this solution of methosulfate was injected, no reversal of the pressor effect of epinephrine was detected. It can be presumed then that the unpurified dibenzyl-β-chloroethyl methylammonium methosulfate previously reported to be active (*4*) in reality contained an appreciable amount of tertiary amine.

The effect of quaternization on the reactivity of the β-chloroethyl compounds listed in Table 1 was determined by measuring the increase in halide ion when the compounds were dissolved in 70% aqueous alcohol containing sodium bicarbonate. No appreciable increase in halide ion concentration was noted over a 24-hr period, whereas, under the same conditions, the organic chloride of Dibenamine is 50% converted into chloride ion in 40 min. We feel that this difference between the tertiary amines and the quaternary salts adequately explains the inactivity of the latter and provides further support for the theory that the adrenergic blocking action of Dibenamine is dependent upon ethylenimonium ion formation in the body. Additional details of this work will be reported elsewhere.

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Differentiation of Minimus Type *C. diphtheriae* by Slow Fermentation of Dextrose

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A distinguishing character of the *minimus* type of *C. diphtheriae* is failure to ferment dextrose promptly or at all, *when first isolated.* After repeated transfer upon artificial media, *minimus* type organisms will manifest normal fermenting ability and will attack dextrose as readily as other strains.

Several workers to whom we have sent cultures of *minimus* type *C. diphtheriae* report that, in their hands, these organisms fermented dextrose as readily as the *mitis* type. As a result of correspondence regarding technical details, we now believe it worth while to give explicit information regarding the method requisite to demonstrate this difference.

The medium used is Difco heart-infusion broth with a pH of 7.8 after autoclaving. Brom-cresol-purple is used as indicator. To each 3.0 ml of this medium, in "Wassermann tubes," is added, aseptically, 0.3 ml of a 10% sterile aqueous solution of chemically pure dextrose. This may be sterilized either by filtration or by autoclaving at 10 lb for 10 min.

[1] From Laboratory Services, Chamblee, Ga.

The organisms to be tested are grown in the same heart-infusion broth as above, without the dextrose or indicator, for 48–72 hr. The dextrose medium is inoculated with one or two drops of this culture, using a capillary pipette or a wire loop. No serum is used in any of the media. Incubation is at 37° C. A *mitis* strain will ferment the dextrose in 24–48 hr, whereas a true *minimus* strain, upon primary isolation, will not ferment before 8–10 days, if at all.

The Histochemical Demonstration of Succinic Dehydrogenase[1, 2, 3]

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Succinic dehydrogenase plays a vital role in respiratory processes of most living cells and forms a link in the chain of reactions concerned with the oxidation of lipids, carbohydrates, and proteins (*1*). In view of the relative importance of this enzyme in physiological processes, it was considered worth while to develop a method for the histochemical demonstration of succinic dehydrogenase in tissue sections.

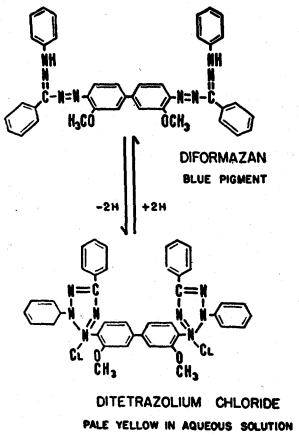


FIG. 1.

The preparation and use of a ditetrazolium chloride[5] (BT, Fig. 1) in the demonstration of specific dehydrogenase activity in extracts of tissue homogenates have been described previously (*2*). In the presence of

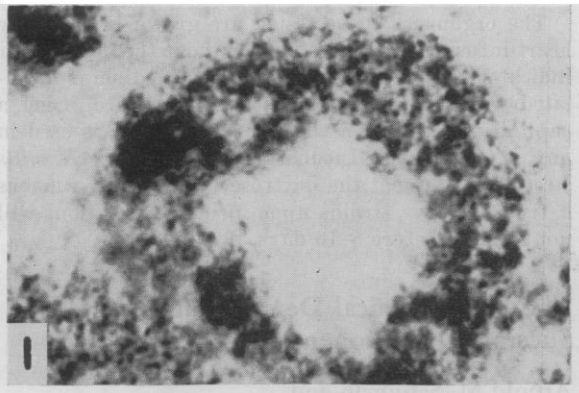
[1] This investigation was supported by a research grant from the National Cancer Institute, National Institutes of Health, USPHS, in part by a grant from the Massachusetts Division of the American Cancer Society, and in part by an institutional grant to Harvard University from the American Cancer Society.

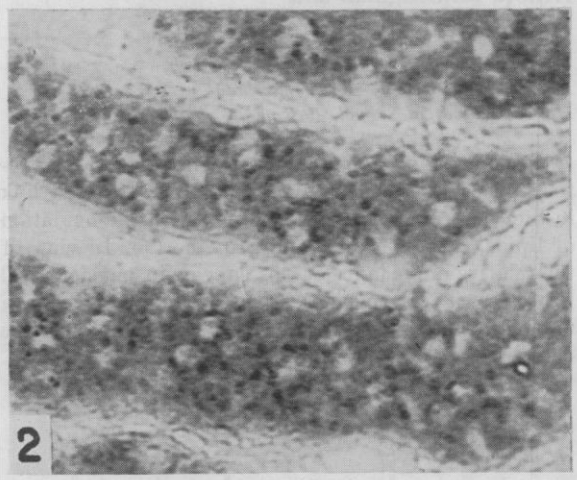
[2] Acknowledgments are due Irving Siegel, Myron Milden, and Selma Rutenburg for technical assistance.

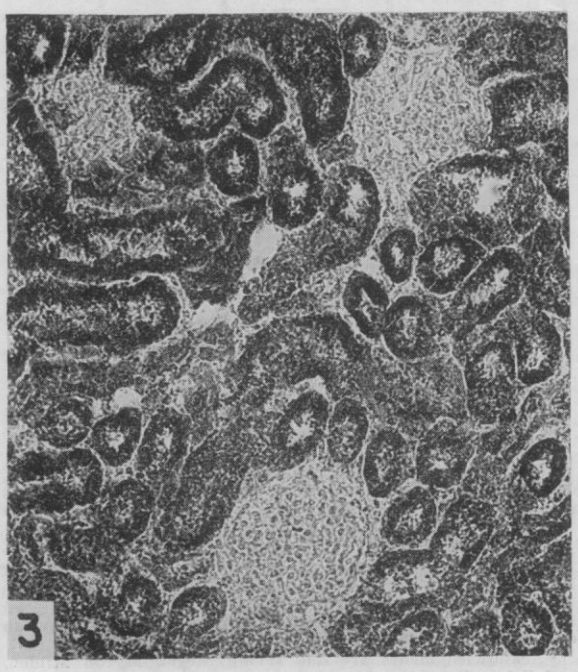
[3] Photomicrographs by Leo Goodman.

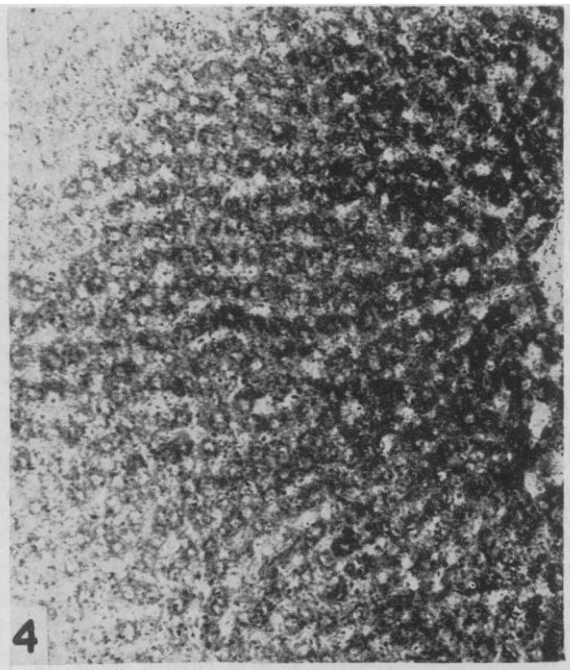
[4] Research fellow in surgery.

[5] The reagents for this method may be obtained from Dajac Laboratories, Monomer-Polymer, Inc., 3430 W. Henderson St., Chicago 18, Ill.









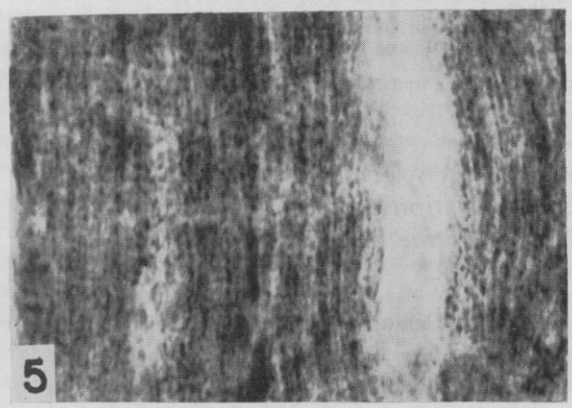


FIG. 2. 1—Rat kidney, cross section of tubule, 30 μ-fresh frozen section. Succinic dehydrogenase demonstrated by granular deposit of pigment. Photographed through a red filter. (×900.) 2—Mouse kidney, longitudinal section of tubules, 10 μ-section. Succinic dehydrogenase activity demonstrated in cytoplasm and most marked in perinuclear deposits. (×800.) 3—Rat kidney, cortex, 20 μ-section. Succinic dehydrogenase activity most marked in tubular epithelium, absent in glomeruli. Photographed through a green filter. (×200.) 4—Rat liver, 20 μ-section. Succinic dehydrogenase activity in cytoplasm and absent from nuclei. Hepatic cells in the periportal region (right) show greater activity than those in pericentral region (upper and lower left). Photographed through a red filter. (×200.) 5—Rat heart, 20 μ-section. Succinic dehydrogenase activity demonstrated by granular and homogeneous deposits of pigment in myocardial cells. (×800.)

appropriate substrates, BT served as a hydrogen acceptor and was reduced to a blue, water-insoluble pigment (diformazan). Oxidation of the diformazan to BT did not occur in air but only with strong oxidizing agents (*2*). It was pointed out earlier (*2*) that BT possessed properties favorable for the development of methods for demonstrating histochemically a variety of dehydrogenases as well as cysteine desulfurase. This communication deals with a description of the method of localization of succinic dehydrogenase

activity in fresh frozen sections with the aid of BT and sodium succinate.

Fresh frozen sections[6] (20 μ) were incubated for 2 hr at 37° C in an aqueous solution prepared with 1 part water and 1 part each of the following solutions: BT (1.0 mg/ml), sodium succinate (0.2 *M*), and phosphate buffer (0.1 *M*), pH 7.6. The sections were washed in saline (0.85%), hardened in 10% formalin for 30 min (optional), and mounted with glycerogel.

Succinic dehydrogenase was demonstrated by the granular deposition of a blue pigment intracellularly (Fig. 2 [1]). The supernatant fluid did not change in color. Complete reduction of BT to a diformazan yielded a dark-blue pigment and corresponded to sites of highest enzymatic activity. In areas where enzymatic activity was low and in the presence of abundant BT, partial reduction of BT to a monoformazan occurred, with resultant reddish-purple color (around central areas of the liver). The reduction potential in these areas may have been lower than in areas stained blue (around portal areas of the liver (Fig. 2 [4]). In the absence of succinate under these conditions, BT was not reduced, although reducing groups such as sulfhydryl, enol, α-aldol, and α-ketol were able to reduce BT in 2 hr at higher pH (9–12). The dye first appeared in tissue sections in about 5–10 min, and the color density progressively increased with the length of incubation. Prolonged incubation (4 hr) resulted in cellular disorganization in these fresh sections. The diformazan, which is blue in aqueous media, appeared purplish-red by transmitted light when dissolved in organic solvents or fat. Fat droplets in close proximity to deposits of the blue pigment appeared to be stained red in some sections. Demonstration of succinic dehydrogenase was dependent upon the thickness of the sections. Very thin sections (8 μ) failed to stain altogether, whereas thick sections (30–40 μ) uniformly showed a deep-blue stain. Thin sections (10 μ) of some tissues (e.g., mouse kidney—Fig. 2 [2]), which had a high order of enzymatic activity, were stained lightly. Sections 10–15 μ in thickness stained irregularly, and many of these sections (40–60%) were not stained at all. Of sections 20 μ thick, about 20% did not stain. Succinic dehydrogenase is inseparable from cell structures (*3*), and cellular integrity, which is disturbed in cut cells, may be required for a significant degree of enzymatic activity in these critically small amounts of tissue as compared to the quantity of tissue used in making extracts of homogenates (*2*). The thin sections, therefore, may have had too high a proportion of damaged cells from which the enzyme diffused into the incubating medium. When blocks of tissue or tissue slices (*4*) are incubated in BT, staining occurs even in the absence of added succinate to a depth of 0.5 mm. Under these circumstances, other dehydrogenases acting on endogenous substrates are responsible for the reaction (*2*).

[6] About 15 freshly cut sections were placed directly into 20 ml of solution.

TABLE 1

SUCCINIC DEHYDROGENASE DISTRIBUTION IN FROZEN SECTIONS OF RAT AND MOUSE TISSUES

Tissue	Succinic dehydrogenase activity*		
	Aerobic		Anaerobic
	Rat	Mouse	Mouse
Heart	+++	+++	++++
Kidney	+++	+++	++++
Liver	++	+	++
Brain	+	+	++
Skeletal muscle	+	0†	++
Stomach	+	±	++
Small intestine	0	0	+
Colon	0	0	0
Pancreas	0†	0	±
Spleen	0†	0†	±
Lung	0	0	0†
Uterus	0	0	±
Ovary	0	0	0
Testis	±	0†	+
Adrenal	0	0	0
Thyroid	0	0	0

* The enzymatic activity was estimated by inspection of sections from 3 to 50 animals of each tissue, studied under uniform conditions. Absence of color is indicated by 0, a trace of color by ±, and degrees of color intensity by + to ++++.

† A rare section showed a trace of color.

No reaction occurred at 0° C. At room temperature enzymatic activity was slow and irregular. The best results were obtained at 37° C. At 50° C enzymatic activity was almost completely destroyed. Greater pigment formation occurred in the more alkaline solutions within the pH range 6.5–7.8 and under anaerobic conditions. The optimum pH was 7.4–7.6. When oxygen was removed from the incubating solution by prior boiling, and when subsequently oxygen was excluded by incubation of the sections in full closed vials, the staining was more rapid and intense, and a higher proportion of thin sections was stained.

Tissue could be stored for 4 hr at 4° C without significant loss in enzymatic activity. Prior fixation of tissues (4 hr) inhibited enzymatic activity as follows: neutral 10% formalin inhibited 100%, cold acetone inhibited 40%, cold absolute ethanol inhibited 50%, cold 70% alcohol inhibited 80%, cold methanol inhibited 70%. The percentage of inactivation was determined colorimetrically by comparison of extracts of homogenates of the fixed tissue with extracts of fresh tissue homogenates (*2*). Roughly similar results were obtained histochemically with sections of tissue exposed to the various fixatives. There was no inhibition of enzymatic activity in tissue sections with sodium fluoride (1.05 mg/ml) or sodium iodocetate (0.15 mg/ml). Sodium malonate (3.7 mg/ml) caused almost complete inhibition. Sodium cyanide (0.125 mg/ml) caused about 50% inhibition aerobically and no inhibition anaerobically. With methylene blue, cyanide did not inhibit succinic dehydrogenase anaerobically (*5*).

The tissues of 6 mammals (mouse, rat, dog, guinea pig, hamster, and rabbit) were examined histochemi-

cally for succinic dehydrogenase. These results will be described in detail elsewhere. Thick sections of heart, kidney, liver, and brain stained consistently, although with variable intensity among the species studied. This is in accord with the findings of others using *in vitro* techniques (*6–8*). The tissues with the greatest succinic dehydrogenase activity (aerobic) are shown in Fig. 2 (1–5). Many tissues showed no evidence of succinic dehydrogenase activity under these conditions aerobically. Some of these same tissues exhibited activity under anaerobic conditions (Table 1). Sections of tissues heavily contaminated with bacteria, such as small intestine and colon, failed to reduce BT. It is therefore unlikely that enzymatic activity associated with 2 hr of bacterial growth was responsible for the results obtained.

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The Validity of Histochemical Phosphatase Methods on the Intracellular Level

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We have recently found that nuclei isolated from rat liver cells contain only a small fraction of the total alkaline phosphatase activity (*1*). This low activity is in sharp contrast to the intense staining of nuclei in sectioned material prepared by the method of Gomori (*2*) and Takamatsu (*3*). Since we could find no evidence of enzyme extraction from the nuclei during our isolation procedure (*4*), we have investigated the possibility that calcium phosphate and/or enzyme was preferentially adsorbed by the nuclei in the course of the histochemical procedure. The results have led us to conclude that the Gomori-Takamatsu technique cannot be relied on to indicate intracellular enzyme sites.

Alkaline Phosphatase Activity by Gomori Method

The procedure used is that of Gomori (*5*). Thin slices of tissue are fixed in ice-cold acetone. Sections, cut at 7 μ, are incubated in the substrate medium containg $1.7 \times 10^{-2} M$ Na glycerophosphate (52% α), $2.3 \times 10^{-2} M$ sodium diethyl barbiturate, $3.1 \times 10^{-3} M$ $MgSO_4$, and $8.4 \times 10^{-3} M$ $CaCl_2$, adjusted to pH 9.4. Incubation is at 37.5°. The sites of calcium phosphate deposition are visualized by treatment first with cobalt nitrate and then with ammonium polysulfide. The same incubation medium is used for "squashes" of *Drosophila* salivary glands prepared in salt solution according to Krugelis (*6*) and fixed in 95% alcohol for 3–48 hr.

Rat liver. Typical results following long incubation (17 hr) are shown in Figs. 1 and 3. Intense stain is seen in the endothelium and lumen of the blood capillaries surrounding the bile ducts. The low-power view shows that the nuclei, cytoplasm, and bile canaliculi stain more darkly in the hepatic cells of the periportal areas than in those surrounding the central veins. Under high power, the dark staining of nucleoli, chromatin, and nuclear membrane is evident.

After short incubation periods (30 min) the periductal capillaries and certain of the sinusoidal cells stain intensely (Fig. 6). The only nuclei which stain are those of these sinusoidal cells and of the bile duct cells adjacent to the darkly stained capillaries. The hepatic cell nuclei are no darker than those of control slides incubated in the Gomori medium from which substrate is omitted.

Guinea pig kidney. The kidneys used are surrounded by a connective tissue capsule formed in response to wrapping with cellophane (*7*). After incubation of the slides in the glycerophosphate medium for 30 min, the brush borders in the proximal convoluted tubules stain intensely black. The nuclei of these tubules are quite dark; those of the distal tubules (lacking brush borders) are only lightly stained. After incubation for 17 hr (Fig. 7) there is a widespread darkening of the cells of the proximal convoluted tubules, and their nuclei are intensely stained. The nuclei of the distal tubules are much lighter. In the perirenal connective tissue capsule, a gradient in staining of fibroblast nuclei is apparent, with the stain fairly intense in the layer adjacent to the dark kidney cortex and absent in the layers furthest removed from the kidney tissue (*7*). Fig. 7 illustrates what may be found occasionally: a fairly dark staining of the nuclei of the distal tubules and perirenal capsule which lie near the dark proximal tubules and a much lighter staining of the nuclei further removed from the proximal tubules.

Drosophila salivary glands. As demonstrated by Krugelis (*6*) and Danielli and Catcheside (*8*), the Feulgen-positive chromosomal bands stain intensely (Fig. 9). The cytoplasm of intact cells stains, sometimes quite deeply (*cf.* Fig. 1 in Krugelis [*6*]). An apparent spread of stain from some darkly staining material present in the "squashes" to the chromosomes of a salivary nucleus is sometimes evident; the bands are visible in the darkened ends of the chromosomes but not at the other ends.

Calcium Phosphate Adsorption

Identical staining may be obtained in the nucleoli and chromatin of rat liver and guinea pig kidney and

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