HISTOCHEMICAL STUDIES OF GLYCOGEN AND PHOSPHATASE IN BLOOD CELLS

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By

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Introduction

Since Hotchkiss (1948) has set his step in the field of polysaccharide staining of various tissues, a great deal of new informations of polysaccharide in the blood cells of men and animals were brought about by many investigators (Wachstein (1949), Wislocki, Rheingold and Dempsey (1949), Gibb and Stowell (1949), Hibino (1950), Eränkö (1950), Ritter and Oleson (1950), and etc.). According to the reports of these investigators, who mainly employed Hotchkiss' periodic acid-Schiff method for glycogen, it was always contained in neutrophilic leucocytes, frequently in lymphocytes and monocytes. Further, Wachstein (1949), Gibb and Stowell (1949) reported that platelets also contained glycogen.

Takeuchi (1949), Horii, Hayashi and Kumashiro (1951), and others studied the alkaline and acid phosphatase in the blood cells of animals, and stated that they appeared in the neutrophilic leucocytes of rabbits, rats, etc., but not in those of snakes, lizards and fishes.

In the present investigation, we have dealt with the appearance of glycogen and phosphatase in the blood cells of vairous kinds of animals, especially in the leucocytes and blood platelets of domestic animals, as the first step of investigations concerning the physiological significance of these substances.

Materials and Methods

As materials, mammals (men, horses, cattle, pigs, goats, rabbits, cats and rats), aves (domestic fowls, ducks), reptiles (tortoises), amphibia (frogs and tritons), fishes (turbots, swellfishes, gibels and loaches) were used. The blood was taken from peripheral blood vessels in the case of large animals and from hearts in the case of small ones and the blood smears were made. The following procedures were employed for each purpose: Glycogen was demonstrated by employing

periodic acid-Schiff method (PAS method) modified by Lillie after fixation in picric-formalin, frequently in 95 per cent alcohol. Identification of glycogen was made by means of salivary test. Alkaline and acid phosphatase were demonstrated by Gomori's revised method by using sodium glycerophosphate as substrate.

The bone marrow smears from rats and fowls were also used for the detection of glycogen and phosphatase by the same methods as described above.

The estimation of blood glucose and of glycogen in the leucocytes was followed by Hagedorn-Jensen's method.

Results

1. *Determination of Fixing Fluid and Staining Method for Glycogen.*

For the demonstration of glycogen, different kinds of fixing fluids and stains have been used by many investigators. Therefore, as the first step of this investigation, we have preliminarily tested the fixatives and staining methods for glycogen. In this case the blood smears of rats were used. The results are given in Table 1.

Table 1. Results obtained by using various fixing fluids and stains for glycogen in neutrophilic leucocytes of rats.

Fixing fluids	Reaction	Best's carmine method	PAS method	Bauer-Feulgen method	Gomori's method
10% Formalin.	Neut.	−	±	−	−
Absolute methyl alcohol	〃	−	−	−	−
95% Alcohol	〃	−	+	−	+
Alc.-form. saturated with sodium acetate (Toryu)	〃	−	++	+	+
Alc.-chloroform with acetic acid (Carnoy)	Acid	−	+	+	+
Picric-form. (picric acid sated. soln. 4, form. 1)	〃	±	++	+	+
Form.-sublimate (form. 1, mercur. chloride sated. soln. 9)	〃	±	++	++	+
Alc.-sublimate (alc. 1, mercur. chloride sated. soln. 2) (Schaudinn)	〃	±	++	++	+
Air dried smears		−	++	++	−

As shown in Table 1, picric formalin, formol-sublimate and alcohol-sublimate proved high satisfactory for glycogen fixatives and PAS method for glycogen staining. Therefore, picric formalin fixation and PAS method were employed throughout the entire course of this investigation.

2. *Results Obtained for Glycogen.*

General description : The neutrophilic leucocytes showed consistent staining reaction for glycogen by using the technic (PAS) already described. The cytoplasm usually contained fine granules of glycogen stained red purple, but occasionally small dustlike or somewhat coarse granules of it. Intergranular

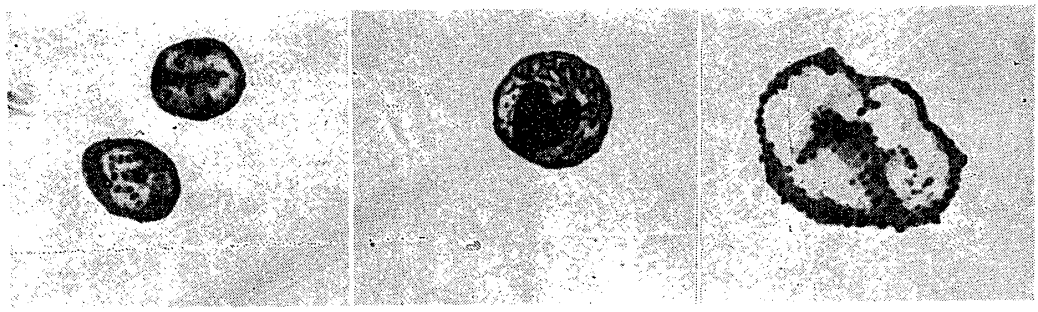

Fig. 1. Fig. 2. Fig. 3.

Fig. 1. Neutrophilic leucocytes of horse. PAS method. ×1000. A great amount of glycogen is contained in the cytoplasm. Fig. 2. Neutrophilic leucocytes (pseudoeosinophilic leucocytes) of fowl. PAS methos. ×1000. Glycogen is not contained in the cytoplasm, but the intergranular substance is PAS reactive. Fig. 3. Neutrophilic leyucoctes of lizard. PAS method. ×1000. A great amount of glycogen is contained in the cytoplasm.

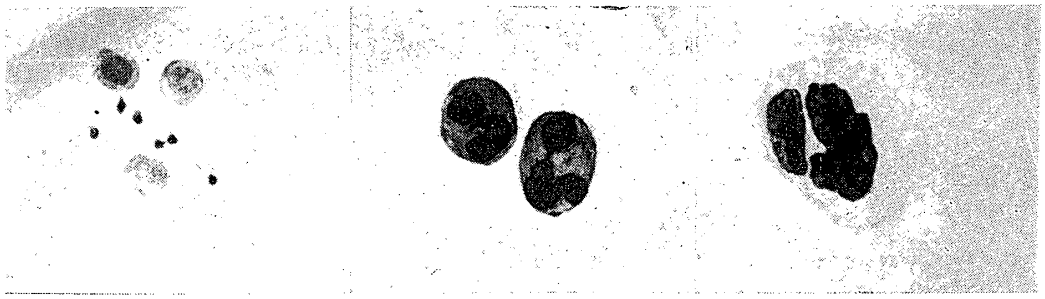

Fig. 4. Fig. 5. Fig. 6.

Fig. 4. Blood platelets of horse. PAS method. ×1000. Glycogen is contained in the cytoplasm and clump. Fig. 5. Neutrophilic leucocytes of horse. Gomori's method. ×1000. Alkaline phosphatase reaction appeares in the cytoplasm. Fig. 6. Neutrophilic leucocytes of lizard. Gomori's method. ×1000. No alkaline phosphatase reaction appears in the cytoplasm.

substance of eosinophilic leucocytes showed very faint staining reaction, but the so-called eosinophilic granules remained unstained. Lymphocytes and monocytes were either negative or showed the faint staining reaction. Erythrocytes did not reveal detectable amount of glycogen. The blood platelets consistently gave a positive reaction, especially in mammals, in centrally located clump (Fig. 4).

Following digestion with a 50 per cent buffered solution of saliva, the PAS reaction in the cytoplasm of the neutrophilic leucocytes, platelets, lymphocytes has completely disappeared, indicating that the PAS recative substance was certainly a pure glycogen. On the contrary, however, since the PAS reaction in the intergranular substance of the eosinophilic leucocytes was not digested

by saliva, the PAS reactive substance in these cells can not be considered as pure glycogen. This will be discussed later on.

Gibb and Stowell (1949) and Hibino (1950) stated that the PAS reactive substance in eosinophilic leucocytes of men was glycogen as well as that in neutrophilic leucocytes, blood platelets and some of lymphocytes. But, Wislocki, Rheingold and Dempsey (1949) reported that the PAS reactive substance in eosinophilic leucocytes of men, rhesus monkeys and rabbits was not glycogen, because this substance was not digested by saliva, agreeing with the results obtained in the warm and cold blooded members by the present authors.

Myeloblasts were almost negative for glycogen staining. In the matured myeloid cells, however, the staining reaction became pronounced in some degree. Most nucleated red cells showed no staining reaction.

Detailed descripton for glycogen in the neutrophilic leucocytes : The detailed results obtained for the appearance of glycogen in the neutrophilic leucocytes of various kinds of animals, together with the results obtained for the amount of liver glycogen and the quantity of blood glucose in those animals as controls, were given in Table 2. In the same Table were also given the results obtained for the alkaline phosphatase activity.

Table 2. Relative amount of glycogen and alkaline phosphatase in the neutrophilic leucocytes of various kinds of animals.

Animals	No. of animals used	Glycogen in neut. leucocyte	Alk. phos. in neut. leucocyte	Liver glycogen	Blood glucose (mg/dl)
Horses	20	⫼	⫼	⫼	
Cattle	10	+ to ⫼	+	+ to ⫼	62
Pigs	12	+ to ⫼	⫼	+ to ⫼	33
Men	3	⫼	⫼		
Goats	10	+ to ⫼	+	⫼	74
Rabbits	13	+	+	+	97
Cats	8	+	+	+ to ⫼	
Rats	10	+	+	+ to ⫼	50
Fowls	10	±	+	+	227
Ducks	1	±	+	+	320
Tortoises	3	⫼	−	⫼	
Tritons	2	⫼	−	⫼	41
Frogs	5	⫼	−	⫼	44
Turbots	1	⫼	−		
Swellfishes	3	⫼	−		
Gibels	1	⫼	−		
Loaches	3	⫼	−		
Commom earth worms	3	⫼	−		

As shown in Table 2, the amount of glycogen in the neutrophilic leucocytes showed a variation among the animals. Mammals such as horses, cattle, pigs,

men and goats contained a great amount of it (Fig. 1), but rabbits, cats and rats contained a slight amount of it. Aves contained no glycogen at all (Fig. 2). Cold blooded members such as tortoises, tritons, frogs, etc. contained a great amount of it without exception (Fig. 3).

It was noticed that the amount of glycogen in the neutrophilic leucocytes was parallel with that in liver with the exception of aves, but inversely proportional to that of blood glucose as shown in Table 2.

As to the fact that the neutrophilic leucocytes (pseudoeosinophilic leucocytes) of aves contained no glycogen at all, it is supposed that these cells are essentially the same in nature with the eosinophilic leucocytes of mammals, but not with the neutrophilic ones of them. To support the view just stated we notice the following relations : First, as is generally known, the cytoplasm of the neutrophilic leucocytes of aves are filled with eosinophilic granules as those of the eosinophilic leucocytes of mammals. Second, as already stated, the cytoplasm of the neutrophilic leucocytes of aves contained no glycogen at all as well as the intergranular substance of cytoplasm of the eosinophilic leucocytes of mammals.

Detailed description for glycogen in lymphocytes : The relative amount of glycogen in the lymphocytes of various animals together with the ratio of lymphocytes containing glycogen and those containing no glycogen are given in Table 3.

Table 3. Relative amout of glycogen in the lymphocytes of various animals together with the ratio of lymphocytes containing glycogen and those containing no glycogen.

Animals	Lymphocytes counted	Relative amonnt of glycogen				Percentage of lymph. containing glycogen
		−	+	++	+++	
Horses	30	30	0	0	0	0
Cattle	30	30	0	0	0	0
Pigs	30	30	0	0	0	0
Men	30	29	0	1	0	3.3
Goats	30	27	2	1	0	3.7
Rabbits	30	29	1	0	0	3.3
Cats	30	30	0	0	0	0
Rats	30	29	1	0	0	3.3
Fowls	30	30	0	0	0	0
Ducks	30	30	0	0	0	0
Tortoises	30	8	2	5	15	73.3
Tritons	30	15	3	3	9	50.0
Frogs	30	15	6	4	5	50.0
Turbots	30	27	0	1	2	3.7
Swellfishes	30	28	0	0	2	6.7
Gibels	30	26	3	1	0	13.3
Loaches	30	23	1	6	0	23.3

As shown in Table 3, in warm blooded vertebrates almost no lymphocytes

containing glycogen were found, but in cold blooded members more or less number of them, especially in reptiles and amphibia such as tortoises, tritons and frogs a relatively large number of them was found, the value being about 50 to 70 per cent.

Gibb and Stowell (1949) reported that glycogen was contained in the majority of lymphocytes in human blood. Wachstein (1949), Wislocki, Rheingold and Dempsey (1949) and Hibino (1950) mentioned that it was appeared in some of them. In the present investigation, however, no glycogen was found in lymphocytes of warm blooded vertebrates as just stated, disagreeing with the results of Gibb and Stowell, but agreeing with those of Wachstein, Wislocki, Rheingold and Dempsey, and Hibino.

3. *Analytical Results of Glycogen in Leucocytes.*

To ascertain the histochemical analysis of glycogen in the leucocytes a chemical analysis was carried out. The methos are as follows: About 200 cc. of blood in each cattle was taken from carotid vein and centrifuged after defibrinated by adding natrium citrate. The leucocytes collected were washed thrice with natrium chloride solution and analyzed by Hagedorn-Jensen's method. The results are given in Table 4.

Table 4. Glycogen content in the leucocytes of cattle.

Cattle No.	Blood sample taken (cc)	Leucocytes collected (mg)	Glycogen content (mg)	Percentage (mg %)
1	200	150	0.213	142
2	200	120	0.132	110
3	200	100	0.090	90
4	200	150	0.252	168
5	200	140	0.196	70
Average	200	132	0.177	116

As will be seen in Table 4, the leucocytes of cattle contained about from 70 to 168 mg% of glycogen. From these results it was clearly confirmed that the substance of leucocytes reactive to the PAS method and digestible in saliva is glycogen.

4. *Results Obtained for Alkaline and Acid Phosphatase Activity.*

a. Alkaline phosphatase.

Alkaline phosphatase activity in the blood cells of various animals was observed with the following results:

Treated with Gomori's revised method using glycerophosphate as substrate, black colored precipitation of cobalt sulfite appeared in the blood cells, showing that the phosphatase reaction occurred in these cells.

As already shown in Table 2, neutrophilic leucocytes of mammals showed alkaline phosphatase reaction. Those of horses showed a most intense reaction

(Fig. 5). It was also noticed that there was a certain parallel of glycogen reaction and phosphatase activity in the neutrophilic leucocytes of mammals. Alkaline phosphatase reaction did not appear at all in the neutrpohilic ones of aves and of cold blooded members (Fig. 6).

Eosinophilic leucocytes of mammals showed faint alkaline phosphatase reaction in their cytoplasm, but not in their granules, agreeing with the localization of the PAS positive substance.

Lymphocytes and monocytes of the cold blooded members showed moderate reaction of alkaline phosphatase in their cytoplasm, agreeing with the appearance of glycogen.

Erythrocytes and blood platelets of all animals did not reveal detectable reaction of alkaline phosphatase. The nuclei of erythrocytes in the cold blooded members showed the reaction, but not in mammals and aves.

Alkaline phosphatase reaction was mostly negative in the myeloid elements of the bone marrow of rats and fowls.

b. Acid phosphatase.

Acid phosphatase reaction was observed by Gomori's method and the results are as follows :

Acid phosphatase reaction was not occurring in both the myeloid elements in the bone marrow and the formed elements in the blood of all animals.

Takeuchi (1949) reported that the alkaline phosphatase was only demonstrated in the neutrophilic leucocytes in rabbits and rats. In our present investigations, a great amount of alkaline phosphatase was contained in the neutrophilic leucocytes of mammals, and also a moderate amount of it in eosinophilic ones of mammals and in lymphocytes of cold blooded animals was found.

Horii, Hayashi and Kumashiro (1951) stated that alkaline and acid phosphatase appeared in neutrophilic leucocytes, eosinophilic leucocytes, lymphcoytes and monocytes of men, guinea pigs, domestic fowls, and etc., but not in those of cold blooded members such as snakes, lizards and fishes, nearly coinciding with the results obtained in our present investigation, as far as the alkaline phosphatase alone is considered. Acid phosphatase was not found at all in the present investigation as already mentioned.

It was a well known fact that the presence of phosphatase indicates an intersignification of metabolic process of glycogen. Thus the glycogen contained in the neutrophilic leucocytes of mammals and cold blooded animals and in lymphocytes of the latter should act as the main source of energy for these cells.

As to the fact that no phosphatase was contained in the neutrophilic leucocytes of the cold blooded members such as reptiles, amphiblians and fishes it may be considered that the phosphatase in the endothelium of th capirallies, the amount

of which is enourmously large, may exhibit an enzymatic action for the glycogen metabolism in place of the phosphatase in the leucocytes of mammals. This will be again considered, if possile, in future.

5. *Results Obtained in Starvation of Animals.*

To obtain physiological significance of glycogen in the neutrophilic leucocytes, further histochemical investigation was made on the blood cells of animals in starvation. Healthy rabbits and rats were starved. The blood was taken at the fixed intervals during starvation. Liver and hearts of these animals were also used as controls. Pieces of these organs were fixed in picric formalin and embbeded in celloidin and sectioned 20 μ thick. The PAS method modified by Lillie was used for glycogen staining. Identification of glycogen was made by means of salivary test. The results are given in Table 5.

Table 5. Relative amount of glycogen in neutrophilic leucocytes of animals in starvation.

Animals	No.	Starvation in days and the amount of glycogen 0 1 2 3 4 5 6 7 8 9 10 11 12 13	Glycogen in liver	Glycogen in impulse conducting system
Rabbits	1	+ + + + + + + − Killed	−	‡
	2	+ + + + + + + − Killed	−	‡
	3	+ + + + + + + + + − Starved to death	−	‡
	4	+ + + + + + + − Revived (−)(−)(±)(+)(+)(+)	+	‡
Rats	1	+ + + + + + + + − Killed	−	‡
	2	+ + + + + + + − Killed	−	‡
	3	+ + + + + + + + ± + Revived (−)(−)(−)(+)	+	‡
	4	+ + + + + + + + + − Revived (−)(−)(±)(+)	+	‡

Note: ( ) — Amount of glycogen after feeding.

As shown in Table 5, the glycogen containing in the neutrophilic leucocytes almost disappeared at 7 to 9 days after starvation. At that time, the liver glycogen also disappeared, but glycogen containing in the impulse conducting system of heart was still found in normal state. The phosphatase reaction in neutrophilic leucocytes of these animals was positive during starvation. In the neutrophilic leucocytes, glycogen reappeared in 3 to 4 days after feeding accompanying with the appearance of glycogen in the liver.

From these results, it is noticed that the glycogen contained in neutrophilic leucocytes may be considered as reserve substance as well as that in the liver differing from the glycogen in the impulse conducting system of heart, the glycogen in which is undoubtedly a functional substance.

Summary

The results obtained in this investigation may be summarized as follows:

1. The amount of glycogen in the neutrophilic leucocytes of mammals showed a wide variation. Namely, the greatest amount of it was found in horses and men, a moderate amount of it in cattle, pigs and goats, and the smallest amount of it in rabbits, cats and rats. Cold blooded animals such as reptiles, amphibians and fishes contained without exception a great amount of glycogen.

2. The amount of glycogen in the neutrophilic leucocytes was almost parallel with that in liver, but not with the content of blood glucose.

3. The glycogen in the neutrophilic leucocytes disappeared at about 7 to 9 days after starvation and reappeared in 3 to 4 days after feeding.

4. Glycogen was also contained in the lymphocytes of cold blooded members and in the blood platelets of mammals, aves and cold blooded members.

5. No glycogen was contained in the eosinophilic leucocytes of all animals used and in the pseudoeosinophilic ones (neutrpohilic leucocytes) of aves.

6. About 70 to 168 mg. per cent of total glycogen was estimated from leucocytes of cattle.

7. In mammals, alkaline phosphatase reaction appeared in the neutrophilic leucocytes accompanying with the appearance of glycogen. In cold blooded members, however, its reaction did not appear at all.

8. Acid phosphatase reaction did not appear in the blood cells of all animals used.

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