ON ARTIFACTS APPEARING IN THE HISTOCHEMICAL FIXATION OF GLYCOGEN

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Plates 102 and 103

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Two major difficulties are encountered in the histochemical procedures used to demonstrate the distribution of glycogen in tissues or cells. The first is that in tissues the glycogen gradually disappears with time postmortem (1), so that it is impossible to demonstrate all the glycogen originally present in the cells, especially in the inner regions of fixed tissues. Secondly, the intracellular distribution of glycogen appears to be modified by the process of fixation. For instance, the so called *Alkohol-flucht* figures, frequently seen in liver cells and muscle fibres, are probably due to glycogen displacement. Glycogen redistribution may take place also in dried smears. It is difficult to believe that the *Alkohol-flucht* in the tissue and the situation found in dried smears represent the natural intracellular distribution of glycogen, and it seems more probable that the appearances mentioned are artifacts of fixation. The present investigation is concerned with the occurrence of these artifacts and with methods that can be used to prevent them. After this study was finished, we became aware of Lison's work (2) on the same problem. Our conclusions confirm and extend those reached by him.

Materials and Methods

Both normal and pathological tissues were used. The normal tissues include liver (mouse, rat); skeletal, cardiac, and smooth muscle (mouse, rat); umbilical cord (human); blood (mouse); and bone marrow (mouse). The pathological tissues consisted of squamous carcinoma of the uterus (human); Yoshida's ascites tumor (rat) (3); and myxosarcoma (fowl).

Slices of fresh tissue were fixed promptly in absolute alcohol, in which glycogen is insoluble, or in Carnoy's fluid, which penetrates rapidly into the tissue. After fixation, the tissues were embedded in paraffin and sectioned at 5 to 7 μ in the usual manner. The sectioned tissues were treated subsequently with the periodate–Schiff's reagent (4).

OBSERVATIONS

Glycogen in Normal and Pathological Tissues.—A point of special interest was to find out whether all or only some of the normal and pathological tissues showed the *Alkohol-flucht*. In general, glycogen displacement, as evidenced by the *Alkohol-flucht*, was observed in tissues of parenchymatous

type, both normal and pathological (group A, Table I), but not in tissues of loose type, either normal or pathological (group B, Table I).

In order to establish the cause of the differences in results, detailed observations were made on liver and umbilical cord. With liver cells, the *Alkohol-flucht* was generally more obvious along the border of the tissue block, and less pronounced or absent in the inner zone (Fig. 1). The cells surrounding large central veins in the inner region of the tissue proved to be an exception in that they also showed the *Alkohol-flucht*. Further, pathological cells of parenchymatous type, for example carcinoma of the uterus, also exhibited the *Alkohol-flucht*.

TABLE I

Occurrence of the Alkohol-flucht of Glycogen in Various Tissues

Group	Type		Tissue	*Alkohol-flucht*
A	Parenchymatous	Normal	Liver (mouse, rat); Skeletal, cardiac, and smooth muscle (mouse, rat)	Present
		Pathological	Squamous epithelial carcinoma (human uterus)	
B	Loose	Normal	Blood (mouse) Bone marrow (mouse) Umblical cord (human)	Absent
		Pathological	Embryonal sarcoma (human kidney) Yoshida's ascites tumor (rat) Myxosarcoma (fowl)	

On the other hand, sections of fixed loose tissues, for example sections of the umbilical cord or myxosarcoma, failed to demonstrate the *Alkohol-flucht* entirely; in addition, the inner region of the tissue block, normal and pathological, was found to be stained as intensely as the border.

These findings seem to indicate that the occurrence of the artifact is related to the structure of tissues, and perhaps to the direction of penetration by the fixative. If so, any procedure which loosens a tissue of parenchymatous type before fixation should result in the elimination of the artifact.

Alkali Treatment before Fixation.—A strong alkaline solution is the most useful agent for artificial loosening of parenchymatous tissues. It is convenient that glycogen is chemically stable in alkali. Slices of mouse liver were placed in NaOH solutions of different concentrations for a period of 5 minutes before alcohol fixation (Table II). The *Alkohol-flucht* was never found if the NaOH

solution was more concentrated than M/3 (Fig. 2). Concentrations higher than M/2 could not be used, because the treated tissue became so hard that it was difficult to obtain sufficiently thin sections. With NaOH concentrations lower than M/5, on the other hand, the *Alkohol-flucht* always appeared, although the staining reaction was generally weak. The most striking results were obtained with a M/3 NaOH solution. It should be added here that M/2 KCN solution had an effect similar to that of NaOH on the artifact production.

There is another artifact which appears in usual liver preparations: namely, in the central region of the liver block the glycogen reaction is less intense than at the periphery. When the preparations were pretreated with NaOH, the difference between these regions disappeared and the glycogen reaction,

TABLE II

Effect of NaOH Treatment upon the Alkohol-flucht and the Intensity of the Glycogen Reaction in the Center of the Block

Mouse liver treated for 5 minutes before fixation with various concentrations of NaOH.

Concentration of NaOH	*Alkohol-flucht*	Reaction of inner regions
M/2	—	+++
M/3	—	++
M/5	+++	+
M/10	+++	+
M/50	+++	+
M/100	+++	+

as a whole, appeared more intense than in the untreated preparations. The latter phenomenon, that is, a stronger glycogen reaction in NaOH-treated preparations, was observed more clearly in the case of the umbilical cord: little glycogen is found in the mesenchymatous cells of the tissue, in untreated blocks, but relatively large amounts of glycogen, distributed all over the cells, are found in blocks pretreated with alkali (Figs. 3 and 4).

The tissue of loose type is rich in ground-substance and poor in number of cells, whereas the tissue of parenchymatous type consists of compactly arranged cells with little intercellular substance. The cells of a tissue of the loose type would be attacked rapidly from all directions since the fixative could presumably penetrate readily the ground-substance. In tissues of parenchymatous type, on the other hand, the penetration of the fixative would chiefly proceed from one direction: *i.e.*, from the margin of the block towards the center. Under such circumstances, intracellular translocation of glycogen may occur in untreated tissue of the parenchymatous type but not in tissues of loose type nor in pretreated tissues of parenchymatous type. These considerations, combined with the loosening effect of NaOH, provide an explana-

tion for the various phenomena described above. If this supposition is correct, it would follow that tissues of loose type, which originally failed to show an *Alkohol-flucht*, might show it upon conversion to parenchymatous type.

Artificial Translocation of Glycogen in Cells of Yoshida's Ascites Tumor.—Takahashi (5) studying glycogen distribution in Yoshida's ascites tumor observed that: (*a*) all the tumor cells contained abundant glycogen when examined 4 to 12 hours after inoculation into the abdominal cavity of a normal rat, (*b*) the glycogen disappeared completely from all the cells 24 to 36 hours after the inoculation, and (*c*) approximately 5 to 6 days after the inoculation, the glycogen began to reappear in some cells. Later the glycogen was evident in larger and larger numbers of cells.

The ascites tumor may be considered a tissue of loose type. In a first experiment, a drop of ascites fluid containing tumor cells rich in glycogen, was smeared on a glass slide and fixed in Carnoy's fluid without previous drying. Glycogen was found to be evenly distributed in the cells with no evidence of fixation artifacts (Fig. 7). In a second experiment, a drop of ascites fluid was placed on a glass slide for a few minutes and allowed to clot. The tumor cells were thereby imprisoned in the fibrin mesh of peritoneal exudate. Conditions in this clot may be considered to resemble in part those prevailing in a tissue of parenchymatous type. When this clot was fixed by Carnoy's fluid, the *Alkohol-flucht* was unmistakably observed along the border of the clot (Fig. 5). It is important to point out that the single tumor cells separated from the clot showed no *Alkohol-flucht*. These observations indicate that in a tissue of originally loose type, a glycogen distribution characteristic of the parenchymatous type can be obtained when the type of tissue is changed to a parenchymatous one by tightly packing its cells. The proposed explanation for these findings is that the fixative did not penetrate all the cell surfaces simultaneously, but entered first the sides next to the margin of the clot. It is not surprising that the isolated cells were free from this artifact since the fixative could penetrate all surfaces of the cells simultaneously, as in the case of tissue of loose type.

Artifacts in Smear Preparations—In dried smears of bone marrow and Yoshida's ascites tumor, the cytoplasm contains numerous glycogen particles, but few or none of them are found on top of the nucleus. The finding is unexpected because these are preparations of whole cells, not sections, and because *in vivo* glycogen particles have presumably an even distribution throughout the cytoplasm, similar to the distribution of basophilic granules in blood cells. Actually, the glycogen particles in dried smears of Yoshida's ascites tumor cells were never found in the cytoplasm above the nucleus,[1] but

[1] Occasionally, a small group of glycogen particles was found on the nucleus. It is supposed that in this case some particles were trapped within a depression on the surface of a lobed nucleus during the drying process.

only in the cytoplasm near the periphery of the cell (Fig. 6). Here the glycogen particles were both large and numerous. Larger granules of glycogen occurred in the peripheral region of the cell in dried smears of thin films. In dried preparations of thick films, glycogen granules were smaller and comparatively evenly distributed in the cytoplasm. If the smears were fixed in Carnoy's fluid without previous drying, glycogen particles of similar size were found uniformly dispersed in the cytoplasm even above the nucleus (Fig. 7).

In dried smears of dividing cells of the ascites tumor, the glycogen particles were homogeneously distributed in the cytoplasm, showing neither appreciable translocation nor variation in size (Fig. 8). The difference from the resting cells will be discussed below.

The displacement of glycogen in the dried smear preparations may be explained as follows: (*a*) during the drying process, the cell is flattened by the surface tension of the surrounding fluid; (*b*) the shape of the nucleus remains approximately the same, although flattened, since the nucleus is comparatively more solid than the cytoplasm of the cell; (*c*) the cytoplasm above the nucleus flows towards the periphery of the cell, carrying the glycogen particles with it; (*d*) the density of the particles will thus be greater near the cell border.

It appears from the findings given above that glycogen particles are translocated in the cells during the drying of the smear preparations, towards the periphery of the cell where they coalesce subsequently into larger granules.

DISCUSSION

A number of investigators have observed that the fixation of glycogen is not free of artifacts as long as absolute alcohol, Carnoy's fluid, alcohol-formol mixtures, Neukirch's fluid (2, 6–8), $MgSO_4$-alcohol mixture (9), KCl-formol mixture (10), picro-alcohol-formalin fixative (11, 12), and others (13) are employed as fixatives. Recently, Lison (2) proposed a new technique which he calls the "*congélation-dissolution.*" Glycogen translocation is prevented in this technique by freezing the tissue at −80°C. and then fixing it, while it thaws, in alcohol at −20°C. Another promising way for preventing the displacement of glycogen may be the separation of the cells by some way or another. Na citrate, which is very useful for separating blastomeres of sea urchin or amphibian eggs on account of its Ca-complexing action, is a well known example of this kind. But unfortunately, it did not help in the present case, presumably because its penetrating power was not sufficient. It is well known on the other hand that a strong alkali solution acts on intercellular substance so as to separate cells from one another. A recent study has shown that protein-bound sulfhydryl and disulfide groups exist in the intercellular substance of tissues; a strong alkali or a KCN solution reduces the protein-bound disulfide on the intercellular cement (14). It is conceivable that in our experiments an

amount of protein-bound disulfide was reduced to sulfhydryl by pretreatment of the tissue with alkali or KCN solution and, as a consequence, the tissue was converted into a tissue of loose type. Other factors may also be involved in the loosening or tightening of a tissue, but at least in the present experiments, it must have been the reduction of protein-bound disulfides that induced the loosening. At any rate, the loose character of the tissue may account for the prevention of artifact formation in pretreated tissues of parenchymatous type since the fixative can rapidly reach and penetrate the cells from all directions as in tissues of original loose type. The glycogen would then be well fixed and evenly distributed in the cytoplasm of the cells without translocation. Additional evidence to support these suppositions was obtained in experiments in which the *Alkohol-flucht* appeared after packing tightly the ascites tumor cells, which originally failed to show the *Alkohol-flucht.*

The observations reported here also indicate that a redistribution of glycogen takes place in dried smears. Accordingly, it is unreasonable to consider that the situation found in dried smears represents the natural distribution of glycogen in living cells. The fact that the distribution of glycogen is homogeneous in dried smears of dividing cells, as compared to resting cells, may be related in part to morphological changes in the nucleus, altered characteristics of the cell membrane, and higher viscosity of the cytoplasm during mitosis (15). Differences in the colloidal properties of the protoplasm may be responsible for the marked artifacts seen in highly active cells, such as those of Yoshida's ascites tumor, as compared to leucocyte and other cell types. Generally, it may be assumed that the variation in the production of artifacts is, in some way, conditioned by some changes in the physicochemical properties of the protoplasm and of the cell membrane, although such changes are not clearly understood. Accordingly, the translocation and coalescence of glycogen particles in the cells of dried smears might indicate a change in these properties. Assuming that the procedure for preparing the smears could be kept constant, detailed observations on the mode of artifact production may lead to better understanding of the physicochemical properties of living protoplasm.

SUMMARY

1. Fixation artifacts associated with glycogen translocation are prevalent in tissues of parenchymatous type and scarce or non-existent in tissues of loose type.

2. Liver tissue treated with M/3 NaOH solution before fixation did not show an uneven distribution of glycogen. This was interpreted as indicating that the liver, a tissue of parenchymatous type, was changed, so to speak, into a loose type of tissue by alkali treatment.

3. The so called *Alkohol-flucht* of glycogen was produced in Yoshida's ascites tumor cells by a procedure which changed a loose type of tissue into a parenchymatous one, that is, by packing the tumor cells tightly.

4. The translocation of glycogen in cells appeared to occur when the fixatives penetrated the cells rapidly from a single direction, but failed to occur when the cells were attacked by the fixative from all directions.

5. In dried smears of Yoshida's ascites tumor cells and bone marrow cells, the glycogen particles are translocated to the peripheral regions of the cells, and coalesce there.

The production of these artifacts is related in some way to the physicochemical properties of the protoplasm and plasma membrane of the cells.

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EXPLANATION OF PLATES

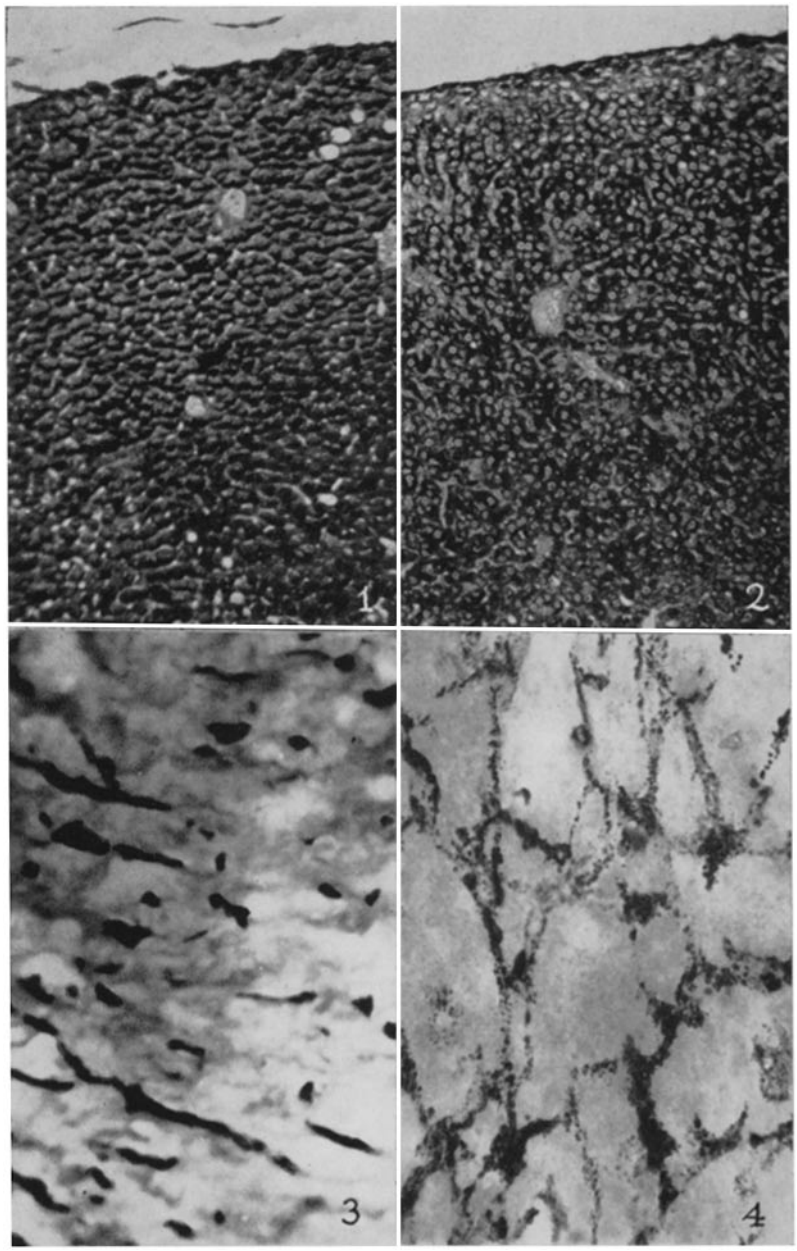
PLATE 102

FIG. 1. Appearance of glycogen in liver tissue fixed directly in absolute alcohol. The *Alkohol-flucht* is visible especially towards the periphery of the block (upper half of the figure). × 150.

FIG. 2. Appearance of glycogen in liver tissue treated with 1/3 M NaOH. Glycogen is distributed homogeneously all over the cytoplasm and shows no *Alkohol-flucht*. × 150.

FIG. 3. Glycogen of umbilical cord tissue fixed in Carnoy's fluid without pretreatment. Glycogen is present only in the central parts of the mesenchymatous cells. × 400.

FIG. 4. Glycogen of umbilical cord tissue treated with 1/3 M NaOH for 5 minutes before fixation. The outline of mesenchymatous cells is indicated by the presence of glycogen not only in the central parts but also in the peripheral regions of the cells. × 400.



(Takahashi and Iwase: Artifacts in histochemical fixation of glycogen)

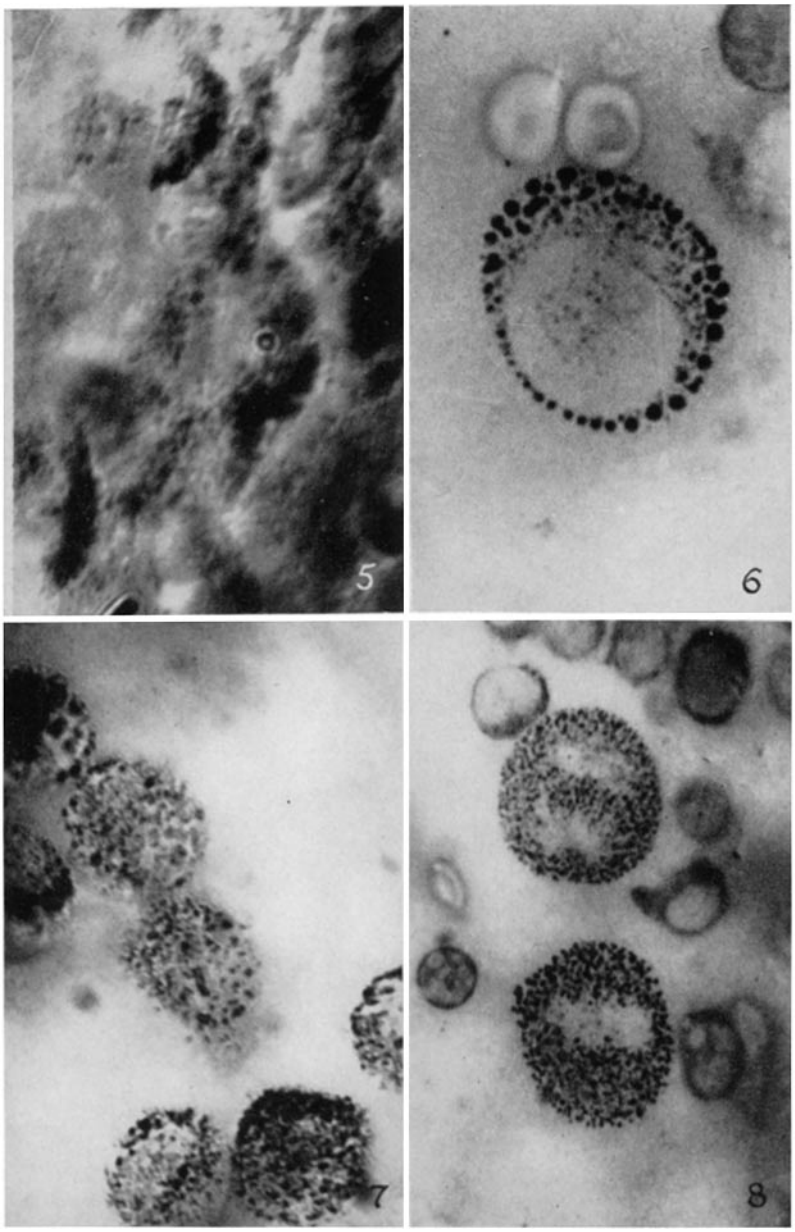
PLATE 103

FIG. 5. Appearance of glycogen in Yoshida's ascites tumor cells imprisoned in a clot of peritoneal exudate. The *Alkohol-flucht* is clearly observed along the border of the clot. × 1350.

FIG. 6. Appearance of glycogen in dried smears of Yoshida's ascites tumor cells. Numerous large particles are present in the peripheral regions of the cytoplasm, very few on the nucleus. × 1350.

FIG. 7. Glycogen appearance in Yoshida's ascites tumor cells in smears, fixed in Carnoy's fluid without previous drying. Glycogen particles of similar size are found uniformly dispersed in the cytoplasm even above the nucleus. × 1350.

FIG. 8. Appearance of glycogen in metaphase and anaphase of Yoshida's ascites tumor cells in dried smear. Glycogen particles are distributed homogeneously in the cytoplasm, showing neither appreciable translocation nor variation in size. × 600.



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