decreased below that obtained at the lower levels of supplementation. In contrast to the results obtained with the cottonseed oil or margarine fat supplements, butter fat at levels of 2% or 10% of the diet did not prolong survival over that on the fat-free ration; nor did it decrease survival time when fed at higher levels in the diet.

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Received November 21, 1960. P.S.E.B.M., 1961, v106.

Histochemical Localization of L-Gulonolactone Oxidase Activity in Tissues of Several Species.* (26319)

RICHARD B. COHEN (Introduced by E. B. Taft)

Department of Pathology, Harvard Medical School, and James Homer Wright Pathology Laboratories, Massachusetts General Hospital, Boston

Oxidation of L-gulonolactone by a specific oxidase is the final step in the pathway of synthesis of L-ascorbic acid from glucose(1). Coenzymes I and II do not participate in the reaction. The transfer of electrons is probably mediated by a flavoprotein the exact nature of which has not been defined. L-gulonolactone oxidase is confined to the liver of mammals, and to the kidneys of amphibia and reptiles(1). According to some investigators it is present only in microsomes(2) while others claim to find some activity in mitochondria(3). It is the absence of L-gulonolactone oxidase and consequent inability to synthesize Vit. C which results in the susceptibility of humans, monkeys, guinea pigs and a few other species to scurvy. In this paper we describe a histochemical method which demonstrates the distribution of the enzyme in tissue slices.

Materials and methods. Thirty adult white rats (250 g) and 10 adult guinea pigs (350 g) on standard laboratory diets were killed by a blow on the head and the tissues immediately removed, blocks 5 mm by 1 cm made, and frozen on dry ice. In addition kidney and liver tissue were obtained from 6 frogs (*Rana pipiens*) and treated similarly. Within 30 minutes of freezing the blocks were cut in a cryostat (−18°) at 20 to 34 μ thickness. Sections were put on coverslips and allowed to rinse for 5-10 minutes in cold (4°C) 0.03 M KCl solution. Then they were removed to an incubating mixture which consisted of 15 mg L-gulonolactone, 2.5 mg of the paranitrophenyl substituted ditetrazolium salt (Nitro-BT) in 4.75 cc of 0.1 M phosphate buffer pH 7.4. The solution was brought up to 5 cc by addition of 0.25 cc of acetone in which was dissolved 0.5 mg of menadione (Vit. K). The incubating mixture was warmed to 37°C before introduction of the sections. The tissues were then incubated at 37°C for 20 to 40 minutes. At termination of the incubation, the medium was drained off and replaced by 10% neutral formalin in which the sections were allowed to fix for 1 hour at room temperature. Sections were then placed on slides using glycerogel, and examined under the light microscope.

Control sections were incubated in solutions which individually excluded substrate and other components of the incubating mixture.

In some experiments sodium pyrophosphate or ethylene diamine tetra-acetic acid (EDTA) at varying concentrations were included in the incubating mixture.

Results. Rat liver was the only mammalian organ studied in which L-gulonolac-

* The research was supported by grant of Nat. Cancer Inst., N. I. H., Bethesda, Md.

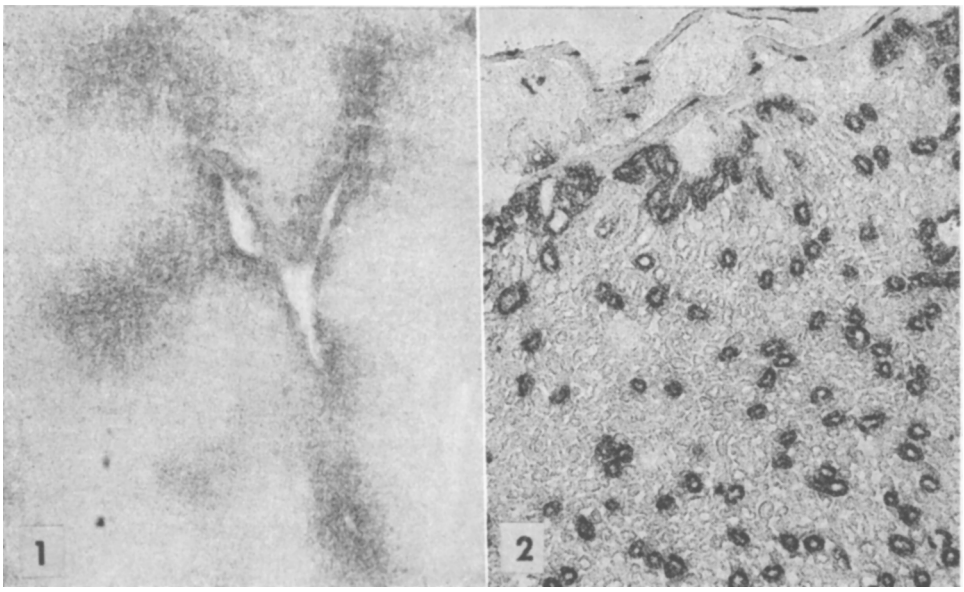

FIG. 1. A section of rat liver showing L-gulonolactone oxidase activity distributed predominately in cytoplasm of epithelial cells around the central vein. Bile ducts, connective tissue and blood vessels are all negative. ×40.

FIG. 2. A section of frog kidney showing selective distribution of L-gulonolactone oxidase activity in cytoplasm of epithelial cells of mesonephric tubules. ×50.

tone oxidase activity was demonstrated. This enzyme was confined largely to the cytoplasm of the centrilobular cells (Fig. 1). Bile ducts, blood vessels and connective tissue were negative as well as kidney, spleen, gastrointestinal tract, lung, ovary, adrenal, uterus and all nervous tissue.

As might be expected all guinea pig organs including liver and kidney were negative.

In the frog only the kidney showed L-gulonolactone oxidase activity. The distribution was confined to the cytoplasm of cells of the more distal portion of the mesonephric tubule, and enzyme activity was completely absent from a considerable portion of the tubule (Fig. 2).

The controls in which the incubating mixture lacked L-gulonolactone were uniformly negative, thus ruling out reduction of the tetrazolium salt *via* the oxidation of endogenous substrates or other endogenous reductants. Menadione definitely increased enzyme activity. EDTA or sodium pyrophosphate enhanced enzyme activity slightly in absence of menadione, but did not increase activity over and above the increase provided by menadione.

Discussion. The mechanisms involved in the histochemical demonstration of oxidative enzyme activity by use of tetrazolium salts as indicators have been discussed elsewhere(4). In the system described here the sequence of events includes oxidation of L-gulonolactone with transfer of electrons through one or more intermediates and ultimate reduction of the Nitro-BT to form a blue granular insoluble substantive diformazan at site of enzyme activity.

It has been demonstrated that the lactone of L-gulonic acid and not the acid itself is the required substrate for the oxidase. L-gulonic acid may be converted alternatively *via* another metabolic pathway to L-xyulose(1). An aldolactonase present in liver and other tissues(5) might conceivably have interfered with the oxidase reaction in our system by splitting the lactone to the acid which would then not have reacted. This, however, can be excluded as a serious limitation because of the apparent excess of substrate. In addition EDTA at concentrations of .001 M which inhibits aldolactonase activity(5) did not appreciably augment the L-gulonolactone oxidase activity as demonstrated by this histochemical technic.

The mechanism of action of menadione in

enhancing enzyme activity is not clear. It has been recently shown that it has a similar enhancing effect on the activity of several other oxidases in the liver(6).

The absence of L-gulonolactone oxidase activity in the liver of guinea pig and amphibians, and its presence in frog kidney as determined by other technics(1) offer an excellent biological control for the histochemical method by the use of which we found an identical general distribution and which provided the advantage of histological localization of activity.

Summary. A histochemical method was described which localized the activity of L-gulonolactone oxidase, the final oxidative step in synthesis of ascorbic acid from glucose. The enzyme was found to be active in the centrilobular cells of the rat liver and in selected portions of the mesonephric tubules of the frog kidney. It was absent from guinea pig kidney and liver, and from frog liver.

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Received November 21, 1960. P.S.E.B.M., 1961, v106.

Latent Viral Infection of Cells in Tissue Culture. IX. Abortive Infection with Psittacosis Virus.* (26320)

JOHN P. BADER AND HERBERT R. MORGAN
Louis A. Wehle Virus Research Lab., Dept. of Bacteriology, University of Rochester School of Medicine and Dentistry, Rochester, N. Y.

A latent infection of L cells with psittacosis virus has been described which is induced by nutritional depletion of cells with a balanced salt solution (BSS) for 2 days prior to infection(1). Following entry into the cell the virus cannot be detected by infectivity tests in chick embryos, but addition of a synthetic medium containing amino acids and vitamins results in reappearance of infectious virus(2). The specific amino acid requirements for conversion of the latent infection to an active one have been delineated(2) and deletion of a single essential amino acid from the medium was shown to be sufficient to render L cells incapable of supporting psittacosis virus propagation and to establish a latent infection.

Since L cells survive longer on a medium deficient in a single amino acid than on BSS alone, studies were initiated to determine if the duration of the latent infection could be extended to permit a more detailed study of its nature.

Materials and methods. Psittacosis (6BC strain) virus stocks were prepared as previously described(3) and diluted in the appropriate amino acid-deficient culture medium for inoculation of the cell cultures. The single dilution method of Golub(4) was used for virus assay in which 0.25 ml of a 10^{-1} dilution of culture fluid was inoculated into the yolk sac of a dozen 7-day-old chick embryos. Virus titers were expressed as the $\log_{10}LD_{50}$ per ml. Cultures of L cells were grown in T-15 flasks in Parker's medium #199 supplemented with 10% horse serum. When a uniform sheet of cells had grown over the glass surface, the growth medium was replaced with a synthetic medium which was deficient in either phenylalanine or isoleucine. Cultures containing approximately 2×10^6 cells were exposed to approximately 10^5 LD_{50} of psittacosis virus after 2 or 3 days' mainte-

* Supported by research grants from Nat. Cancer Inst., U.S.P.H.S., and N. B. Delavan Fund for Virus Research.