

FACTORS LIMITING THE HISTOCHEMICAL REACTION OF CHOLINE DEHYDROGENASE ACTIVITY

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INTRODUCTION

It has long been regarded that histological procedures, including fixation, embedding *etc.*, are generally inhibitory to the histochemical reactions of dehydrogenases. The exact mechanism of this inhibition, however, was not fully understood²⁾. Recently it has been reported that the inhibitory effects of the histological procedures, included in fixation in absolute acetone and embedding in soft paraffin, upon the histochemical demonstration of mitochondrial dehydrogenase activity are due to the extraction of membranous phospholipids and coenzyme Q¹³⁻¹⁵⁾. In 1960 Wattenberg *et al.* showed that the histochemical demonstration of succinic dehydrogenase and α -glycerophosphate dehydrogenase activity require the presence of an adequate amount of coenzyme Q in the tissue sections¹⁸⁾. Recently, Wolman *et al.* and Horwitz *et al.* have demonstrated that the mitochondrial membrane phospholipids and coenzyme Q are the limiting factors in the histochemical reaction of succinic dehydrogenase activity^{9,21)}. As a result of the previous studies on the histochemistry of mitochondrial oxidative enzymes it was speculated that some membrane lipid substances must participate in the histochemical reaction of choline dehydrogenase activity.

The present communication is concerned with the presence of two factors limiting the histochemical demonstration of choline dehydrogenase activity.

MATERIALS AND METHODS

Male adult albino rats of Wistar strain weighing 200 g were used in the present experiments. Immediately after killing the animals small pieces of tissue were collected from liver, kidney and heart, and were quickly frozen with the application of dry-ice acetone. Ten μ thick tissue sections cut in the cryostat, maintained at -18°C , were used for histochemical detection of enzyme activity.

Incubation media were slightly modified after Farber *et al.*⁵⁾ as follows;
Medium I.

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 salt (4 mg/ml)	0.25 ml
Dist. water	0.35 ml

Medium II,

Choline chloride (0.1 M or 2%)	0.1 ml
Tris-HCl buffer (0.2 M, pH 7.4)	0.35 ml
Nitro-blue tetrazolium salt (4 mg/ml)	0.25 ml
Dist. water	0.35 ml

The concentration of coenzyme Q₁₀ solution and menadione solution in acetone were 0.1 per cent. In some experiments sodium succinate and disodium α -glycerophosphate were used as substrates in place of choline chloride.

The hepatic mitochondrial fractions were prepared in 0.25 M sucrose solution according to Schneider's procedure¹⁶) from 5 g of liver, and heart mitochondrial fractions were prepared after Tyler *et al.*s procedure¹⁷). The mitochondrial pellets were extracted with 50 ml of absolute acetone, and with 10% water-in-acetone respectively for 10 to 15 minutes at 25°C. Then the mitochondrial pellets were discarded and the extracts were concentrated to 1/10 of the original volume at 25°C. The concentrated extracts were of bright-yellowish color and were designated as the mitochondrial extracts.

Extracts from tissue sections were prepared as follows: A hundred tissue sections of 10 μ thickness of liver and of heart (10 \times 5 mm²) were extracted with 50 ml of absolute acetone, and with 10% water-in-acetone respectively for 10 to 15 minutes at 25°C. The tissue sections were discarded and the extracts were concentrated to 1/20 of the original volume at 25°C. The concentrated extracts had a bright-yellowish color and were designated as the section-extracts.

Phenazine methosulfate, menadione, nitro-blue tetrazolium salt and coenzyme Q₁₀ were purchased from Sigma Chemical Company.

Control experiment.

Control non-pretreated sections were incubated in Medium 1 or Medium 11 for 20 minutes at 37°C with, or without coenzyme Q₁₀, menadione, phenazine methosulfate, section-extracts and mitochondrial extracts.

Experiment 1.

Prior to incubation the tissue sections were treated with absolute acetone at 25°C, 2°C and -15°C for ten minutes respectively, then incubated in medium I for 20 minutes at 37°C.

Experiment 2.

The tissue sections were treated as in Experiment 1, then incubated in medium 11 for 20 minutes at 37°C.

Experiment 3.

Prior to the incubation the sections were treated with 10% water-in-acetone at 25°C, 2°C and -15°C for ten minutes respectively, and incubated in medium I for 20 minutes at 37°C.

Experiment 4.

The tissue sections were treated as in Experiment 3, and then incubated in medium II for

20 minutes at 37°C.

Experiment 5.

Prior to incubation the tissue sections were treated with absolute acetone at 25°C, 2°C and -15°C for ten minutes respectively and covered with either the acetone extract of the tissue sections (liver or heart) or 10% water-in-acetone extract of the sections. Then the tissue sections were incubated in medium II for 20 minutes at 37°C.

Experiment 6.

Prior to incubation the tissue sections were treated with 10% water-in-acetone at 25°C, 2°C and -15°C for ten minutes respectively and covered with extracts as in Experiment 5. Then incubated in medium II for 20 minutes at 37°C.

Experiment 7.

Prior to incubation the tissue sections were treated with absolute acetone at 25°C, 2°C and -15°C for ten minutes respectively and covered with coenzyme Q₁₀ solution. Then incubated in medium II for 20 minutes at 37°C.

Experiment 8.

Prior to incubation the tissue sections were treated with 10% water-in-acetone at 25°C, 2°C and -15°C for ten minutes respectively and covered with coenzyme Q₁₀ solution. Then incubated in medium II for 20 minutes at 37°C.

Experiment 9.

As in Experiment 7, substituting menadione solution for coenzyme Q₁₀ solution.

Experiment 10.

As in Experiment 8, substituting menadione solution for coenzyme Q₁₀.

Experiment 11.

Prior to incubation the tissue sections were treated with absolute acetone at 25°C, 2°C and -15°C for ten minutes respectively and covered with the acetone extract, or 10% water-in-acetone extract either from the hepatic mitochondrial fraction or the heart mitochondrial fraction. Then the tissue sections were incubated in medium II for 20 minutes at 37°C.

Experiment 12.

Substituted 10% water-in-acetone in Experiment 11 pretreatment phase.

RESULTS

Control staining: In the control sections of liver and kidney incubated in medium II (no phenazine methosulfate) slight formazan formation was recognized. On the other hand, the sections incubated in medium I (with phenazine methosulfate) showed very strong staining for enzyme activity both in liver cells and urinary tubular cells. The heart sections, however, revealed no positive staining either in the presence or absence of phenazine methosulfate. Therefore, it was concluded that no significant amount of the enzyme was present in the heart muscle cells. The enhancing effect of phenazine methosulfate upon the histochemical reaction of the enzyme was in agreement with previous reports of Farber *et al.*^{5,6)} Menadione, coenzyme Q₁₀, section-extracts and mitochondrial extracts also enhanced the staining reaction for choline dehydrogenase, like phenazine methosulfate. The results with menadione and coenzyme Q₁₀

are similar to those in studies of succinic dehydrogenase and α -glycerophosphate dehydrogenase ^{9,18,19,21}. The extracts (section and mitochondrial, hepatic and heart) with absolute acetone had the same enhancing effects as those with 10% water-in-acetone.

Exp. 1 and Exp. 2

Tissue sections from liver and kidney treated with absolute acetone prior to incubation at 25°C showed only moderate staining reaction for the enzyme in the presence of phenazine methosulfate. Tissue sections from liver and kidney treated with absolute acetone for ten minutes either at 2°C or -15°C showed strong staining for the enzyme with phenazine methosulfate. Sections treated with absolute acetone prior to incubation at 25°C failed to show even the slightest reaction of the enzyme in the absence of phenazine methosulfate. Tissue sections, however, treated with absolute acetone at 2°C showed slight staining with medium II (no phenazine methosulfate). Moderate staining was recognized in the tissue sections treated with absolute acetone at -15°C. Lowering the temperature of the pretreatment absolute acetone significantly preserved the enzyme activity.

Phenazine methosulfate was effective in restoring enzyme activity inhibited by pretreatment of the sections with absolute acetone, especially at lower temperature.

Exp. 3 and Exp. 4

Tissue sections treated with 10% water-in-acetone at 25°C prior to incubation revealed no positive staining either in the presence or absence of phenazine methosulfate. Sections treated at 2°C showed occasionally very slight and irregular staining for enzyme activity both with and without phenazine methosulfate. Tissue sections treated similarly at -15°C revealed slight staining.

Exp. 5 and Exp. 6

Sections of liver and kidney covered with section-extract (by either absolute acetone or 10% water-in-acetone) after treatment with absolute acetone at 25°C showed moderate staining reaction, and at 2°C and -15°C, strong staining reaction. On the other hand, sections treated with 10% water-in-acetone at 25°C revealed no trace of staining reaction even when covered with section-extracts. Sections covered with section-extracts after treatment with 10% water-in-acetone at 2°C showed slight staining and those treated at -15°C showed moderate staining.

Exp. 7 and Exp. 8

Tissue sections of liver and kidney treated with absolute acetone at 25°C showed moderate staining for enzyme activity when covered with coenzyme Q₁₀ solution. However, sections treated with 10% water-in-acetone at 25°C revealed no staining even when covered with coenzyme Q₁₀ solution. The enhancing effect of coenzyme Q₁₀ upon the staining of the sections treated with either absolute acetone or 10% water-in-acetone at lower temperatures was similar to that of section-extracts.

Exp. 9 and Exp. 10

Tissue sections of liver and kidney covered with menadione after treatment with absolute acetone at 25°C revealed moderate staining reaction. However, sections treated with 10% water-in-acetone at 25°C showed no staining even with menadione solution covering the sections. The enhancing effect of menadione upon the staining at lower temperatures was similar to that of coenzyme Q₁₀ and the section extracts,

Exp. 11 and Exp. 12

Tissue sections of liver and kidney treated with absolute acetone at 25°C showed moderate staining reaction when covered with any of the mitochondrial extracts. But those sections treated with 10% water-in-acetone at 25°C showed no staining. The enhancing effect of the mitochondrial extracts upon the staining of sections treated with solvents at lower temperatures was similar to that of coenzyme Q₁₀, menadione and the section-extracts.

The activity of succinic dehydrogenase and α-glycerophosphate dehydrogenase was lost by pretreatment of the sections with either absolute acetone or 10% water-in-acetone at 25°C. The inhibitory effect of pretreatment upon enzyme activity decreased as the temperature of the solvents fell. The activity of the two enzymes lost in pretreatment with absolute acetone was restored by coenzyme Q₁₀, menadione and the bright-yellow extracts, but that lost in pretreatment with 10% water-in-acetone was not restored by the agents.

Table 1 summarizes the experimental results.

Table 1. The effects of pretreatment and of addition of various chemicals upon the histochemical reaction of the enzyme.

Addition Treatment	CoQ ₁₀	Menadione	Extract from sections by absolute acetone	Extract from sections by 10 % water-in-acetone	Extract from mitochondrial fraction by absolute acetone	Extract from mitochondrial fraction by 10 % water-in-acetone	Phenazine methosulfate	No addition
No treatment	(+)	(+)	(+)	(+)	(+)	(+)	(+)	($\frac{+}{-}$)
absolute acetone 25°C	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(-)
absolute acetone 2°C	(+)	(+)	(+)	(+)	(+)	(+)	(+)	($\frac{+}{-}$)
absolute acetone - 15°C	(+)	(+)	(+)	(+)	(+)	(+)	(+)	(+)
10 % water-in-acetone 25°C	(-)	(-)	(-)	(-)	(-)	(-)	(-)	(-)
10 % water-in-acetone 2°C	($\frac{+}{-}$)	($\frac{+}{-}$)	($\frac{+}{-}$)	($\frac{+}{-}$)	($\frac{+}{-}$)	($\frac{+}{-}$)	($\frac{+}{-}$)	(-)
10 % water-in acetone - 15°C	(+)	(+)	(+)	(+)	(+)	(+)	(+)	($\frac{+}{-}$)

(+) : positive reaction
 (-) : no reaction
 ($\frac{+}{-}$) : slight positive reaction

DISCUSSION

Treatment of tissue sections with absolute acetone, which extracts coenzyme Q₁₀¹⁰, and with 10% water-in-acetone which is claimed to extract more than 80% of mitochondrial phospholipids^{8,11}, provides a useful approach to the problem of mechanism of inhibitory effect of acetone fixation of tissue blocks upon staining for enzyme activity. The results of experiment 1 showed clearly that pretreatment of tissue sections with absolute acetone was not inhibitory to the histochemical reaction of the enzyme in the presence of phenazine methosulfate. It was

shown in experiment 2 that in the absence of phenazine methosulfate, pretreatment of the sections with absolute acetone at 25°C was completely inhibitory. As the temperature of pretreatment reagent was lowered, the inhibitory effect was diminished. These results indicate that a certain endogenous factor participating in the histochemical reaction of the enzyme was extracted during pretreatment of the sections with absolute acetone, and that the extraction was influenced by temperature. Phenazine methosulfate could substitute for the factor. The histochemical reaction of choline dehydrogenase is similar to that of succinic dehydrogenase and α -glycerophosphate dehydrogenase in that it was inhibited by acetone treatment of the sections^{18,19}).

In experiments 3 and 4 it was shown that even in the presence of phenazine methosulfate the histochemical reaction was entirely inhibited by pretreatment of the sections with 10% water-in-acetone at 25°C. When the temperature of 10% water-in-acetone was lowered, the reaction was recognized in the presence of phenazine methosulfate. In the absence of phenazine methosulfate there was no reaction. Even when the temperature of 10% water-in-acetone fell the reaction was very slight in the absence of phenazine methosulfate. These results show that a certain endogenous factor participating in the reaction was lost during pretreatment with 10% water-in-acetone and that the extraction of the substances was dependent upon the temperature. Judging from the fact that pretreatment of the sections with absolute acetone at 25°C was not inhibitory to the reaction in the presence of phenazine methosulfate but that pretreatment with 10% water-in-acetone at 25°C was entirely inhibitory, it was concluded that the substance which was extracted with absolute acetone was different from that extracted with 10% water-in-acetone. Based upon speculation that pretreatment of tissue sections extracted some important endogenous substances, concentrated extracts from tissue sections were used as substitutes for phenazine methosulfate. Tissue sections covered with section extracts after pretreatment with absolute acetone at 25°C revealed restoration of the staining reaction. The reaction lost in pretreatment with 10% water-in-acetone was not restored by the concentrated extracts. Since both the absolute acetone extracts and the 10% water-in-acetone extracts were similar in the restoration of the staining for choline dehydrogenase lost by pretreatment of the sections with absolute acetone, it was concluded that 10% water-in-acetone extracted the substance extractable with absolute acetone in addition to the substance extractable only with 10% water-in-acetone. From the results of experiments 5 and 6 it was also concluded that changes in the molecular arrangements of the mitochondrial membrane induced by pretreatment of the tissue sections with absolute acetone could be corrected by returning the concentrated extracts. However, pretreatment of the sections with 10% water-in-acetone produced more severe and irreversible changes to the membrane molecular arrangement than did absolute acetone. Acetone is known to extract α -tocopherol and coenzyme Q₁₀ from mitochondrial preparations^{7,10}. It is known that acetone-extracted mitochondria show an absolute requirement for coenzyme Q₁₀ in the restoration of succinoxidase activity, though no requirement for α -tocopherol^{1,3,7}. On the other hand, acetone containing 10% water is known to extract mitochondrial phospholipids^{8,11}. Farber *et al.* demonstrated that incubation of frozen sections of rat kidney with lipase abolishes the staining reaction for succinic dehydrogenase⁵. They concluded that the succinic dehydrogenase system contains carriers of lipid nature. The results of the experi-

ments with 10% water-in-acetone suggest that the choline dehydrogenase system contains phospholipids and that the presence of phospholipids is required for staining reaction of the enzyme. The functional roles of phospholipids and coenzyme Q₁₀ have been reported in the mitochondrial electron transport system^{1,3,4,7,8,11,20} and the present experimental data are in agreement with the results of these studies.

In experiments 7 and 8 it was shown that coenzyme Q₁₀ was able to restore the reaction lost by pretreatment of the sections with absolute acetone but not effective in restoring that lost by pretreatment with 10% water-in-acetone. Therefore, it was concluded that the effect of coenzyme Q₁₀ upon the enzyme reaction is similar to that of the section extracts. Judging from the fact that coenzyme Q₁₀ is a constituent of the respiratory chain of the mitochondrial membrane the results of experiments 7 and 8 are of great significance in identifying the chemical nature of the substance which is participating in the reaction and extractable with absolute acetone.

In experiments 9 and 10 it was shown that menadione also had the same effect upon the reaction of the enzyme as that of the tissue extracts and coenzyme Q₁₀. Menadione is known to act as an electron carrier in some metalloflavoproteins¹²⁾ and the results are in agreement with the previous studies.

It was assumed that since choline dehydrogenase is known as a mitochondrial enzyme the factors limiting the histochemical reaction of choline dehydrogenase activity may be present in the mitochondrial structures. Liver and heart were selected as the organs from which the mitochondrial fractions were prepared. Because it was shown in the control experiments that while liver sections show strong staining for enzyme activity, heart sections showed no staining. Since the heart sections showed no histochemical staining for enzyme activity it was thought impossible that the enzyme itself could be the factor extracted from mitochondria. This was confirmed by the fact that the extract from the heart mitochondrial fraction was effective in restoring the staining lost by pretreatment with absolute acetone. This assumption was also applied to the preparation of the extracts from tissue sections.

In experiments 11 and 12 it was clearly shown that the extracts both from liver mitochondrial fractions and from heart mitochondrial fractions, extracted either with absolute acetone or 10% water-in-acetone, were effective in the restoration of staining for enzyme activity lost by pretreatment with absolute acetone. The extracts could not, however, restore the staining lost by pretreatment with 10% water-in-acetone.

Staining for succinic dehydrogenase activity and α -glycerophosphate dehydrogenase activity showed the same response to pretreatment of the sections as that for choline dehydrogenase activity. These results are of particular significance in identifying the factors in connection with previous studies^{18,19}.

From the results of the present experiments it is concluded that there are at least two endogenous factors which play significant roles in the histochemical reaction of choline dehydrogenase, a mitochondrial enzyme. The first is soluble in absolute acetone and the second is soluble in 10% water-in-acetone. The first is probably coenzyme Q₁₀ which is a constituent of the normal mitochondrial respiratory chain. The second may be phospholipids which constitute the mitochondrial membrane, holding the respiratory chain in proper functional state.

ABSTRACT

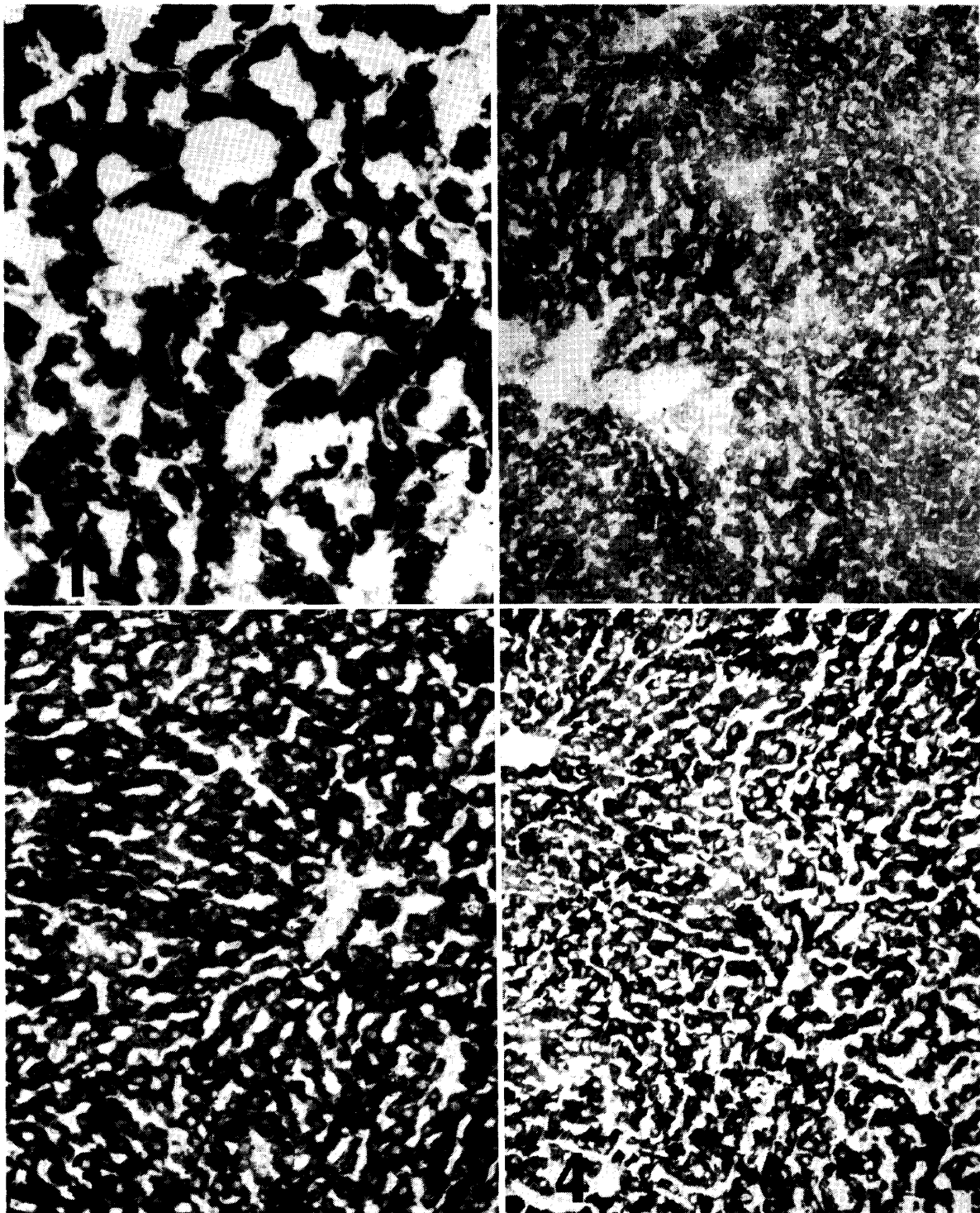
The histochemical reaction of choline dehydrogenase was completely inhibited by the pretreatment of cryostat sections with either absolute acetone or 10% water-in-acetone at room temperature (25°C). The temperature of the organic solvents was critical in the inhibition of the staining for choline dehydrogenase. As the temperature was lowered, the inhibitory effects of the pretreatment decreased. The staining lost by absolute acetone was restored by coenzyme Q₁₀, phenazine methosulfate, menadione, section-extracts and mitochondrial fraction extracts. On the other hand, the staining lost by 10% water-in-acetone was not restored. Both absolute acetone and 10% water-in-acetone produced the same inhibitory effect upon the staining. There was, however, a difference between the effect of absolute acetone and that of 10% water-in-acetone in the restoration of the staining lost by the pretreatment of the sections. Coenzyme Q₁₀ and phospholipids are suggested as limiting factors in the histochemical reaction of choline dehydrogenase.

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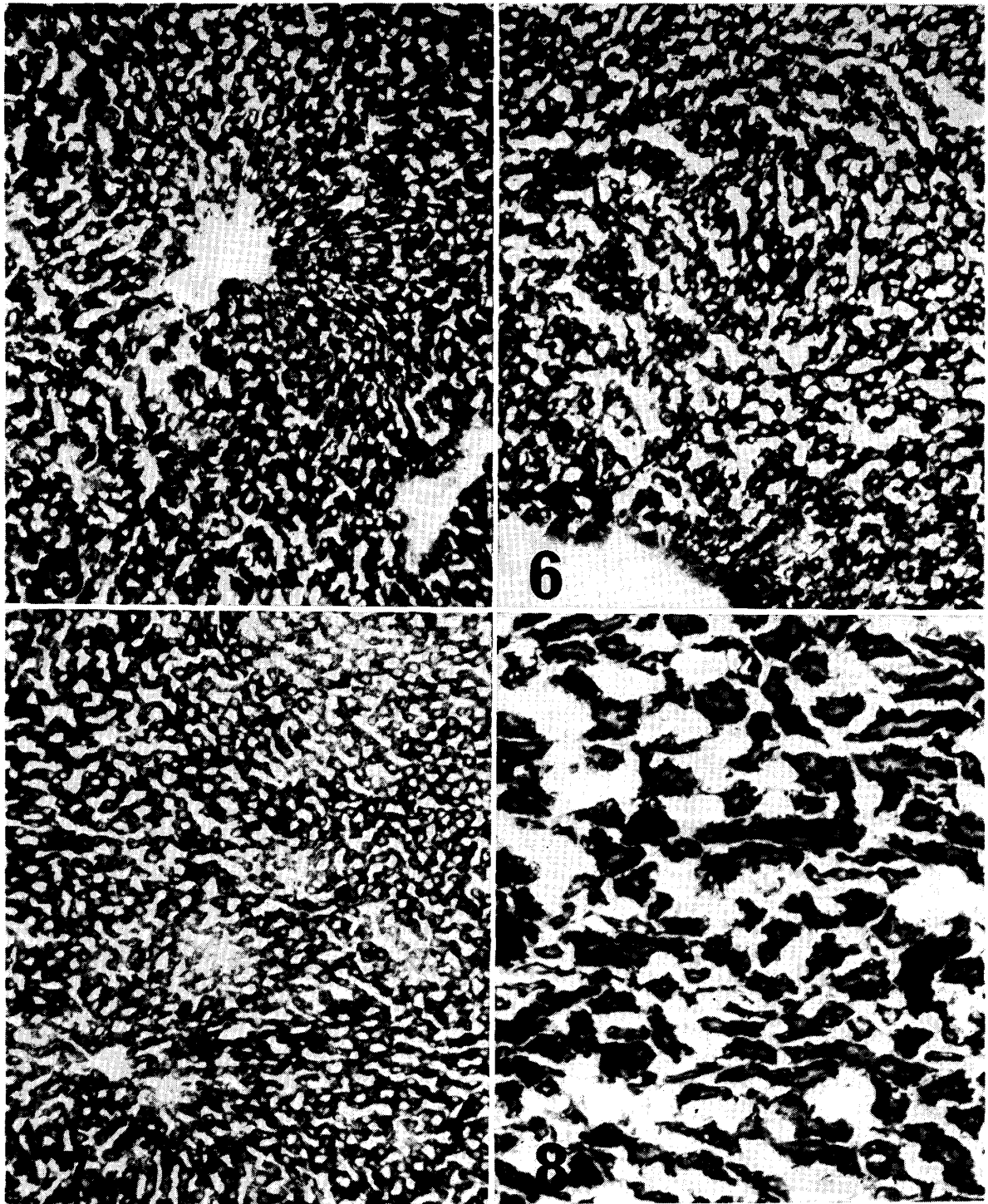
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All figures show the localization of choline dehydrogenase activity.



- Fig. 1** Kidney. Activity in the section incubated in medium I (with phenazine methosulfate) after pretreatment with absolute acetone at -15°C . Strong staining is seen in the tubular epithelial cells. $100\times$
- Fig. 2** Liver. Activity in the section incubated in medium II (without phenazine methosulfate) after pretreatment with 10% water-in-acetone at -15°C . Slight staining is seen. $100\times$
- Fig. 3** Liver. Activity in the section covered with liver mitochondrial fraction extract after pretreatment with 10% water-in-acetone at -15°C . Strong staining is seen. $400\times$
- Fig. 4** Liver. Activity in the section covered with coenzyme Q_{10} solution after pretreatment with absolute acetone at -15°C . Moderate staining is seen. $100\times$



- Fig. 5** Liver. Activity in the section covered with section-extract of liver after pretreatment with absolute acetone at -15°C . Strong staining is seen. $100\times$
- Fig. 6** Liver. Activity in the section covered with heart mitochondrial extract after pretreatment with absolute acetone at 25°C . Moderate staining is seen. $100\times$
- Fig. 7** Liver. Activity in the section covered with section-extract of heart after pretreatment with absolute acetone at 25°C . Moderate staining is seen. $100\times$
- Fig. 8** Kidney. Activity in the section covered with menadione after pretreatment with absolute acetone at -15°C . Strong staining is seen in the tubular epithelial cells. $100\times$