XCIV. ULTRACENTRIFUGAL STUDIES OF COMPOUNDS OF PROTEINS WITH POLYSACCHARIDES. COMPOUNDS BETWEEN PROTEINS AND GLYCOGEN

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UNTIL recently proteins and polysaccharides have been considered as separate entities of the living cell, but during the past few years evidence has been accumulated for the theory that these compounds are able to form complex compounds with one another. What relation these complexes may have to the already well known conjugated proteins, the glycoproteins, remains to be seen.

It is rather difficult to imagine that the colloidal components present in the cell should remain in contact with each other without exerting any mutual influence. Significant are the facts accumulated from several lines of investigation. First, it is very difficult to obtain the individual components in the pure state from the organism, e.g. a lipoid-free protein [Macheboeuf, 1928; Sandor, 1933; Sørensen, 1930]. Secondly, many complex compounds which play an important role in the organism have been isolated (yellow enzyme, pyridine-nucleotide of Warburg, vitamin D, myosin-glycogen etc.). Finally investigations carried out *in vitro* have shown that many possibilities of combination exist between such compounds as proteins and polysaccharides.

It has not been possible to fix any hard and fast rules governing the formation and existence of such complex compounds. Studies which help to elucidate the nature of the binding forces and the types of combination are of prime importance.

Przylecki & Majmin [1931] distinguish the following types of binding between proteins and other substances, viz.:

(*a*) The binding of non-ionized polar groups of proteins with the polar groups of other substances (Willstätter's symplexes).

(*b*) The binding of ionized groups of proteins with the ionized groups contained in other substances.

With regard to combinations between proteins and polysaccharides Przylecki [1936] distinguishes the following groups:

(*a*) The symplexes due to binding through auxiliary valencies, e.g. serum globulin-amylose and clupein-dextrin. Since only arginine and tyrosine, of the amino-acids present in the protein molecule, are responsible for the binding, this group can be divided into two subgroups: one caused by arginine binding and the other by tyrosine binding.

(*b*) Salt-type binding, e.g. ovalbumin-glycogen.

(*c*) Mixed types, in which both auxiliary valencies and main valencies appear.

The experiments on binding were conducted chiefly on proteins in the gel state. However, it was possible to show that such combinations also exist in solution [Przylecki *et al.* 1935; Przylecki & Mystkowski, 1935]. These authors found that the turbidity of a mixture of serum globulin with glycogen or with starch as measured nephelometrically differs much from the sum of turbidities of both components when measured separately, the same being true of viscosity.

Such was not the case when serum albumin was used instead of serum globulin, for in this case the turbidity of the mixture corresponded to the sum of the individual components. It was concluded from these experiments that the changes in the turbidity were caused by the combination between proteins and polysaccharides. Cataphoresis experiments [Przylecki & Mystkowski, 1935] have shown that in the presence of guanidine, amylose shows a strong positive charge and migrates towards the cathode together with guanidine. Amylose itself when in solution shows a slight negative charge. The change in charge and the migration towards the cathode were assumed to depend on the binding of amylose with guanidine.

The ultracentrifugal method of Svedberg [1934, 1, 2, 3[1]] appeared to offer great possibilities for studying the problem of the combinations existing among colloidal compounds in solution. It has been shown by Svedberg and collaborators that the native proteins are generally monodisperse in solution, giving a sedimentation diagram characteristic for a protein under given conditions; any combination product in a mixture of protein and polysaccharide should find expression in this diagram and then it would be possible to secure data on such combination products by calculating the sedimentation constants and the concentration of compounds present in solution.

Unfortunately we know very little about the structure and the physical state of water-soluble polysaccharides; only starch has been examined in the ultracentrifuge [Lamm, 1934]. In the present experiments glycogen was used because of its relatively greater simplicity as compared with starch. According to earlier estimations the molecular weight of glycogen is about 100,000, but this figure cannot be accepted as reliable owing to the imperfect methods which were applied. Haworth & Percival [1932] estimated that the minimum molecular weight of glycogen is about 2500. Oakley & Young [1936] using osmotic pressure measurements found that the value for the mean particle weight of glycogen was about 2×10^6. These authors pointed out that their values were only mean ones and that there was no evidence as to whether glycogen in solution was monodisperse or not. They suggest that the glycogen particle in water may be an aggregate of a large number of chemical molecules. In order to get a clear picture of the behaviour of the glycogen in the ultracentrifuge, before studying mixtures of it with proteins, a number of preliminary experiments using glycogen only were carried out. Difficulties similar to those found by Lamm [1934] in his investigations on starch were encountered. Of the two components of starch, the amylopectin, which is closely related to glycogen in its behaviour and chemical structure, showed greater polydispersity than amylose. The molecular weight of amylopectin as calculated by Lamm was about 300,000. In these experiments glycogen showed a polydispersity similar to amylopectin.

Experimental

Serum, serum globulin, lactoglobulin and glycogen were used in these experiments. Glycogen was prepared from rabbit liver by the method which was found by the author to be the most satisfactory for obtaining a very pure sample of glycogen in a short time. The animal was killed and the liver was removed as quickly as possible and crushed in a mortar with an equal amount of 4% trichloroacetic acid in order to precipitate the proteins; the solution was filtered. This was repeated three times using the same liver paste. To the collected fluid an equal amount of alcohol was added, and after 24 hours

[1] A bibliography of literature will be found in these papers.

the glycogen was separated by centrifuging and dissolved in a small amount of water. To the solution, which still contained a small amount of proteins, trichloroacetic acid was added once more. After several precipitations with alcohol and finally with ether a snow-white powder very soluble in water was obtained; it was stored in a desiccator.

Serum globulin was prepared by the method described by McFarlane [1935] and was stored in small bottles at 4° as a 0·56 % solution. Lactoglobulin was prepared according to the method of Palmer [1934]. Serum was dialysed against 0·2 M NaCl solution for 12 hr. in an ice-box.

The general ultracentrifugal technique was similar to that used at the Upsala laboratory [Svedberg, 1934, 1, 2, 3]. For recent improvements see papers by McFarlane [1935] and Pedersen [1936].

The refractive index method of Lamm was used. All solutions were examined in a cell 12 mm. thick: the amount of the solution in the cell was 1·0–1·1 ml., a β-methylaesculetin filter ($\lambda=436$) and Imperial Process plates were used; the exposure times were 20 and 25 sec., except in the case of horse serum where, because of a very strong light absorption, a Wratten No. 77 filter ($\lambda=546\,m\mu$) and orthochromatic plates were used, and the exposure time was 1 min.

To either the solution of serum globulin preparation or to serum diluted 5 times with 0·2 M NaCl solution an amount of glycogen sufficient to make a 1 % solution of the latter was added. After standing 2–4 hr. the cell was filled with this solution and the sedimentation of both components was observed in the ultracentrifuge. The first exposures were taken at low speed (15,000–20,000 r.p.m.) in order to record the sedimentation diagram of glycogen. The centrifuge was then brought to a speed of 58,000–60,000 r.p.m., at which it was kept for 2–3 hr. During this time exposures were taken every 20 min. The control runs were made with protein solution without glycogen. Reference scale runs, corresponding to proper runs, were carried out as described by Pedersen [1936]. In these runs the cell was filled with the same amount of buffer solution as in the proper run, the exposures being taken at 20 min. intervals.

The sedimentation constants and the concentrations of the components were calculated and compared with corresponding data from control runs.

Results

(1) *Glycogen.* Two samples of glycogen were examined, one from rabbit liver and the other a commercial sample (Kahlbaum). The liver glycogen sediments as early as 17,000–25,000 r.p.m. and gives rise to a diagram showing a great polydispersity. One cannot speak of molecules in their proper sense here, since the huge particles corresponding to this diagram are evidently aggregates of various sizes. The concentration of glycogen as calculated from the diagrams was 0·5–0·65 % instead of 1·0 %, which indicates that besides those aggregates, which give rise to the curve, there must be many other particles scattered throughout the solution, part of them sedimenting even at lower speeds and part perhaps of lower molecular weight. The commercial sample was even more heterogeneous—we were not able to obtain any definite curve from it. It was impossible to give more attention to glycogen because of very limited time, yet it would be very interesting to try to obtain a more nearly monodisperse preparation of glycogen or to separate it into more distinct fractions. These experiments show the great polydispersity of glycogen in water and its tendency to form aggregates. Using the methods available at present it is impossible to speak of the molecular weight of glycogen; osmotic pressure measurements, moreover, deal with large aggregates of different sizes.

(2) *Serum*. Using pig serum diluted 5 times with 0·2 *M* NaCl solution diagrams similar to those obtained by McFarlane with horse serum and cow serum were obtained. The concentration of albumin calculated from diagrams was 0·85 %, whereas the concentration of globulin was 0·26 %. In the presence of glycogen the corresponding figures were 1·05 and 0·40 % respectively. This corresponds to an apparent increase of concentration of albumin of 23·5 % and of globulin of 53 %. The sedimentation constant of globulin in the original serum was $7 \cdot 1 \times 10^{-13}$ and in the presence of glycogen $7 \cdot 8 \times 10^{-13}$.

In the case of horse serum the concentration of globulin obtained from the control diagrams was 0·45 %, and in the presence of glycogen 0·6 %, which means an increase of 33 %. The corresponding sedimentation constants were 7·1 and $7 \cdot 5 \times 10^{-13}$. The concentration and sedimentation constant of albumin were not calculated in this case.

In the case of pig serum we were unable to find any marked change in the shape of the peaks from normal and control runs. However, in the case of horse serum the diagrams were more like those obtained with the preparation of serum globulin, the curve obtained from the sedimentation of the mixture showing a greater polydispersity.

(3) *Serum globulin*. Table I shows the results obtained with mixtures of serum globulin with glycogen at different *p*H values. From this table we can see that within the *p*H range 6·2–7·5 there is an apparent increase in the

Table I

		Concentration %			$s_{20} \cdot 10^{13}$	
*p*H	Buffer solution	Globulin	Globulin +glycogen	Difference %	Globulin	Globulin +glycogen
4·0	0·2 *M* CH_3COOH, 0·008 *M* NaCl, 0·032 *M* CH_3COONa	0·35	0·34	—	7·1	7·1
6·2	0·2 *M* KH_2PO_4, 0·008 *M* NaCl, 0·032 *M* Na_2HPO_4	0·30	0·38	+27	7·1	8·1
6·8	0·2 *M* NaCl	0·45	0·51	+13	7·1	7·4
7·1	0·2 *M* NaCl, 0·025 *M* NaOH	0·43	0·49	+14	7·1	7·1
7·5	0·2 *M* NaCl, 0·027 *M* NaOH	0·34	0·4	+18	7·1	7·3
8·2	0·2 *M* NaCl, 0·03 *M* NaOH	Very irregular diagrams, impossible of calculation				

concentration calculated for globulin in the presence of glycogen in comparison with the concentration calculated from the diagrams from the control runs. At the same time the sedimentation constants have also increased. There was no change in either at *p*H 4·0.

The sedimentation diagram obtained in the presence of glycogen shows a greater polydispersity than the serum globulin alone. In the control runs there was a large curve caused by the principal components, but there were also a certain number of small peaks corresponding to the larger and faster sedimenting molecules of serum globulin. In the presence of glycogen the number of these small peaks and the area included by them were always greater. It was impossible to take them all into account and make any exact calculations, but they seem to correspond to combination products between the larger molecules of serum globulin and glycogen.

If only a part of glycogen of lower particle weight goes into combination with protein this should have no great influence on the shape and the area of the glycogen curve. However, in many cases where we found evidence of a combination the glycogen curve was widely disturbed and in many cases it was even impossible to find any distinct sedimenting boundary.

(4) *Lactoglobulin*. There was no change in concentration and sedimentation constants in runs either with or without glycogen.

Discussion

The results of this investigation show that in the presence of glycogen the concentration of serum globulin calculated from the diagrams is greater than the concentration calculated from diagrams obtained from control runs. This increase in the case of serum is 30–50 % and in the case of serum globulin preparation 13–27 %. At the same time the sedimentation constants show a similar increase. This apparent increase of concentration and simultaneous increase of sedimentation constants must be caused by the particles of greater mass than the serum globulin itself and must be due to a combination product of globulin with glycogen. The globulin, after being bound with glycogen, forms a complex which sediments with a greater velocity than globulin alone and has a larger area and a greater polydispersity.

Calculating roughly from the increase of concentration the possible increase of mass of particles is found to be about 10–30 %; one would expect a corresponding increase of the sedimentation constant of about 5–10 % which is experimentally almost true. This all goes to show that only small particles of glycogen combine with globulin, their particle weight being far below the molecular weight of globulin.

The results obtained in this paper agree with those obtained by Przylecki *et al.* [1935] on the same protein and polysaccharide.

Summary

1. Ultracentrifugal investigations have shown the great polydispersity of glycogen solutions.
2. In mixtures of glycogen with serum globulin evidence of combination between these two components was found.
3. Only a part of glycogen of lower particle weight combines with protein.
4. No combination of lactoglobulin with glycogen could be detected.

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References

Haworth & Percival (1932). *J. chem. Soc.* 2277.
Lamm (1934). *Kolloidzschr.* **69**, 44.
McFarlane (1935). *Biochem. J.* **29**, 407, 660.
Macheboeuf (1928). Thèse de doctorat, Paris.
Oakley & Young (1936). *Biochem. J.* **30**, 868.
Palmer (1934). *J. biol. Chem.* **104**, 359.
Pedersen (1936). *Biochem. J.* **30**, 948.
Przylecki (1936). *Mh. Chem.* (in the Press).
—— Andrzejewski & Mystkowski (1935). *Kolloidzschr.* **71**, 325.
—— & Majmin (1931). *Biochem. Z.* **240**, 18.
—— & Mystkowski (1935). *Biochem. Z.* **281**, 420.
Sandor (1933). Thèse, Paris.
Sørensen (1930). *Kolloidzschr.* **53**, 102.
Svedberg (1934, 1). *Science*, **79**, 327.
—— (1934, 2). *Chem. Rev.* **14**, 1.
—— (1934, 3). *Naturwissenschaften*, **22**, 225.