A METHOD FOR THE PREPARATION OF GLYCOGEN AND A STUDY OF THE GLYCOGEN OF THE ABALONE, HALIOTIS RUFESCENS, SWAINSON.*

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The methods by which glycogen is commonly prepared involve treatment with hot concentrated alkali. It may be questioned, therefore, whether the product thus obtained is glycogen as it occurs in the tissues or a derivative formed by the action of the strong alkali. The question assumes new importance now that it has been shown clearly that glycogen, at least from certain sources, contains phosphoric acid (1). It seems important, therefore, to determine whether the phosphoric acid content reported is the true content or whether phosphoric acid is lost in the process of preparation, for phosphate plays some sort of rôle in the intermediary metabolism of carbohydrate in muscle, although opinions differ concerning the nature of this rôle (2). In the present paper a method is described for preparing glycogen from the abalone, a local edible mollusc, without the use of powerful reagents, and analyses of the phosphoric acid and ash content of the glycogen thus prepared are recorded.

* Since the abalone meat was bought in a butcher shop, the species from which it was derived was not definitely determined. However, Halioitis rufescens, Swainson, the red abalone, is the one commonly used for food about San Francisco. Other species that are utilized in California either for food or for mother-of-pearl, etc., are Halioitis fulgens, Phil., the green abalone, Halioitis wallelensis, Stearns, the northern green abalone, Halioitis cracherodii, Leach, the black abalone, and Halioitis corrugata, Gray, the pink abalone (cf. Thompson, W. F., Calif. Fish and Game, 6, 45 (1920); and Goode, G. B., The fisheries and fishing industries of the United States, sect. 5, Washington, 623 (1887).
The question of the phosphorus content of glycogen is also of interest in connection with the question of the ash content. Obviously, if phosphorus is regularly present, it should be impossible to obtain ashless preparations, yet many investigators have reported obtaining preparations free from ash, for example Gatin-Gruzewska (3). Harden and Young (4) found "no weighable amount of ash" in yeast glycogen prepared without the use of strong alkali. Slater (5), more recently, reported no ash in one sample of glycogen from the mussel, *Mytilus*, and 0.02 per cent in another. Indeed, the absence of ash has been regarded as a criterion of purity by many. On the other hand, Stohmann and Schmidt (6) found 0.045 per cent of ash in a sample of rabbit liver glycogen, prepared by Voit without the use of alkali. More recently, Samec and Isajevic (1) found dog liver glycogen to contain ash even after electrodialysis with ultrafiltration. Meier and Meyerhof (7) purified commercial *Mytilus* glycogen and found over 1 per cent of ash, although the preparations had been dialyzed over 2 hours. A sample of frog muscle glycogen had nearly 1 1/2 per cent of ash. McDowell (8), working in this laboratory, found *Mytilus* glycogen to contain a few hundredths of 1 per cent of ash, even after prolonged electrodialysis with ultrafiltration.

There are then discrepancies on record in regard to the ash content of *Mytilus* glycogen and, by implication, in regard to its phosphorus content. However, the descriptive phrases, "free from ash" or "without weighable amount of ash," are relative terms. A small percentage of ash, such as McDowell found, might be disregarded, especially if the quantity of substance used for the incineration was small. Furthermore, hitherto only two kinds of glycogen have been examined for phosphorus, that from dog liver (Samec and Isajevic) and that from *Mytilus* (McDowell), and of these the liver glycogen contained 0.721 per cent of \( P_2O_5 \) and the *Mytilus* glycogen, 0.036 per cent of \( P_2O_5 \). Until more is known concerning the effect of strong alkali in the preparation of glycogen, it is impossible to say whether these differences in ash and phosphoric acid content are specific and indicative of the existence of different glycogens, or whether they are due to the methods of preparation. It seemed, therefore, important in the present investigation to determine also to what extent the ash
content of abalone glycogen may be reduced by electrodialysis and especially to what extent the phosphorus may be removed thereby.

The abalone was chosen as raw material, since in California the flesh of this mollusc may be purchased in butcher shops when in season. These abalone steaks consist of thin slices of the large pedal muscle with which the mollusc adheres to its support and with which it creeps about upon the rocky bottom of the sea coast. As offered in the shops, the steaks have been thoroughly pounded to render them tender. These steaks are perhaps the most readily available local source of glycogen, for Albrecht (9) found their glycogen content high, although he does not seem to have put quantitative determinations on record. The method here reported of preparing abalone glycogen is applicable to other similar materials.

The method itself is nothing other than the method described by Levene nearly 30 years ago for the preparation of nucleic acids (10). 5 pounds of abalone meat, purchased from the iced stock of a local dealer, were minced in a food chopper and then thrown into boiling water and the extraction conducted for about 15 minutes. The liquid was filtered through folded cheesecloth, the meat then ground to a finer consistency, and again extracted. A third extraction completed the process.

Since the steaks contain an appreciable amount of acid which imparts a very decided acid character to the extraction mixture, the resulting danger of hydrolyzing the glycogen present was minimized by maintaining the mixture neutral to litmus during extraction through the addition of small quantities of NaOH.

To the cold, combined, slightly acidified (with acetic acid) filtrate, totaling about 1.5 liters, was added an excess of aqueous concentrated picric acid solution, thereby precipitating proteins. Picric acid should be added as long as a precipitate forms and a little in excess of that quantity. If insufficient picric acid is added at this stage, filtration is difficult and resulting preparations are not free from substances giving the biuret reaction.

From the filtrate, glycogen is precipitated by the addition, with stirring, of twice its volume of 95 per cent alcohol. After standing overnight, as much as possible of the clear liquid is decanted off and the remainder of the material filtered on a Buchner funnel. It is then washed with 60 per cent alcohol;
but the yellow color of picric acid cannot at this stage be removed entirely by washing. The precipitate, which is usually a grayish tan color, is taken up in the least possible quantity of water and reprecipitated by a double volume of alcohol. This process is repeated four or five times. As the glycogen approaches the pure state, double volumes of alcohol fail to precipitate it without the addition of electrolytes. A small crystal or two of ammonium acetate should then be introduced into the solution.

The glycogen at this stage is free from the picric acid color and, if a sufficient quantity of picric acid has been added, is free from biuret-reacting substances. If it is not, the glycogen must be dissolved in water, picric acid solution again added, the precipitate filtered off, and precipitation with alcohol as above described repeated until the picric acid color has been removed.

The precipitated glycogen is then first washed with 95 per cent alcohol, next with anhydrous ether, and finally dried to constant weight in a vacuum desiccator over CaCl₂ at room temperature. Nearly 2 weeks are required for the product to reach equilibrium. The resulting product is a snow-white powder. However, the physical properties of the preparation are closely connected with the manner in which it is precipitated from solution by alcohol, as well as with the efficiency with which it is washed with alcohol and ether. Lastly, the way in which it is dried is a factor. Too rapid drying, in the early stages particularly, produces a hard, gritty product instead of an extremely light, fluffy one. According to Bizio (11), glycogen dried at atmospheric pressure over CaCl₂ is a half hydrate. In evaluating the ash and phosphoric acid determinations reported below, it should be kept in mind that the preparations were probably not completely anhydrous.

Abalone glycogen, thus prepared, forms opalescent solutions in cold water and gives the usual coloration with iodine. It does not give the Lassaigne test for nitrogen. The yields obtained varied from 8 to 25 gm. per pound of fresh muscle. These variations are due presumably in part to varying richness in glycogen of the original material, but probably even more to varying losses in the process of purification, depending upon the number of reprecipitations. No effort was made to determine the conditions for obtaining maximum yields nor was any attention given to making the method quantitative.
The ash content of abalone glycogen thus prepared varied. Preparation A contained 0.6 per cent of ash and 0.26 per cent of \( \text{P}_2\text{O}_5 \), as estimated by the method of Embden with, as in all cases, about 1 gm. of substance for each analysis. Preparation B, on the other hand, had an ash content of only 0.069 per cent and a \( \text{P}_2\text{O}_5 \) content of 0.039 per cent. As will be shown below, it was not possible to reduce the ash content much below this value. Preparation B was one in which insufficient picric acid had been added in the first place, for it was found when tested before drying to give the biuret reaction. It was, therefore, again put through the picric acid process and a biuret-free preparation finally obtained. It had, thus, been reprecipitated more frequently than Preparation A, and this presumably accounts for its lower ash content. As recorded by McDowell (8) for \textit{Mytilus} glycogen, inorganic phosphoric acid could not be detected in neutral or slightly acidified solutions by means of Embden’s reagent.

The ash is nearly white with a pale bluish green tint, suggesting the presence of copper. The ash of Preparation B was tested for this metal with negative results, although calcium, magnesium, and iron, as well as phosphoric acid, were identified. The ash of Preparation A was not analyzed.

A 10 per cent solution of Preparation A was electrodialyzed with ultrafiltration several hours in the Bechhold-König apparatus, the direction of the current being changed from time to time. The membranes used in these and all the other experiments were made from 10 per cent acetic acid-collodion and the current was 110 volts d.c. When a constant low reading on the ammeter had been obtained, the glycogen was reprecipitated with the addition of a trace of ammonium acetate, dried, and analyzed. It was found that dialysis had reduced the ash content from 0.6 per cent to 0.065 per cent and the \( \text{P}_2\text{O}_5 \) content from 0.26 per cent to 0.061 per cent. It will be noted that the ash content is but slightly greater than the \( \text{P}_2\text{O}_5 \) content, so that there can be little other than \( \text{P}_2\text{O}_5 \) in the ash. It is possible that in the ash determinations some \( \text{P}_2\text{O}_5 \) may have been volatilized. The \( \text{P}_2\text{O}_5 \) determinations were not made upon the ash; but for this purpose a separate portion of substance was used and the organic material destroyed in the wet way with sulfuric acid.

100 cc. of a 6 per cent solution of Preparation B were next
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electrodialyzed with ultrafiltration as above, but without changing the direction of the current. After 7 hours, the ammeter readings having become constant, a sample was withdrawn for analysis. The ash content had only dropped from 0.069 per cent to 0.065 per cent. The total phosphorus had only dropped from 0.039 per cent to 0.037 per cent.

The dialysis was continued for another 7 hours. During the 14 hours of dialysis the ammeter reading fell over a range of less than 10 ma. The dialysis was then concluded and the glycogen again analyzed. The ash content was then 0.065 per cent and the total P$_2$O$_5$, 0.037 per cent. Electrodialysis for 14 hours, therefore, had left the ash and P$_2$O$_5$ content practically unchanged. The ash of the dialyzed glycogen was pure white and gave positive tests for iron, calcium, and phosphates.

The experiment was repeated, but instead of the Bechhold-König apparatus one improvised in the laboratory was used. This consisted of a short Pyrex glass tube, about 4 cm. in diameter held horizontally and closed at either end with rubber stoppers. Through holes in the stoppers were inserted two small porcelain balloons bearing the membranes. The platinum wire electrodes were inserted into the balloons which were closed with perforated rubber stoppers, provided with an arrangement to remove the dialysate as fast as formed. The electrodes were about 2 cm. apart. In the upper border of the Pyrex glass tube was a hole through which the liquid undergoing treatment between the two balloons might be sampled or stirred. This apparatus differs from the Bechhold-König set-up in that it permits the liquid undergoing electrodialysis to be observed through the glass tube and in that the current passes horizontally through the liquid instead of vertically. Hence any separation occurring in the liquid may be observed.

In this apparatus a second portion of Preparation B was dialyzed without change of direction of the current. In the course of the

1 Gatin-Gruzewska (3) attributed the presence of iron in some of her preparations to contamination with the iron contained as an impurity in the KOH used in the process of preparation. This, of course, fails to account for the traces of iron contained in the abalone glycogen preparations. In this connection, it is perhaps significant that Albrecht (9) found a large amount of iron in abalone tissues.
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Dialysis the liquid separated into an upper water-clear layer, not appreciably opalescent, and a lower denser opalescent layer, in which was no flake formation—simply a deepening of the opalescence. A slight agitation of the apparatus restored the dispersion of the glycogen throughout the liquid and no attempt was made to analyze the layers separately. This curious phenomenon will be discussed at greater length below.

After 7 hours of dialysis in this apparatus, the ash content of the glycogen was 0.057 per cent and the total P₂O₅, 0.047 per cent. After 7 additional hours of dialysis, the ash content was 0.055 per cent and the total P₂O₅, 0.040 per cent.

A third portion of Preparation B was then subjected to dialysis in the above apparatus for 14 hours. The ammeter reading at the beginning of the operation was 12 ma. As glycogen eventually decreases the permeability of the membranes, though it could not be detected in the dialysate, the process was interrupted at the end of 14 hours. The membranes were then removed and the balloons cleaned with acetic acid under suction. The cleansing fluid became cloudy. Fresh membranes were then put on the balloons and the glycogen solution subjected to electrodialysis for 17 hours longer. The ammeter reading had then dropped to 4.5 ma. The glycogen contained 0.05 per cent of P₂O₅; ash was not determined. The dialysate of the last 17 hours was concentrated and tested for P₂O₅ by Embden's reagent with negative results.

It is clear then that abalone glycogen does contain a small amount of P₂O₅ in a form that cannot be removed by electrodialysis with ultrafiltration. The average of twelve determinations made upon two different preparations dialyzed under a number of different conditions was 0.047 per cent. The lowest value found was 0.036 per cent; the highest, 0.068 per cent. This high value was obtained for a sample relatively high in ash that had been dialyzed only several hours. It is difficult to conceive of the P₂O₅ of abalone glycogen being thus firmly held unless it be an integral part of the molecule. If it is indeed a part of the molecule, then the molecular weight must be huge. It is possible, however, that glycogen like starch consists of two fractions, one containing phosphorus, the other free from it. In that event, the percentage of P₂O₅ found would, by itself, furnish no basis for estimating the molecular weight.
The average P₂O₅ content of 0.047 per cent found is but little greater than that found by McDowell for the edible mussel (*Mytilus*), 0.036 per cent. The difference is small and may not be significant. Since it is so small and since the *Mytilus* glycogen was prepared with hot, concentrated KOH, whereas the abalone glycogen was subjected to no more destructive reagents than hot water and a slight excess of picric acid, the presumption is created that strong, hot KOH does not split off phosphoric acid from glycogen—at least from that of molluscs. The observations made in this investigation do not warrant a decision as to whether or not the two glycogens are identical or different chemical individuals. Besides the small difference in P₂O₅ content, the only other difference between the two glycogens observed was that in equal concentrations abalone glycogen solutions were much more opalescent than those of *Mytilus* glycogen. Differences in the opalescence of solutions of glycogen from different sources have been reported by others. Clautriau (12) found yeast glycogen about one-fourth as opalescent as that from fungi or rabbits. Harden and Young (4) found oyster glycogen 2.5 times as opalescent as yeast glycogen, while rabbit liver glycogen was slightly more opalescent than oyster glycogen. Meier and Meyerhof (7) found *Mytilus* glycogen less opalescent than frog muscle glycogen. They regard it as less aggregated than frog muscle glycogen.

The settling of glycogen during electrodialysis in the apparatus of Pauli (13) has been observed by Samec and Isajevic (1), but their observations are not comparable with those here reported, because they used solutions of dog liver glycogen that had been heated to 120°. They found that such solutions separate into two parts. This is analogous to the behavior of similarly heated starch solutions which these investigators found separated into a lower gel layer of amyllopectin, so called, containing about 80 per cent of the starch substance, and an upper layer of amylose, containing most of the rest of the substance. Practically all the P₂O₅ is found in the lower gel layer (14). Glycogen solutions, however, they found separate into a lower layer containing only 20 per cent of the glycogen and only a minor fraction of the P₂O₅ present, and a clear upper layer containing the rest of the glycogen. Samec and Isajevic believe, therefore, that electrodialysis fractionates dog liver glycogen into two distinct components. Samec and Haerdtl
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(14) and Samec and Mayer (15) attribute the fractionation of starch by electrodialysis to the fact that amylopectin, being a phosphoric acid ester, is more electronegative than amylose. For the behavior of glycogen in which the gel fraction separated by electrodialysis was found to contain less P₂O₅ than the original substance no explanation is presented by Samec and Isajevic.

The writers, in their experiments in which electrodialysis was conducted with electrodes placed at the same level so that the path of the current was horizontal between them, found the upper strata of the liquid began to clear up soon after the current was turned on and the separation into an upper clear layer and a lower opalescent one became marked in a little while. There was, however, no evidence that the glycogen was thereby divided into two fractions for the water-clear upper layer gave no color whatever with iodine. All the iodine-reacting substance must have descended into the opalescent lower layer. If no current was sent through the electrodes, no separation occurred. The line of demarcation between the two layers was horizontal and ultimately dropped below the level of the balloons. It is not likely that the disappearance of glycogen from the clear layer was due to its removal either by deposition upon one of the membranes or by passage through either of them. As already stated, no notable deposit was found on the membranes nor could more than traces of glycogen be detected in the dialysate. There is undoubtedly a sinking of glycogen into the lower layer, for this becomes manifestly much more opalescent than the original solution. Furthermore, if the whole solution be removed from the apparatus, mixed, brought to its original volume, its opalescence is the same as that of a portion of the solution that was never introduced into the apparatus and never dialyzed, as determined with a nephelometer. There is no sign of flocculation or coagulation, at least of so gross a character as might be detected by such simple means. Moreover, when the dialyzed solution was removed from the apparatus, mixed, and allowed to stand in a tall vessel, as little separation occurred as in undialyzed glycogen solutions.

Svedberg offers the suggestion for this rather frequent behavior of colloids that "The phenomenon is probably due to cataphoresis of the colloid, which makes the solution more concentrated at one
membrane and produces clear liquid at the other. On account of
the difference in density the former flows down to the lower part
of the vessel and the latter rises to the upper part" (16). The
writers have convinced themselves that this is the correct ex-
planation by the following experiment: A septum of mica was
introduced midway between the two balloons in such manner
that there was free communication through the upper half of the
tube but not through the lower half. The glycogen wandered to
the anode side of the septum so that the cathode side of the septum
became clear and the glycogen as it accumulated on the anode side
sank to the bottom on that side. Ultimately, nearly all the gly-
cogen accumulated as a very opalescent lower layer on the anode
side of the septum. The liquid in all the rest of the tube, except
a thin layer at the bottom of the cathode side, was clear. It is
possible that the introduction of a septum in this manner may
turn out to be a practical improvement in the technique of con-
centrating colloids by the electrodialysis method of Pauli, who
discovered this phenomenon as exhibited by proteins (13).

As shown above, ordinary opalescent glycogen solutions do not
behave in the same manner as the autoclaved solutions of Samec
and Isajevic. This is also true for unheated opalescent starch
solutions. The writers electrodialyzed opalescent potato and
maize starch solutions prepared by suspending dry ground starch
in cold water (17) and filtering, and found that the opalescent
material settled out in a lower layer beneath a clear upper layer.
The clear upper layer could not have contained much amylose, since
on addition of iodine it gave a greenish coloration which turned
blue only on standing for some time. Even then the blue
coloration was not intense. These experiments are not com-
parable with those of Samec and Haerdtl, since the starch had not
been subjected to heat or chemical reagents, whereas Samec and
Haerdtl used autoclaved starch. In view of the observations here
recorded and in view of the importance of the conclusions drawn
by Samec and Haerdtl from their electrodialysis experiments, it
would seem that the behavior of untreated starch in electrodialysis
requires further investigation.

SUMMARY.

A method is described for the preparation of glycogen without
the use of powerful reagents.
Glycogen was prepared by this method from the red abalone, *Haliotis rufescens*, Swainson.

Abalone glycogen was found to contain 0.047 per cent of $P_2O_5$ (average of twelve analyses) that could not be removed by prolonged electrodialysis with ultrafiltration.

It was found impossible by electrodialysis to obtain abalone glycogen free from ash. The ash of glycogen that had been thoroughly electrodialyzed contained, besides $P_2O_5$, very small amounts of calcium and iron.

During electrodialysis of opalescent glycogen solutions, the glycogen sank to the lower part of the containing vessel without visible flocculation.

No evidence was obtained that electrodialysis separates unheated glycogen into two fractions, one poor in or free from $P_2O_5$, the other rich in $P_2O_5$. No evidence was obtained that abalone glycogen is a mixture of chemical individuals.

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