustrate the effect of increasing phenol concentrations added to known quantitities of albumin on O.D. values + Cu, — Cu and Δ.

It is evident that in presence of even small quantities of phenol, the error in albumin, estimating according to O.D. + Cu, is very important. For exemple, for albumin at 50 µg in presence of 10 µg of phenol the value found would be 115 µg, that is 230 % of the real quantity. In presence of 20 µg of phenol, the overestimation would reach 378 %. Though if calculated according to Δ, the values obtained (Table 1) are in good agreement with the real quantities of albumin. This procedure can be applied to phenol levels up to 30 µg. At higher phenol concentrations, the values found for the albumin are underestimated. So it appears that 40 µg of phenol, the quantity admitted by Potty (4), is indeed too high.

In the presence of such quantities of phenol, direct determination is no longer possible. Either we have to eliminate the excess of phenol by retaining it on ion exchange resins (10) or precipitate the proteins (by alcohol or trichloracetic acid), centrafigue, wash and redissolve them in NaOH.

The use of polyvinyl pyrrolidine is to be advised against, as its capacity for absorbing phenol compounds is very small and moreover, it is not without action upon the enzymes (10).

Concerning phenol levels, they can be estimated with precision according to — Cu values, that is by subtraction from the registered — Cu value, the — Cu value corresponding to albumin alone. The latter is easy to find according to Δ.

For example, for 100 µg of albumin + 20 µg of phenol the O.D. values registered are :

+ Cu = 0.331 ; — Cu = 0.212 ; so Δ = 0.119.

According to Fig. 1, to Δ = 0.119 corresponds — Cu value = 0.032, so 0.212 — 0.032 = 0.180 which corresponds, according to Fig. 2, to 19.3 µg of phenol.

Such a phenol estimation readly allows us to see if a direct determination of proteins by Potty's procedure is applicable.

Sucrose interferences

As shown in Figure 3a sucrose alone gives a weak color development with +-Cu reagent which becomes appreciable at a concentration of about 15 %. The color intensity increases regularly with increasing sucrose concentrations. With — Cu reagent there is practically no color reaction except for very high concentration (over 50 %).

The effect of increasing amounts of sucrose on the color reaction given by albumin is illustrated in Figures 3 b, c, d.

It is apparent that the error made in protein estimation according to + Cu values depends on the amount of protein. At a protein level of about 50 µg practically no interferences are observed (Fig. 3 b). The two opposing sucrose effects compensate one another : the color development by surcrose alone and the inhibitory effect of sucrose on the color development by albumin.

For 100 µg and still more for 150 µg of protein (Fig. 3 c, d), a depressive effect of sucrose can be observed. The error becomes significant from the sucrose concentration of about 10 % and increases with increasing disaccharide

TABLE I. — **Estimation of albumin in presence of increasing concentrations of phenol.**

µg Phenol added	µg Albumin added																						
	0	50							100							200							
	O.D.	O.D.			Value found				O.D.			Value found				O.D.			Value found			
					Albumin[a]		Phenol[b]					Albumin[a]		Phenol[b]					Albumin[a]		Phenol[b]	
	-Cu	+Cu	-Cu	Δ	µg	%	µg	%	+Cu	-Cu	Δ	µg	%	µg	%	+Cu	-Cu	Δ	µg	%	µg	%
0	0	0,081	0,016	0,065	50	100	-	-	0,152	0,032	0,120	100	100	-	-	0,274	0,058	0,216	200	100	-	-
10	0,093	0,172	0,106	0,066	51	102	9,7	97	0,243	0,122	0,121	101	101	9,7	97	0,368	0,150	0,218	202	101	9,9	99
20	0,186	0,262	0,198	0,064	49	98	19,6	98	0,331	0,212	0,119	99	99	19,3	97	0,454	0,240	0,214	198	99	19,6	98
30	0,279	0,363	0,300	0,063	48	96	30,7	102	0,433	0,315	0,118	98	98	30,4	101	0,543	0,334	0,209	192	96	29,9	100
40	0,372	0,452	0,393	0,059	44	88	40,6	102	0,520	0,412	0,108	89	89	41,3	103	0,622	0,432	0,190	170	85	41,1	103
60	0,558	0,633	0,586	0,047	35	70	61,8	103	0,685	0,595	0,090	73	73	61,5	103	-	-	-	-	-	-	-

[a] according to Δ [b] according to -Cu registred corrected by -Cu corresponding to albumin

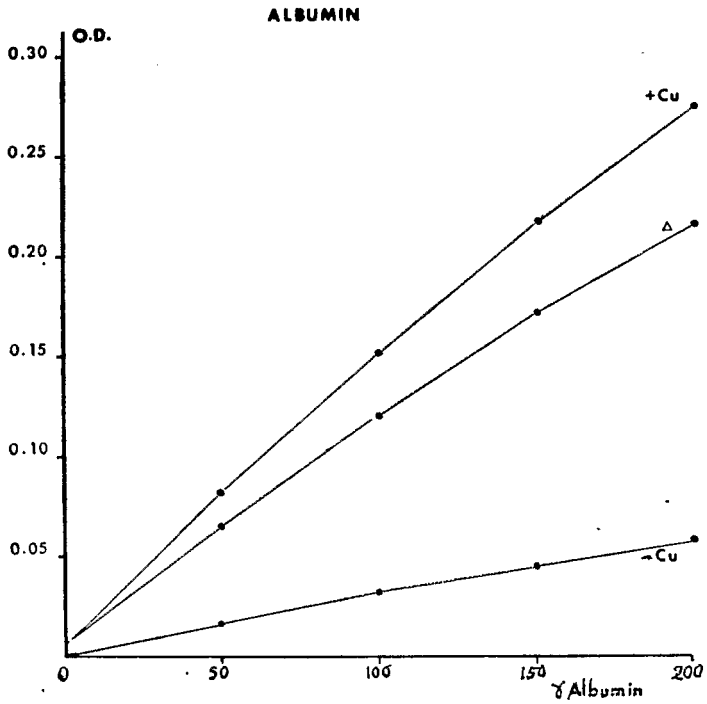

FIGURE 1. — **Standard curves of albumin.**

amounts. So, 150 µg of albumin in the presence of 30 % sucrose gives a value corresponding to 119 µg = 79 % of the real protein amount.

In general, our results are concordant with those reported by Gerhardt and Beevers (5). Though the interferences we have noted are a little less important.

Calculating Δ in the case of sucrose brings no solution as it was the case for phenol. Values calculated according to Δ are affected with a still more important error. This is probably due to a reaction of the sucrose with both Cu ions and protein groups.

So, a correct protein determination is only possible when the sucrose concentration is lower than 10 %. At higher sucrose levels a dilution is necessary. This is particularly important in the case of sucrose gradient fractions.

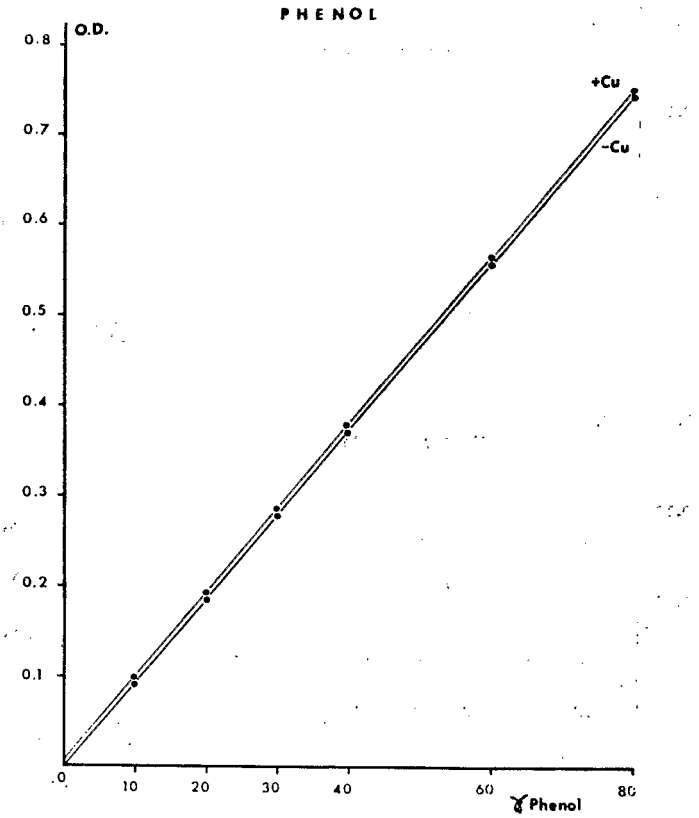

FIGURE 2. — **Standard curves of phenol.**

Manitol interferences

Mannitol alone produces a color development with + Cu reagent but not with — Cu reagent (Fig. 4 a). So Δ values = + Cu values.

The determination of albumin in the presence of 5 % mannitol (concentration in mannitol of extraction medium frequently used for subcellular fractionating) gives O.D. + Cu and Δ increased by a constant value when compared with those registred for albumin alone (Fig. 4 b and 1). This additional value corresponds to O.D. value given by 5 % mannitol.

So, the correct evaluation of proteins in the presence of a known and constant amount of mannitol is quite possible. The only condition is the inclusion of the same mannitol amount into standard.

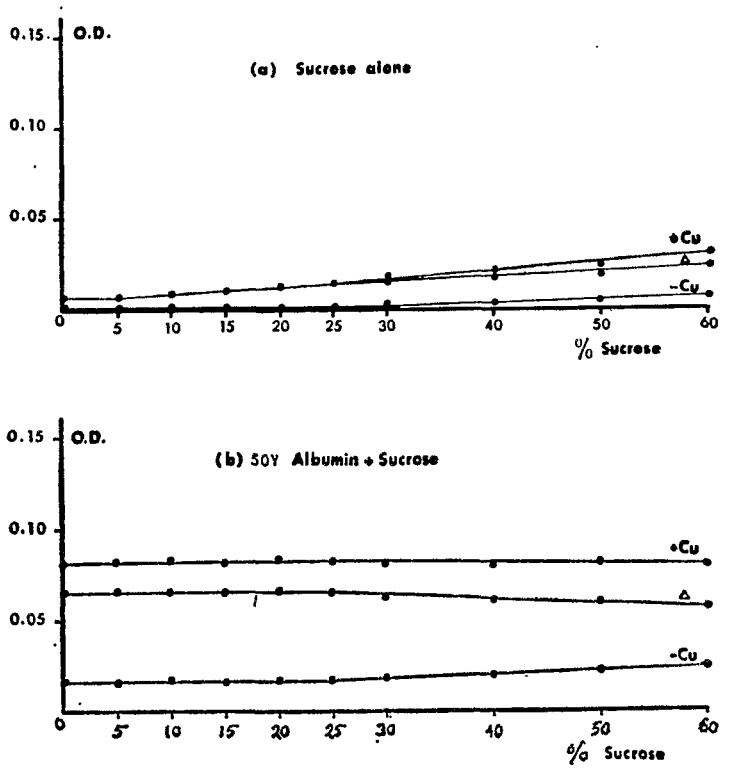

FIGURE 3. — **Effect of increasing amounts of sucrose on determination of different amounts of albumin (a, b).**

Trihydroxy methyl amino methane interferences

Tris is often used as a buffer of extraction medium and it is not rare to find in publications results of protein determinations made in the presence of Tris. So we have investigated its interferences.

As schown in Figure 5 a, Tris alone at a concentration as low as 0.2 mg/ml gives already a color reaction with + Cu reagent.

At the concentration of 3 mg/ml (25 mM solution), that is that of extraction medium frequently used for separation of subcellular particles, the color becomes important.

On the contrary there is on detectable reaction with — Cu reagent and Δ = + Cu.

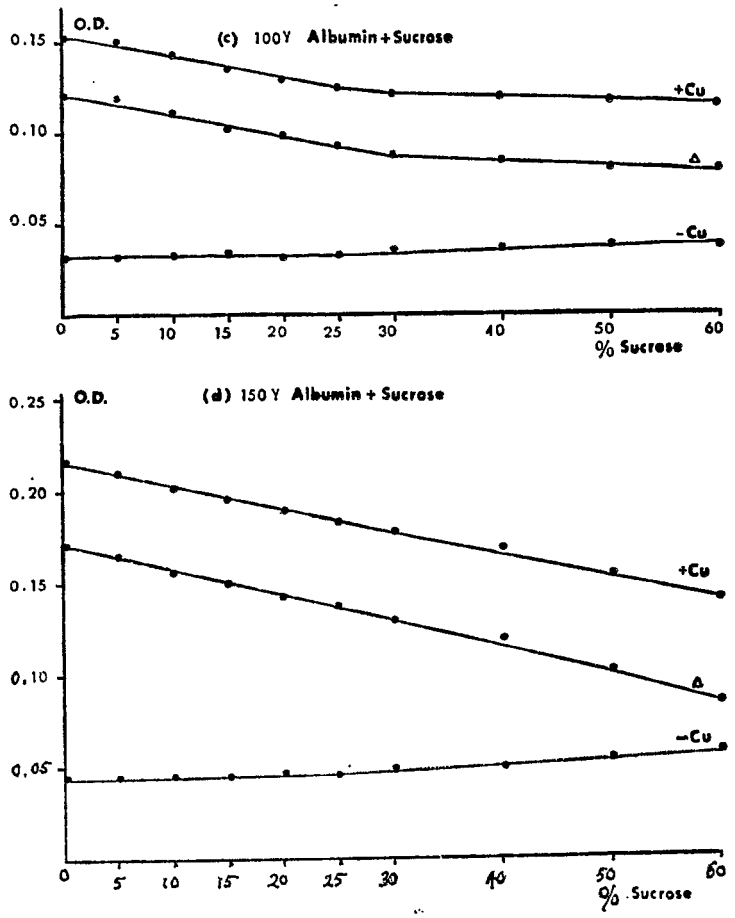

FIGURE 3. — (c, d).

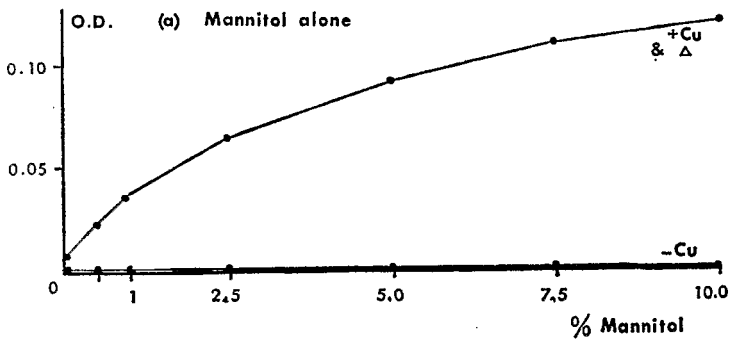

FIGURE 4. — **Effect of mannitol on determination of albumin (a).**

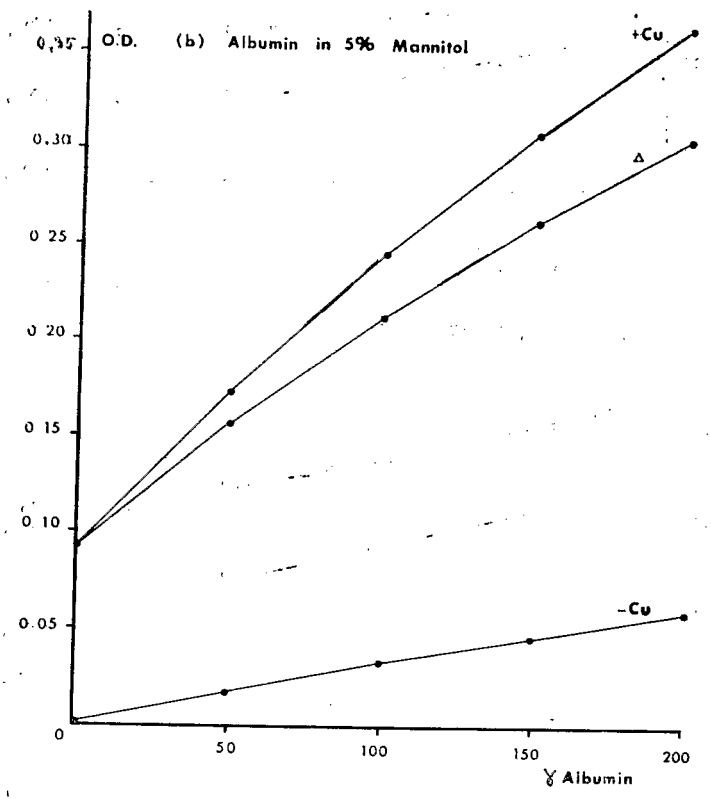

FIGURE 4. — **(b).**

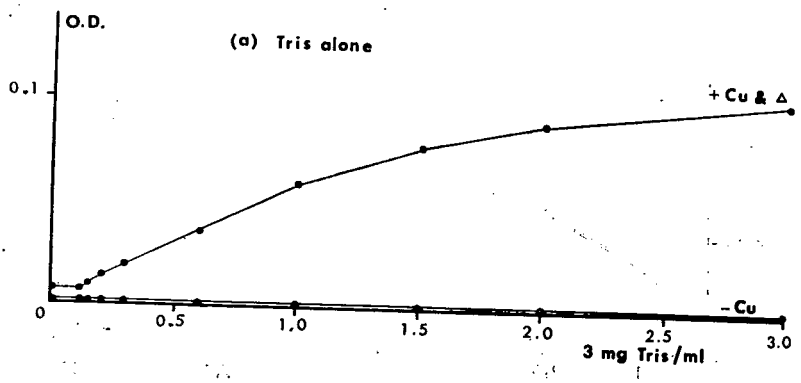

FIGURE 5. — **Effect of Tris on determination of albumin (a).**

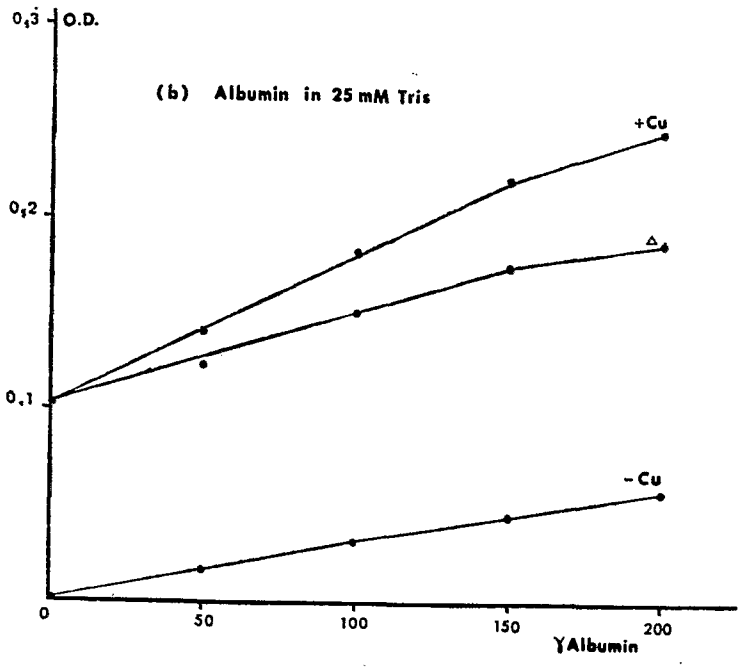

FIGURE 5. — **(b).**

The determination of albumin in the presence of Tris at 3 mg/ml (Fig. 5 b and Table II) leads to erronoeus values. The error depends on the amount of protein in the sample. For lower concentrations, up to 100 µg of albumin, the values are overestimated. For 150 µg the value found is correct. For higher concentrations, of about 200 µg of albumin, the values are underestimated.

So no correction can be brought to eliminate the error and the direct determination of proteins in the presence of Tris is not possible.

Reducing sugars interferences (glucose and fructose)

In the essays with the reducing sugars we tested the color reaction given by each of those compounds with the Folin reagent + Cu and — Cu in the absence of protein (Fig. 6 a and b).

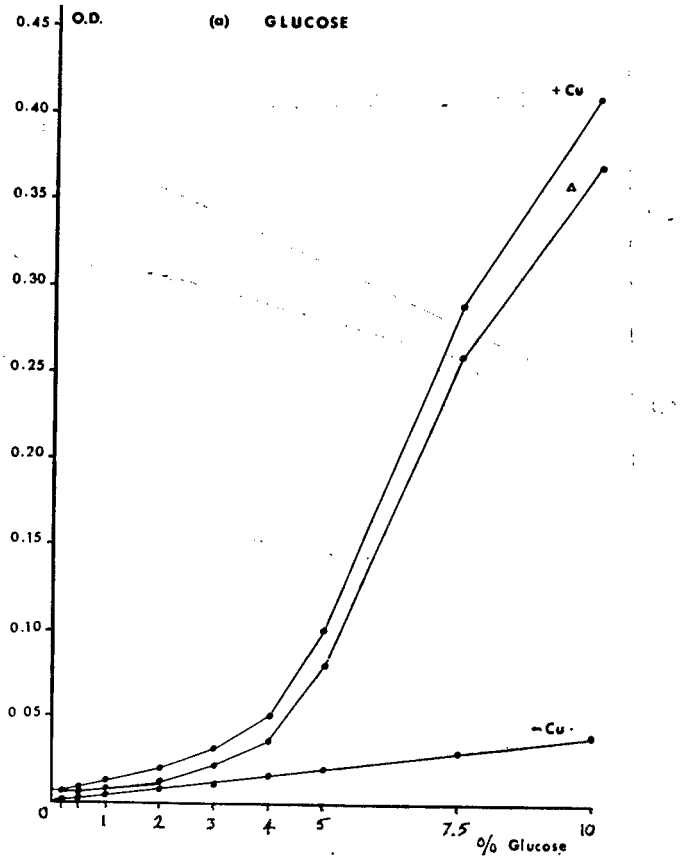

FIGURE 6. —**Color reaction produced by reducing sugars with Folien reagent (a).**

TABLE II. — **Estimation of albumin in presence of Tris at concentration of 3 mg/ml.**

µg albumin in the sample	Albumin found			
	according to +Cu		according to Δ	
	µg	%	µg	%
0	(65)	-	(84)	-
50	91	182	104	208
100	123	123	129	129
150	153	102	153	102
200	175	87,5	167	83,5

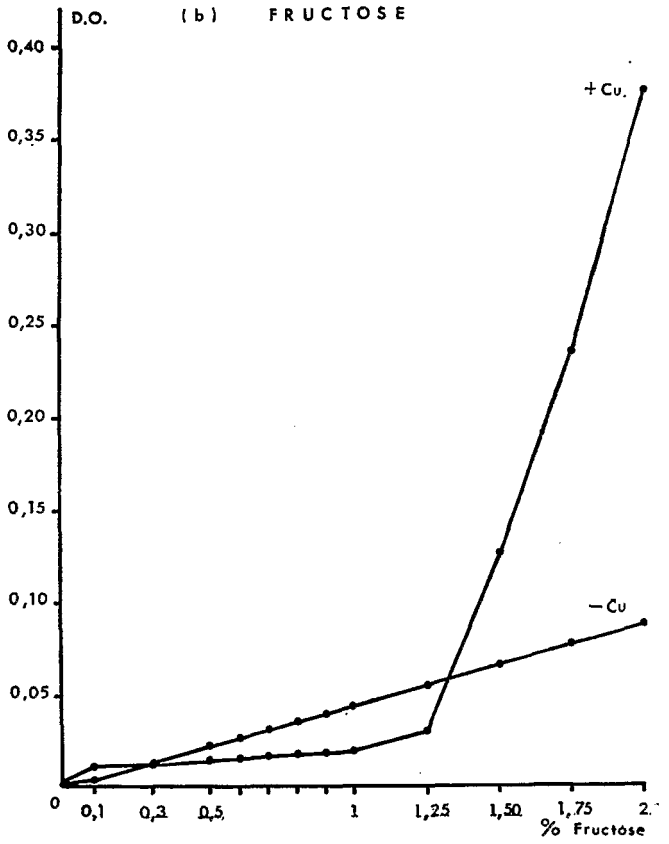

FIGURE 6. — **(b)**.

As could be expected the reducing sugars give a strong color with + Cu reagent. With — Cu reagent there is also a color development.

The reaction sensitivity is different for the two sugars. The color given by fructose is far more intense than that produced by glucose. This is true for both + Cu and — Cu reagents. Thus, in the case of fructose, interferences can be expected already at the low concentration of about 0,1 % whereas in the case of glucose this concentration will be of about 1 %.

Furthermore it appears that only the O.D. without copper is a linear function of the sugar concentration in the range studied, the O.D. with copper is not.

In conclusion, serious interferences are to be feared in protein determinations carried out on the material rich in reducing sugars.

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