been mentioned no very marked changes in the brain of the dog have been previously observed following chronic barbiturism. Lack of histologic change, however, does not necessarily preclude the possibility of a derangement of physiological processes, and the ataxia of the barbital dogs is a good indication of this fact. Failure to observe further neurological signs or withdrawal symptoms may very well be due to the dosages used which, as can be seen in Table I, were not large when compared to the daily intake in human barbiturate addiction. Table I also shows that large doses of Presidon were needed to produce sleep as compared to small doses of the barbiturates, yet the sleep after the former was much more uniform without cumulative symptoms or after effects such as ataxia or disorientation. The presence of the amyloid-like bodies could not in this experiment be related to any neurological signs for they were present in the dogs receiving Presidon as well. However, their appearance only in those animals treated for one year does suggest a relationship to prolonged use of these drugs.

Whether or not there are species differences in reactions to these drugs is of course not determined. However, in comparing our results with the literature, it would seem such differences, at least with regard to the nervous system, would be a matter of degree. With regard to the blood, on the other hand, species differences may be a factor in the development of dyscrasias following the use of drugs. The dog appears to have a much more active hemapoietic system than man and conceivably could be more resistant to drug effects.

Summary. (1) Twelve dogs receiving barbital, phenobarbital or Presidon daily for six months to one year were observed for neurological disturbances and alterations in the histology of the viscera, blood and brain. (2) Dogs receiving barbital for one year developed a mild ataxia. These dogs and one receiving phenobarbital for one year had small splenic tumors at autopsy. All other post mortem findings were negative with respect to histologic changes in the viscera and blood. Small structures similar to amyloid bodies seen in man were found in the brain stem and cortex of all dogs treated one year, but not in dogs treated six months. These structures could not be correlated with neurological changes in behavior, nor is their significance known.

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Mechanism of Enzymatic Reduction of Triphenyl Tetrazolium Chloride.* (19018)

ERNEST KUN.†

From the Department of Medicine, University of Chicago.

Although considerable attention is being paid to tetrazolium salts in various fields of biology(1,2) the mechanism of their reduction by mammalian tissues requires further elucidation. It can be assumed that in an enzymatic reducing system, composed of an enzyme protein, pyridine-nucleotide and flavo-protein, the immediate electron donor of triphenyl tetrazolium chloride (TTC) is the dye-reducing flavo-protein of Straub(3). This mechanism was suggested by Bieling, *et al.*(4) and Kun(1) and clearly demonstrated by Brodie and Gots(5) with yeast triose phos-

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† Present address: Tulane University of La., Departments of Medicine and Biochemistry.

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phate dehydrogenase. The present paper deals with the mechanism of the intracellular enzymatic reduction of TTC by rat tissues.

Materials and methods. Enzymes. The dialyzed cytoplasmic centrifuged fraction of tissue homogenates (in 0.15 *M* KCl, containing ⅕ volume of 0.1 *M* phosphate buffer of pH 7.4) served as a source of soluble dehydrogenases. Amino acid dehydrogenases(6-8) were extracted from rat kidney cortex, glycolytic enzymes (enzymes of type A) from rat liver or muscle. Mitochondria were prepared from rat liver homogenates according to Schneider and Hogeboom(9) and extracts of mitochondria by the isobutanol procedure, as described previously(10), except that the extraction was carried out at 37°C for 45 minutes (Enzyme B). The procedure of Racker was employed for the preparation of yeast alcohol dehydrogenase(11). *The potassium salt of fructose-1-6-diphosphate* (HDP) was prepared from a commercial sample of the calcium salt (Schwarz). The inhibitory effect of eventually occurring heavy metal contaminants could be abolished by the use of histidine hydrochloride, in a final concentration of 10^{-2} *M*(12). The TTC was a purified sample, generously given by Dr. George Gomori. *Enzyme activity* was determined spectrophotometrically as described previously (13). The enzyme activity is expressed in terms of optical density x 10^3 of the acetone filtrate as measured at 484 mμ on the Beckman spectrophotometer in 1 cm Corex cells (3 ml content). Distilled water served as a blank.

Results. Typical flavoproteins, like amino-acid dehydrogenases(6-8), present in kidney extracts, directly reduce TTC. The rate of reduction in the presence of l-alanine or d-aspartic acids is 6-12 times greater under anaerobic conditions, than in air. Unlike aminoacid dehydrogenases, triose phosphate dehydrogenase, present in liver or muscle extracts (Enzyme A) does not react with TTC either aerobically or in N_2 atmosphere. Mitochondria prepared by the sucrose procedure(9) do not reduce TTC with HDP as substrate. Rapid reduction of TTC occurs, however, when mitochondria are added to the glycolytic enzyme systems (Enzyme A). With an excess of glycolytic enzymes present, the rate of TTC reduction is a function of the amount of mitochondrion particles. It is apparent that the reduction of TTC by triose phosphate dehydrogenase (present in Enzyme A) occurs by way of mitochondria. Cytochrome C reductase activity is also known to be bound to mitochondrion particles(14). It was therefore of interest to inquire whether or not the TTC and cytochrome C reducing systems have a catalyst in common. It was previously reported that the cytochrome reductase system of mitochondria could be extracted by treatment with isobutanol at 0°C (10). This cytochrome C reductase system did not reduce TTC. On the other hand, when the extraction of mitochondria was carried out at 37°C, TTC reductase was obtained, and cytochrome C reductase activity lost. The catalytic properties of the mitochondrion extracts prepared at 0°C and at 37°C can be compared, by taking the activity of intact mitochondria as 100 (Table I).

TABLE I.

	TTC reduction	Cytochrome C reduction
Mitochondria	+ (100)	+ (100)
Cold (0°C) isobutanol ext.	—	+(40–70)
Warm (37°C) "	+(40–60)	—

The role of mitochondria and that of the warm isobutanol extract of mitochondria (Enzyme B) in TTC reduction is summarized in Table II, which represents typical experi-

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TABLE II. The Glycolytic Reduction of TTC.

Enzyme	TTC reduction in terms of optical density × 10^3	Incubation time
Rat muscle ext. (Enzyme A type) (1)	0	30′ aerobic
Rat liver mitochondria (2)	46	30′ ″
Muscle ext. (1) + mitochondria (2)	500	15′ ″
Rat liver ext. (Enzyme A type) (3)	20	15′ anaerobic
Mitochondrion ext. (37°C) (Enzyme B) (4)	0	15′ ″
Rat liver ext. (3) + mitochondrion ext. (4)	720	15′ ″

Enzyme preparations: (1) = muscle ext. equivalent to 0.5 g fresh rat muscle (Enzyme A). (2) = rat liver mitochondria equivalent to 0.140 g fresh liver. (3) = rat liver ext. equivalent to 0.2 g fresh liver (Enzyme A). (4) = mitochondrion ext. equivalent to 0.3 g fresh liver (Enzyme B).

The glycolytic system is composed of: 60 μM HDP, 40 μM nicotinamide, 40 μM KF, 0.6 μM DPN and 3 μM TTC in a final volume of 3 ml made up with 0.1 M phosphate buffer. Temp. 37°C.

mental results. Essentially the same results were obtained when the triose phosphate dehydrogenase system of tissue extracts was replaced by crystalline yeast alcohol dehydrogenase(11). The reduction of TTC by the alcohol dehydrogenase system was measured anaerobically at pH 7.4, with semicarbazide hydrochloride (10^{-2} M) as carbonyl reagent. Here again, TTC reduction occurred only when mitochondria, or the extract of mitochondria (Enzyme B) were present in addition to the yeast enzyme, Table II.

Discussion. In animal tissues TTC can be reduced by a variety of dehydrogenases. Soluble flavoproteins, like aminoacid dehydrogenases, readily transfer electrons to TTC under anaerobic conditions. The retardation of TTC reduction by aminoacid dehydrogenases in air is probably due to a competitive mechanism between O_2 and TTC, particularly noticeable in homogenous systems (solutions). It was shown that an obligatory component of the glycolytic TTC-reducing system is present in mitochondria. This component appears to be a flavoprotein part of the cytochrome C reducing system(10). The fact that intact mitochondria mediate electrons to both cytochrome C and TTC, while the two reductase activities can be separated by extraction, requires further elucidation.

Summary. The conditions of the reduction of TTC by cytoplasmic fractions of rat tissue homogenates were determined. Rat kidney extracts, upon addition of aminoacids, reduce TTC anaerobically. Glycolytic enzymes of liver and muscle reduce TTC only when supplemented by mitochondria. An extract of mitochondria can be obtained which will supplement the glycolytic TTC reducing system.

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Retardation of Growth of Walker Rat Carcinoma 256 by Administration of Diethyl Riboflavin.* (19019)

H. VASKEN APOSHIAN AND JOHN P. LAMBOOY. (Introduced by E. S. Nasset.)

From the Department of Physiology and Vital Economics, University of Rochester, School of Medicine and Dentistry, Rochester, N. Y.

In recent years the effects of riboflavin deficiency on the growth of neoplasms have received increasing attention. Morris and Robertson(1) found that the growth rate of spontaneous mammary carcinomas in C3H mice was decreased by severe riboflavin de-

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