2. The position of the free hydroxyl group in the hydroxydimethoxybenzoic acid indicates that the methoxylated benzene ring in the three substances mentioned above are united with the remainder of the molecule through a carbon atom and an indifferent oxygen atom which are ortho to each other.

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PHOSPHORUS IN GLYCOGEN[1]

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Glycogen from various sources has phosphorus closely associated with it.[2] A part of this phosphorus dialyzes out readily through a collodion membrane and is obviously phosphate or a nearly related phosphorus-bearing ion, while the remainder is not so removed. This residual phosphorus may be insoluble and non-diffusible either because of its attachment to an organic residue or because it is in the form of an insoluble inorganic salt. The results of this investigation show that the major amount of non-dialyzable phosphorus is attached to a nitrogenous residue.

That this bound phosphate is firmly held to some system is evidenced by the fact that the severe treatment with hot aqueous alkali in the usual Pflüger method[3] for the isolation of glycogen from animal tissue does not hydrolyze all the material to give simple phosphate ion.

For example, a specimen of rat liver glycogen prepared by the Pflüger method and subjected to long dialysis still contained 0.024% of "bound" phosphorus, while two different specimens of sea scallop (muscle glycogen) similarly treated still contained, respectively, 0.013 and 0.018% phosphorus. An undialyzed sample of Kahlbaum's glycogen contained 0.1% phosphorus.

Sources of Glycogen.—At first an attempt was made to obtain the material from the liver of calves and sheep by removing the organ immediately after slaughtering and dropping it into liquid air. The resulting friable mass was ground in a mortar and carried through the Pflüger method.[3] The yields on these test runs were negligible, due either to the starved condition of the animals or to fear, both factors causing depletion of the storage carbohydrate.

Liver fairly rich in glycogen was obtained finally by decapitating adult

[1] The material for this paper is taken from the first part of a dissertation submitted by J. J. McBride to the Faculty of Pure Science of Columbia University in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[2] Samec and Isajevič, *Compt. rend.*, **176**, 1419 (1923); Petree and Alsberg, *J. Biol. Chem.*, **82**, 385 (1929).

[3] Pflüger, *Pflüger's Arch.*, **93**, 81 (1903).

albino rats (*mus norvegicus*), removing the organ immediately and dropping it into alcohol. The ratio of liver tissue to alcohol was adjusted so that the final concentration of alcohol became 60% by volume. The mixture was boiled under a reflux condenser for one hour on a steam-bath, the liquid drained off and the residue finely ground in a meat chopper. Four extractions of the residue with alcohol and one with ether followed. The resulting desiccated powder was used as the raw material from which the glycogen was later isolated.

In order to have a representative sample of muscle glycogen, the sea scallop (*pecten tenuicostatus*) was selected. The muscle fiber was removed very shortly after the shell had been opened, cut into thin slices and treated as the previous sample of rat liver was treated. This gave also a mass of dry powdery crude material.

Experimental

Method of Extraction I.—Three extractions of 58 g. of each of the previously mentioned crude materials were made with 200 cc. of water at room temperature, pouring the extract through a filter. To the clear filtrate concentrated hydrochloric acid was added until the maximum turbidity was reached. The mixture was then centrifuged in a gold-plated container at 40,000 r. p. m. in a Sharples laboratory centrifuge for five minutes. To the clear liquid in the cup twice its volume of alcohol was added, causing a precipitate which after filtration was dissolved in water. This solution was dialyzed through a collodion membrane until salt free. To the salt-free solution a mixture of equal volumes of acetone and ether was added, causing the precipitation of the glycogen. Samples from both liver and scallop were thus obtained. Still a different method was used for the isolation of some glycogen from the scallop muscle in order to compare the effect of the reagents used, on the phosphorus content of the resulting glycogen.

Method II.—Eight pounds of thinly sliced scallop muscle tissue was added slowly to two liters of boiling water and the mixture maintained at the boiling point for one-half hour. After filtration the residue was finely ground in a meat chopper and extracted with boiling water by decantation through a filter. To the warm filtrate portions of lead subacetate were added until there was a strong test for lead ion with hydrogen sulfide in the supernatant liquid. A filtration removed the solids brought down by the lead subacetate and the filtrate was treated with hydrogen sulfide to remove the excess lead ion and refiltrated. This filtrate was then treated with twice its volume of alcohol, giving a precipitate which was subsequently taken up in water and dialyzed until free of salts.

These procedures gave three samples of glycogen material, namely, two from sea scallop (methods I and II) and one from rat liver (method I).

For control purposes a sample of each material was treated by the Pflüger method.[3]

The behavior of the products of the above treatment was the same polarimetrically and toward acid hydrolysis as the samples of the desiccated powder with the Pflüger treatment.

Inasmuch as it is impossible to characterize glycogen as a chemical substance by the usual methods or to isolate a phosphorus-bearing carbohydrate or other compound, it was decided to follow the value of the *ratio between phosphorus and nitrogen* in glycogen samples subjected to

various treatments. The investigation of the value of this ratio seemed to hold promise because qualitatively *all samples that contained phosphorus at the same time contained nitrogen.*

The treatment decided upon was an electrophoretic one having for its purpose the possible fractionation of the easily dispersed glycogen from the probably more polar nitrogenous material, for preliminary experiments showed that there was migration of such dispersions when placed in a suitable cell.

A modification of the electrophoretic cell previously used by one of us was employed here,[4] the upper cathode chamber being provided with a stopper through which passed an intake and outlet tube for the circulation of water during the operation of the apparatus. When a direct current potential of 220 volts was applied across the cell with water in the electrode compartments and a dilute solution of glycogen (1.5 to 3 g. per 100 cc. but not higher) in the inner compartment, there was considerable bubbling at the electrodes and the electrolyte including the phosphate ion in the mixture dialyzed out. Later the bubbling ceased and the current flowing through the cell fell to about a mil. During this time the opalescent material in the cell began to migrate toward the lower or anode compartment, forming a sharp boundary between it and the clear supernatant liquid. Incidentally, on evaporation of the clear liquid and ignition of the very small residue, there were no evidences of organic material.

If the current passed for a sufficient time, all the organic material packed down on the lower membrane. When this material was re-suspended by stirring into newly added distilled water which replaced the supernatant liquid and the potential again applied a different phenomenon was observed. There was no boundary formed but white material gradually built up on the lower membrane during the course of twelve hours. The operation was stopped and the supernatant liquid, still cloudy, pipetted off and that together with the residue from the membrane was each analyzed for nitrogen and phosphorus. Even after application of the potential for four days the solution had not lost its opalescence. Two experiments (1 and 2) were made using, respectively, rat-liver crude glycogen and scallop crude glycogen isolated as previously described. The P_H was about 4 at the outset and about 6 at the finish, the adjustment being a function of the flow of water through the compartments. In one case (3), however, the P_H of the dispersion was made to be 9 and after partial deposition on the membrane and removal of the supernatant liquid, the residue was stirred back three successive times in distilled water to which dilute alkali was added to give the P_H of 9.

The amount of nitrogen and phosphorus in each fraction and the ratio of these two elements to each other was determined.

[4] Taylor, Braun and Scott, *Am. J. Physiol.*, **74**, 539 (1925).

Experimental

The material in the supernatant liquid was precipitated out by saturating the water with a mixture of acetone and ether (50–50 by volume) while the solids on the membrane were removed mechanically and then washed with the acetone and ether mixture.

Nitrogen Determinations.—Samples of glycogen, dried to constant weight over phosphorus pentoxide, containing approximately 0.1 mg. of nitrogen were digested in a small Kjeldahl flask with the usual Kjeldahl–Gunning mixture with added 30% hydrogen peroxide. The digested mixture was transferred to a Pregl vacuum-jacketed Kjeldahl apparatus where caustic soda was added and the mixture heated. The ammonia distilled over into a 25 cc. graduated cylinder containing 0.3 cc. of 1:1 sulfuric acid. When the volume of the distillate had reached, during the course of fifteen minutes, about 10 cc. the outlet tube was raised and the distillation continued so that with washings the final volume was 18 cc. To this distillate there was added 6 cc. of Nessler's solution made according to Koch and McMeekin[5] and after making up to 25 cc. with water the color was compared in a Klett colorimeter[6] with a standard made in the same manner containing 0.1 mg. of nitrogen as ammonium sulfate. Tests with pure hippuric acid showed that the method was capable of handling from 0.05 to 0.13 mg. of nitrogen with an accuracy of about 2.3%.

Phosphorus Determinations.—The samples were digested in hot concentrated nitric acid to which was added from time to time a small amount of "perhydrol" (40% H_2O_2) until the mixture was colorless. In this digested mixture the phosphate ion was determined by the colorimetric method of Fiske and Subbarow.[7] The method was followed in all detail except that the quantities of reagents were decreased by one-half and the final solution made up to 25 cc. instead of 50 cc. and the color compared in the Klett colorimeter. For comparison solutions of potassium dihydrogen phosphate were used. The error when working with samples containing from 0.016 to 0.070 g. of phosphorus was not greater than 5%. The result of the analysis is given below.

RATIO OF NITROGEN TO PHOSPHORUS IN GLYCOGEN MATERIAL AFTER FRACTIONATION

Expt.	Solution in cell	Source of fraction		N, %	P, %	Ratio N/P
				0.74	0.46	
		Anode		.73	.42	
		membrane		.77		
			Av.,	0.74	0.44	1.69
1	Rat liver (Method I)				.31	
	1.5 g. per 100 cc.	Supernatant		0.55	.34	
		liquid		.57	.33	
			Av.,	0.56	0.32	1.75
				0.22	0.060	
		Anode		.22	.056	
		membrane		.25		
			Av.,	0.23	0.058	3.9

[5] Koch and McMeekin, THIS JOURNAL, **46**, 2066 (1924).

[6] Beaver, *J. Opt. Soc. Am.*, **18**, 41 (1929).

[7] Fiske and Subbarow, *J. Biol. Chem.*, **66**, 375 (1925).

Expt	Solution in cell	Source of fraction	N, %	P, %	Ratio N/P
2	Sea scallops (Method I)				
	3 g. per	Supernatant	0.120	0.025	
	100 cc.	liquid	.130	.028	
		Av.	0.125	0.027	4.3
		Anode	0.062	0.012	
		membrane	.057	.011	
		Av.	0.059	0.011	5.3
3	Sea scallops (Method II)				
	3 g. per	Supernatant	0.046		
	100 cc.	liquid	.046		
		Av.	0.046	0.008	5.7

Samples taken from the anode membrane and the supernatant liquid and hydrolyzed with aqueous acid showed by the presence of reducing sugar that glycogen was to be found in both places. Although qualitatively there was much less in the supernatant liquid, most of the solids being on the membrane, there was still some carbohydrate there also.

It is obvious that in all cases the supernatant liquid is poorer in both nitrogen and phosphorus than the material that deposits on the membrane. The *ratio of nitrogen to phosphorus* is the *same* however in both cases. If the nitrogen and phosphorus were part of the glycogen molecule, the percentage of these two elements should stay the same in both fractions although their absolute amount might change. Such is not the case. Even in Experiment 3 where the high alkalinity causes the removal of nitrogen and phosphorus to the point where there is very little left, the ratio N/P remains constant. Furthermore, it is significant that the nitrogenous material itself that was associated with scallop glycogen and removed from it in the previous preparation of the sample, contains nitrogen and phosphorus in the ratio N/P = 5. In all cases the inorganic phosphate ion has already been removed.

From these experiments it seems that the phosphorus is not part of the glycogen molecule at least with respect to the carbohydrate from the two sources used here and that the major part of the "bound" phosphorus is attached to some nitrogenous residue of constant composition.

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