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## ORIGINAL REPORT

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# HISTOCHEMICAL STUDIES ON MITOCHONDRIAL OXIDATIVE ENZYME ACTIVITY IN PARAFFIN SECTIONS WITH SPECIAL REFERENCES TO SOME ARTIFACTS

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## INTRODUCTION

Acetone has long been used in enzyme histochemistry as a fixative for the demonstration of some hydrolytic enzyme activity such as that of alkaline phosphatase and esterases (Gomori, 1952; Barka et al., 1963).

On the other hand, acetone was not successfully used for preparing paraffin sections in which the histochemical demonstration of dehydrogenase activity was possible. The inhibitory effect of histological procedures upon the activity of dehydrogenases, including acetone fixation and paraffin embedding, however, was recently demonstrated to be due to the extraction of phospholipids and coenzyme Q from cellular membranous structures (Ohkawa, 1964; Ohkawa, 1965a). It was also shown that the activity of some dehydrogenases reduced by prolonged acetone fixation could be restored sufficiently to permit histochemical demonstration by the addition of exogenous phospholipids and coenzyme Q to the incubation media (Ohkawa, 1964).

In an attempt to improve the technique for preparing paraffin sections two types of false staining were recognized. The present paper is concerned with possible mechanisms of the occurrence of these artifacts.

## MATERIALS AND METHODS

Experimental animals used in the present studies were adult male albino rats of Wistar strain. The animals were divided into two groups: (1) animals fed on laboratory chow until killing, and (2) animals fasted for 24 hours until killing. Immediately after killing the animals by decapitation small pieces of tissues were removed from the liver, kidney and interscapular brown adipose tissue. The size of the tissue blocks to be fixed was about  $1 \times 2 \times 2$  mm<sup>3</sup>. Tissue blocks were quickly put into absolute acetone which had been previously chilled at 2°C. The fixation time was 90 minutes. Absolute acetone was changed

several times during the fixation. Clearing of the small tissue blocks was carried out with either xylol or a mixture (4 : 1, v/v) of xylol and cedar-wood oil for 45 minutes with three changes of the reagent. Melting point of the paraffin used for infiltration and embedding was 48°C. Bees-wax was added to the paraffin so that the final mixture contained bees-wax at the concentration of 25 per cent. Paraffin infiltration was carried out with three changes of the paraffin-bees-wax mixture during a 45 minute period in an oven maintained between 50°C and 52°C. After complete infiltration the tissue blocks were embedded in the paraffin-bees-wax mixture. Tissue sections of 4 to 6  $\mu$  thickness were cut and floated on a minimal amount of distilled water which had been spread on clean slides. Then, the slides were slightly warmed to spread the sections in order to eliminate any creases. Immediately after spreading the sections any excess water was quickly and carefully removed with filter paper, and the sections were dried at room temperature for one to two hours. Dewaxing was performed with either xylol or a mixture (4 : 1, v/v) of xylol and cedar-wood oil. Immediately after dewaxing, the tissue sections were either transferred to distilled water after quick removal of the dewaxing reagent by rinsing in absolute acetone, or dried without transferring the sections to distilled water. The dried tissue sections from which the absolute acetone was evaporated were covered with coenzyme Q<sub>10</sub> solution in absolute acetone (0.1%) and the absolute acetone was again evaporated.

*The compositions of the incubation media were as follows:*

- |   |         |
|---|---------|
| (1) Succinic dehydrogenase                                  |         |
| a) Sodium succinate (0.5 M, pH 7.4)                         | 0.1 ml  |
| Phenazine methosulfate (5 mg/ml)                            | 0.05 ml |
| Tris-HCl buffer (0.2 M, pH 7.4)                             |         |
| or phosphate buffer (0.2 M, pH 7.4)                         | 0.25 ml |
| Nitro-blue tetrazolium (2 mg/ml)                            | 0.25 ml |
| Distilled water   | 0.35 ml |
| b) Sodium succinate (0.5 M, pH 7.4)                         | 0.1 ml  |
| Tris-HCl buffer (0.2 M, pH 7.4)                             |         |
| or phosphate buffer (0.2 M, pH 7.4)                         | 0.25 ml |
| Nitro-blue tetrazolium (2 mg/ml)                            | 0.25 ml |
| Distilled water   | 0.40 ml |
| (2) Non-NAD linked $\alpha$ -glycerophosphate dehydrogenase |         |
| a) Disodium $\alpha$ -glycerophosphate (1 M)                | 0.1 ml  |
| Phenazine methosulfate (5 mg/ml)                            | 0.05 ml |
| Tris-HCl buffer (0.2 M, pH 7.4)                             | 0.25 ml |
| Nitro-blue tetrazolium (2 mg/ml)                            | 0.25 ml |
| Distilled water   | 0.25 ml |
| b) Disodium $\alpha$ -glycerophosphate (1 M)                | 0.1 ml  |
| Tris-HCl buffer (0.2 M, pH 7.4)                             | 0.25 ml |
| Nitro-blue tetrazolium (2 mg/ml)                            | 0.25 ml |
| Distilled water   | 0.30 ml |
| (3) Choline dehydrogenase                                   |         |

a) Choline chloride (0.1 M or 2%)	0.1 ml
Phenazine methosulfate (5 mg/ml)	0.05 ml
Tris-HCl buffer (0.2 M, pH 7.4)	0.25 ml
Nitro-blue tetrazolium (2 mg/ml)	0.25 ml
Distilled water	0.35 ml
b) Choline chloride (0.1 M or 2%)	0.1 ml
Tris-HCl buffer (0.2 M, pH 7.4)	0.25 ml
Nitro-blue tetrazolium (2 mg/ml)	0.25 ml
Distilled water	0.4 ml
(4) $\beta$ -Hydroxybutyric dehydrogenase	
$\beta$ -Hydroxybutyric acid (1 M)	0.1 ml
NAD (10 mg/ml)	0.2 ml
Tris-HCl buffer (0.2 M, pH 7.4)	0.25 ml
Nitro-blue tetrazolium (2 ml/ml)	0.25 ml
Distilled water	0.2 ml

The incubation time for the histochemical demonstration of the enzyme activity ranged from 5 minutes to 20 minutes. Periodic acid Schiff (PAS) staining was performed in some of the tissue sections from livers.

## RESULTS

### *The nature of the histochemical reactions for the enzyme:*

- (1) Succinic dehydrogenase: The incubation medium containing phenazine methosulfate showed intensive staining in the tissue sections of the liver and kidney which were either covered or not covered with coenzyme Q<sub>10</sub> solution. Incubation medium, however, lacking phenazine methosulfate showed intense staining only in tissue sections which were covered with coenzyme Q<sub>10</sub> solution. As a result it was concluded that the histochemical demonstration of succinic dehydrogenase activity required the presence of either phenazine methosulfate in the incubation medium or coenzyme Q<sub>10</sub> in the tissue sections. Nuclei did not show any positive staining and the staining was entirely cytoplasmic.
- (2) Non-NAD linked  $\alpha$ -glycerophosphate dehydrogenase: The phenazine methosulfate-containing incubation medium showed intense staining in tissue sections of kidney and brown adipose tissue which were either covered or not covered with coenzyme Q<sub>10</sub> solution. The incubation medium lacking phenazine methosulfate showed intense staining only in tissue sections which were covered with coenzyme Q<sub>10</sub> solution. In this case, too, the histochemical reaction was dependent upon the presence of either phenazine methosulfate or coenzyme Q<sub>10</sub>. However, sections from interscapular brown adipose tissue, which were not covered with coenzyme Q<sub>10</sub> solution, exceptionally showed moderate staining with the incubation medium containing no phenazine methosulfate. The intracellular staining pattern was of cytoplasmic nature.
- (3) Choline dehydrogenase: Phenazine methosulfate-containing incubation medium showed intense staining in tissue sections either covered or not covered with coenzyme Q<sub>10</sub>

solution. Incubation medium lacking phenazine methosulfate showed intense staining only in tissue sections which were covered with coenzyme Q<sub>10</sub> solution. The histochemical reaction for choline dehydrogenase had the same property as that of the above-mentioned two enzymes in that it requires the presence of either phenazine methosulfate or coenzyme Q<sub>10</sub>. The cytoplasmic staining pattern was seen in this case, too.

(4)  $\beta$ -Hydroxybutyric dehydrogenase: The incubation medium showed intense staining in tissue sections which were either covered or not covered with coenzyme Q<sub>10</sub> solution. In other words, the histochemical reaction for  $\beta$ -hydroxybutyric dehydrogenase did not require the presence of coenzyme Q<sub>10</sub>. The staining was of cytoplasmic nature.

*The differences in the staining pattern between the tissue sections from the animals normally fed until killing and those from the animals fasted for 24 hours prior to killing:*

Tissue sections, especially of the livers, from animals normally fed until killing showed a peculiar distribution of the end-product, formazan dye. This artifact consisted in the staining of one side of the cells. The staining pattern of the individual cell showed a crescent appearance. It was almost the same as that seen in PAS staining in tissue sections fixed in either acetone or ethanol. The tissue sections from the animals fasted for 24 hours prior to killing showed either minimal or no artifact. It was significant in speculation on the occurrence of this artifact that fasting the animals prior to killing improved the microscopic appearance of the staining.

*The staining pattern of the activity of succinic dehydrogenase, non-NAD linked  $\alpha$ -glycerophosphate dehydrogenase and choline dehydrogenase in tissue sections incubated in phenazine methosulfate-containing incubation media and in media lacking phenazine methosulfate:*

Phenazine methosulfate-containing incubation media gave rise to an artifact in the

**Fig. 1** The effects of phenazine methosulfate and CoQ<sub>10</sub> upon histochemical dehydrogenase activity reactions.

	With phenazine methosulfate		Without phenazine methosulfate	
	With CoQ <sub>10</sub>	Without CoQ <sub>10</sub>	With CoQ <sub>10</sub>	Without CoQ <sub>10</sub>
SDH	(+)	(+)	(+)	(-)
$\alpha$ -GPD	(+)	(+)	(+)	(-)
Ch. D	(+)	(+)	(+)	(-)
$\beta$ -HBD			(+)	(+)

SDH : Succinic dehydrogenase  
 $\alpha$ -GPD:  $\alpha$ -Glycerophosphate dehydrogenase  
 Ch. D : Choline dehydrogenase  
 $\beta$ -HBD:  $\beta$ -Hydroxybutyric dehydrogenase  
 CoQ<sub>10</sub> : Coenzyme Q<sub>10</sub>  
 (+) : Positive reaction  
 (-) : Negative reaction

staining pattern, especially in sections of the kidney. This artifact consisted of the staining of basal portions of the urinary tubules and staining of regions adjacent to the sites of the enzyme activity including the brush borders. There was no such staining artifact in tissue sections covered with coenzyme Q<sub>10</sub> solution and incubated in the absence of phenazine methosulfate. From the histochemical view point incubation media containing no phenazine methosulfate were better than those containing phenazine methosulfate, particularly with respect to the kidney. However, in sections of brown fat there was almost no artifact due to phenazine methosulfate. The effects of phenazine methosulfate and coenzyme Q<sub>10</sub> upon the reactions for dehydrogenase activity are summarized in Fig. 1.

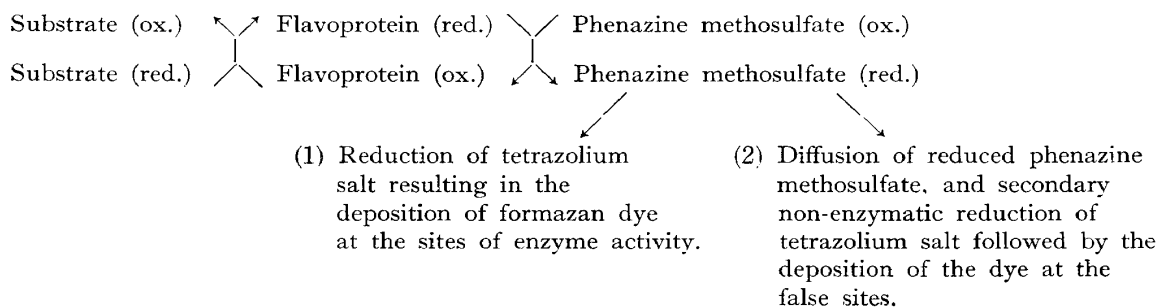
## DISCUSSION AND CONCLUSION

In the field of histochemistry of oxidative enzymes, freshness of tissue sections has long been regarded as an essential requirement in practice for the demonstration of these enzymes (Pearse, 1960; Barka et al., 1963). However, it was reported that the activity of dehydrogenases such as that of succinic dehydrogenase could be demonstrated in cryostat sections fixed in cold acetone (Novikoff et al, 1960).

The mechanisms of the inhibition of oxidative enzyme activity by cold acetone fixation and paraffin embedding have become known recently. In 1964 and 1965 it was reported from this laboratory that exogenous phospholipids and coenzyme Q<sub>6</sub> or coenzyme Q<sub>10</sub> added to the incubation media could enhance the staining of some mitochondrial oxidative enzyme activity, even after dewaxing, in tissue sections prepared from tissue blocks fixed in cold absolute acetone and embedded in paraffin (Ohkawa, 1964; Ohkawa, 1965 b). It was also demonstrated that the inhibitory effect of histological procedures of acetone fixation and paraffin embedding is due to the extraction of phospholipids and coenzyme Q from mitochondrial membranes (Ohkawa, 1965 a, c). The present investigation is primarily concerned with the above-mentioned subjects.

The artifact similar to the so-called "polarization artifact" seen in the case of PAS staining was minimized or completely prevented by fasting the animals prior to killing. It is significant for understanding the possible mechanism of this kind of artifact to consider the following facts. (1) The pattern of the artifact staining resembled closely the so-called "polarization artifact" of PAS staining. (2) Fasting of the animals was strikingly effective in preventing the occurrence of the artifact staining. (3) PAS staining of the tissue sections of the liver from animals fasted for 24 hours prior to killing decreased markedly. Moreover, it has long been known from electron microscopy that the content of cytoplasmic glycogen granules will rapidly decrease at an early stage of fasting. Therefore, it was concluded that the occurrence of the artifact was due to the secondary dislocation of mitochondria to one pole of the cell with the primary derangement of the cytoplasmic glycogen granules induced by possible streaming of cytoplasm during fixation.

The occurrence of the artifact observed in the staining with incubation media containing phenazine methosulfate is due to the use of phenazine methosulfate itself because the use of coenzyme Q<sub>10</sub> instead of phenazine methosulfate is effective in preventing the artifact.

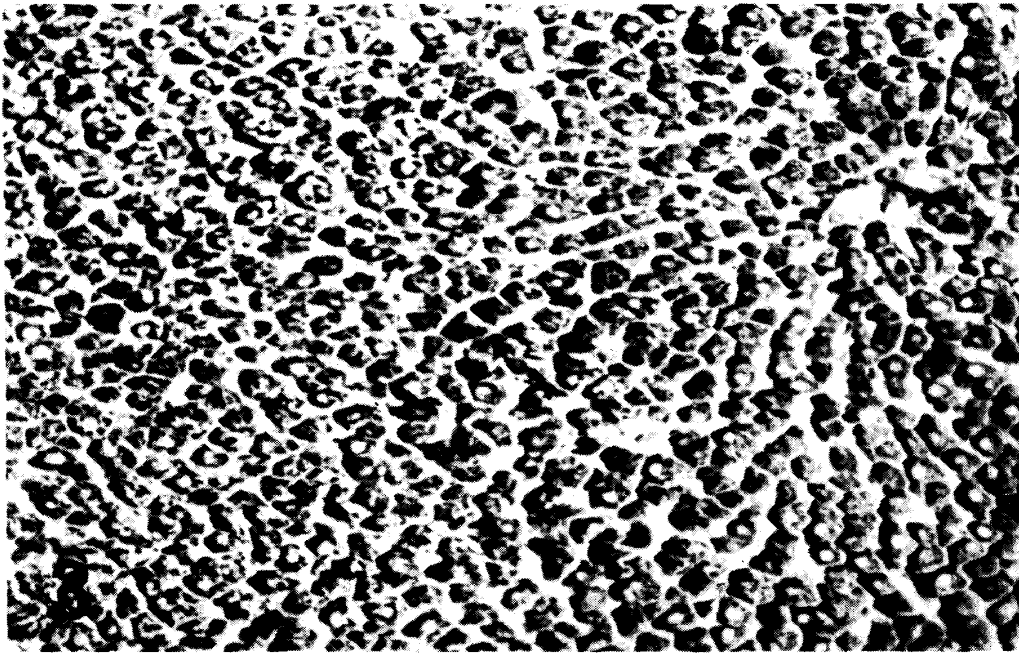
**Fig. 2** A possible mechanism of artifact due to phenazine methosulfate.

This shows that diffusion of reduced phenazine methosulfate may be responsible for the occurrence of artifact staining due to phenazine methosulfate.

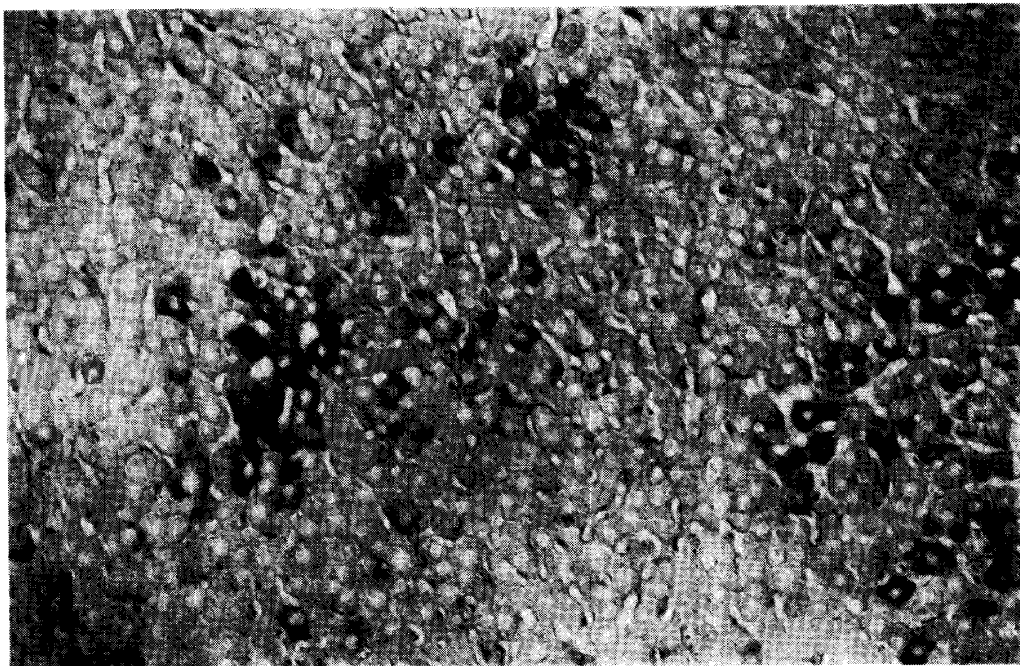
The mechanism of the artifact is not known at present. It is, however, speculated to be as illustrated in Fig. 2. Both the oxidized and the reduced forms of phenazine methosulfate are soluble in water. The solubility of reduced phenazine methosulfate in water may be a possible main factor leading to the occurrence of the artifact. The soluble reduced phenazine methosulfate may diffuse from the sites of enzyme activity at which phenazine methosulfate was reduced enzymatically. Then the reduced phenazine methosulfate will be adsorbed to certain cellular structures, and tetrazolium salt will be reduced at the false sites, resulting in the artifact staining.

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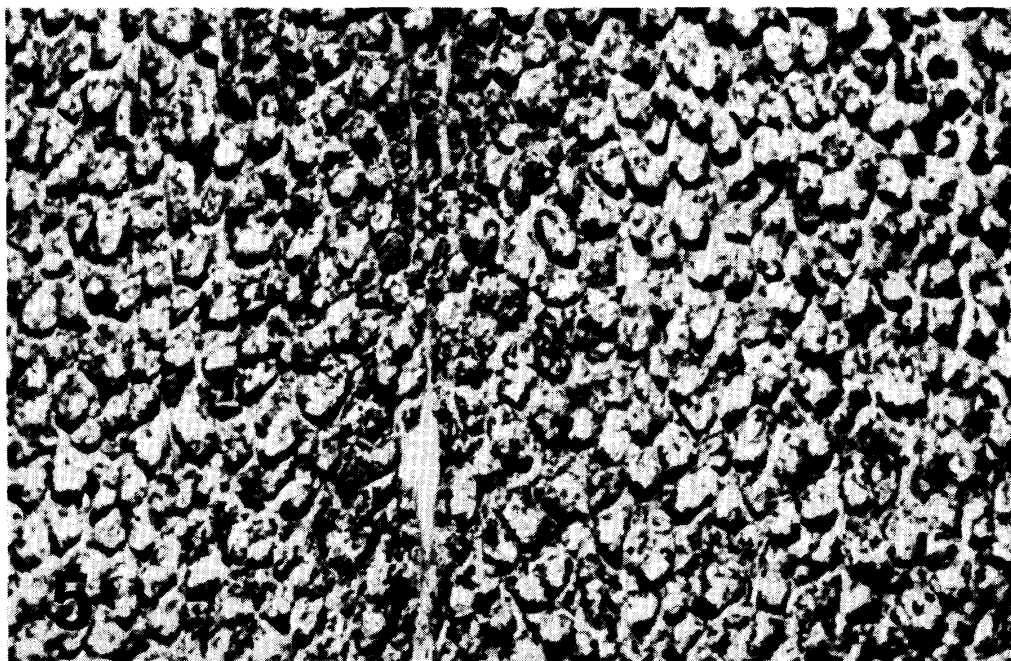
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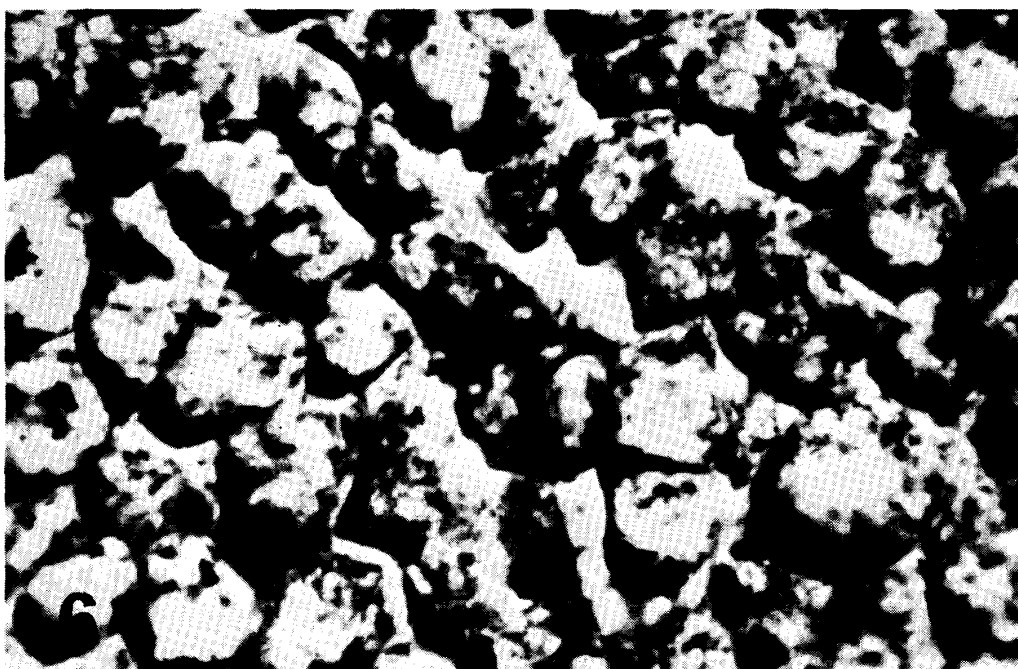
**Fig. 3.** Periodic Acid Schiff (PAS) staining of normally fed rat liver paraffin section. "Polarization artifact" staining is clearly shown. 100 $\times$ .



**Fig. 4.** PAS staining of rat liver fasted for 24 hours before killing. The staining is patchy. 100 $\times$ .

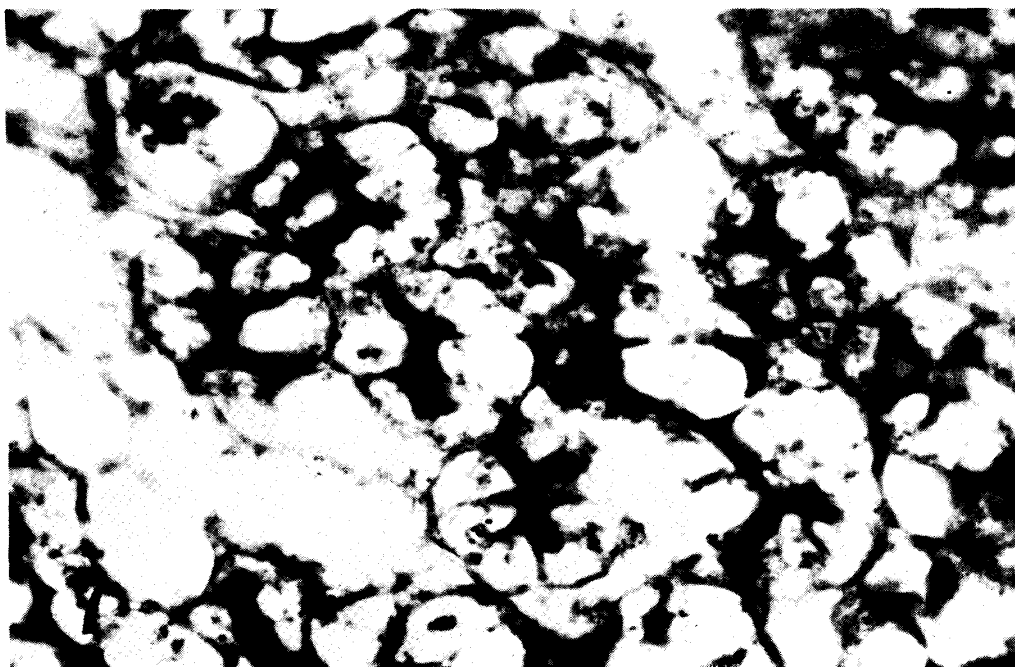


**Fig. 5.** Succinic dehydrogenase in normally fed rat liver. "Polarization artifact" is as clear as in Fig. 3. 100 $\times$ .

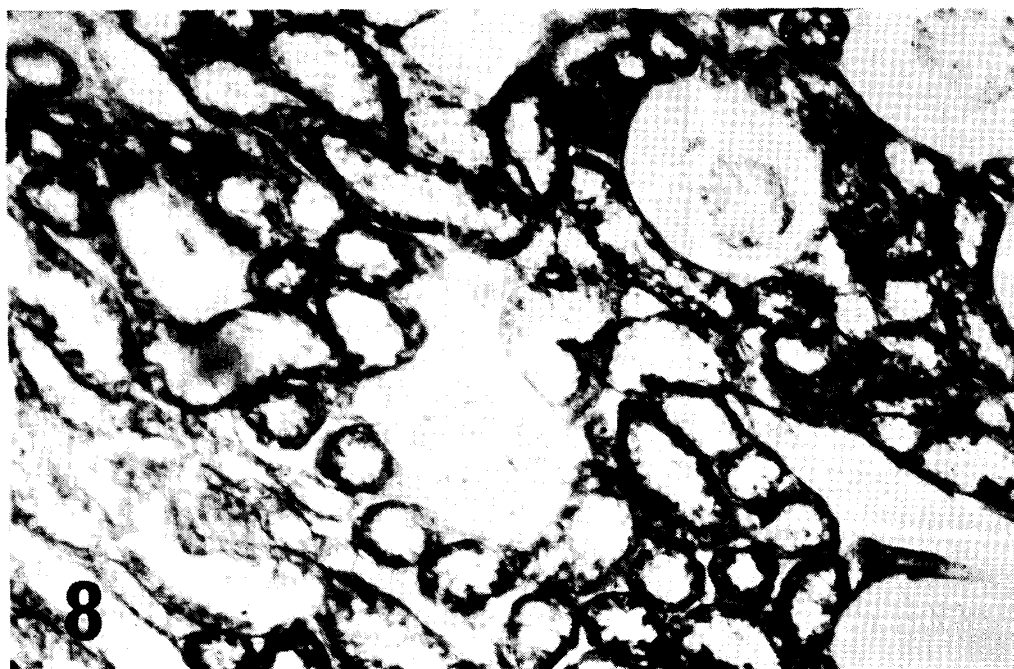


**Fig. 6.** Succinic dehydrogenase. Higher magnification of Fig. 5. "Polarization artifact" is evident in each hepatic cells. 400 $\times$ .

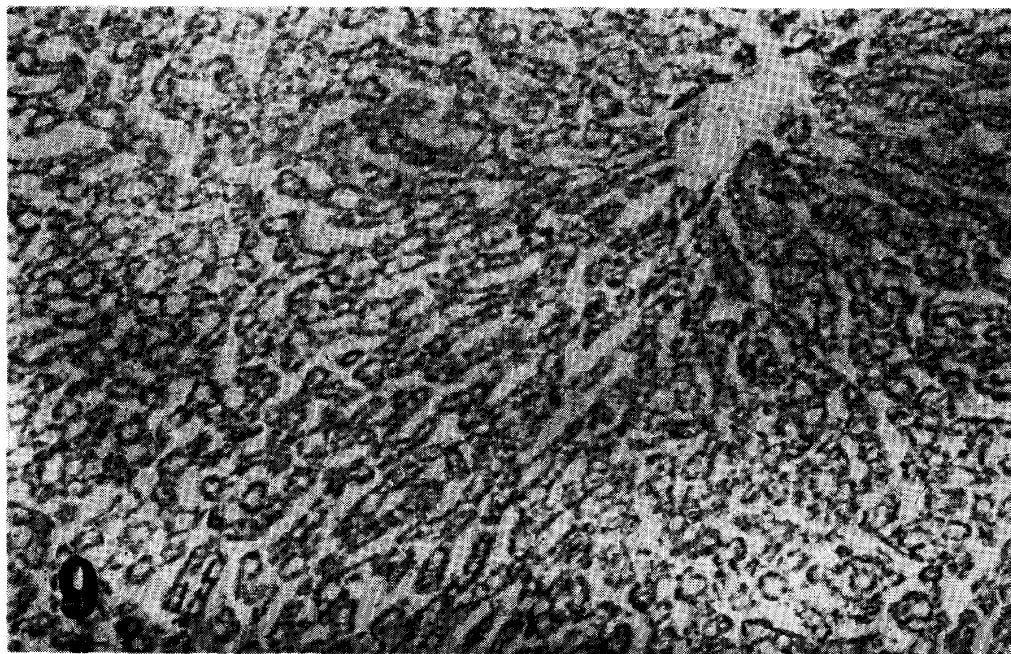




**Fig. 7.** Non-NAD linked  $\alpha$ -glycerophosphate dehydrogenase in kidney section incubated in medium containing phenazine methosulfate. In this picture non-specific staining of brush borders of the epithelial cells is evident. 400 $\times$ .



**Fig. 8.** Non-NAD linked  $\alpha$ -glycerophosphate dehydrogenase in kidney section. The basal parts of urinary tubules show non-specific staining. 100 $\times$ .



**Fig. 9.** Choline dehydrogenase of fasted rat liver. No "polarization artifact" is seen. 100×.



**Fig. 10.** Choline dehydrogenase of the CoQ<sub>10</sub>-covered kidney section incubated in medium devoid of phenazine methosulfate. There is no artifact staining. 100×.