adrenal cortex other steroids which may be more effectively anti-scorbutic than cortisone. More probably, some of the activities of vitamin C may be independent of the adrenal cortex.

The similarities between scurvy and rheumatic conditions are in many respects very striking. Consequently, the DCA-treated scorbutic guinea pig or the chronically scorbutic animal would seem to be excellent test subjects for the screening of compounds with anti-rheumatic activity. The advantage of such a test animal is the more evident as it now becomes apparent that adrenal glycogenic and anti-arthritic properties can be dissociated.

Summary. Cortisone inhibits many of the manifestations of scurvy in the guinea pig while desoxycorticosterone aggravates the condition. These activities of the hormones probably depend on their action on mesenchymal tissues. The scorbutic guinea pig, untreated or treated with DCA, may be a valuable test subject for the assay of anti-arthritic compounds.

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Effect of Tolerance on Inhibition of Respiration of Brain Homogenates by Thiopental. (17908)

THEODORE F. HUBBARD* AND LEO R. GOLDBAUM
(Introduced by Paul K. Smith)

From the Department of Basic Science and Department of Chemistry and Physics, Army Medical Department Research and Graduate School, Washington, D.C.

In a previous paper it was shown that mice become tolerant to the daily intraperitoneal injection of thiopental sodium, 45 mg per kg. This tolerance became maximal in 5 to 6 days and was indicated by shortened sleeping times and the ability of the experimental animals to awaken at higher brain levels of the barbiturate than controls(1). Since it has been proposed that barbiturates induce sedation by interference with the carbohydrate metabolism of the brain(2,3,4) it seemed important to study the inhibitory effects of barbiturates on the *in vitro* carbohydrate metabolism on the brains of control and tolerant mice.

Experimental. Male mice, weighing 23 to 25 g were made tolerant by the daily intraperitoneal injection of thiopental sodium, 45 mg per kg, for from 7 to 10 days; controls, kept under identical conditions, were given daily injections of saline. Records of the sleeping times of the experimental animals were kept, and the animals were used when their sleeping times were between 50 and 60% of their original sleeping times. For each experiment the whole brains from 5 control and 5 tolerant mice were rapidly removed and homogenized in cold distilled water in a glass homogenizer. No more than 15 minutes were allowed to elapse from the time the animals were killed until the homogenates were placed in the reaction vessels. The method of Reiner (5) was used in the study of glucose, pyruvate, and lactate oxidation. Since it seemed possible that the addition of the cofactors in these fortified homogenates might mask some difference in the metabolism of the control and tolerant mouse brains, isotonic homoge-

* Current address: Mayo Clinic, Rochester, Minn.

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TABLE I.
Effect of Thiopental on the Respiration of Brain Homogenates from Control and Tolerant Mice.

Thiopental sodium conc. (mg per cc)		0	83.3	166.6	333.3	666.6
Glucose oxidation	Control	6.22*	6.15	4.63	3.11	1.86
	Tolerant	6.14	6.21	4.69	3.06	1.73
Pyruvate oxidation	Control	7.80	7.68	4.68	3.12	1.90
	Tolerant	7.67	7.77	4.53	3.16	1.79
Lactate oxidation	Control	6.88	6.80	4.51	3.03	1.76
	Tolerant	7.02	6.88	4.38	2.97	1.88
Glucose anaerobic glycolysis	Control	5.55†	5.60	5.49	5.53	5.34
	Tolerant	5.39	5.55	5.62	5.60	5.42
Succinate oxidation	Control	3.85	3.78	3.80	3.70	3.89
	Tolerant	3.74	3.86	3.69	3.89	3.66
Cytochrome oxidase	Control	3.35	3.43	3.46	3.38	3.40
	Tolerant	3.39	3.30	3.33	3.30	3.36

* Values are given as ml O_2 per mg of wet wt of tissue per hr.

† Values for anaerobic glycolysis are in ml CO_2 per mg of wet wt of tissue per hr.

Temp. 36.3°C; gas phase, air; 0.1 cc of 30% KOH in center well; conc. of thiopental sodium as shown above. Contents of flasks; (a) Glucose oxidation—glucose .028 M; hexose diphosphate Ba, .005 M; ATP, .0007 M; DPN, .001 M; nicotinamide, .04 M; cytochrome C, .00006 M; K_2HPO_4-KH_2PO_4 buffer, pH 7.4, .04 M; .5 cc 1:4 mouse brain homogenate; final volume 3 cc. (b) Pyruvate and lactate oxidation—pyruvate .028 M, or lactate .028 M in place of glucose, otherwise as in (a). (c) Anaerobic glycolysis of glucose—Temp. 36.3°C, gas phase 95% N + 5% CO_2; conc. of sol.: glucose, .028 M; hexose diphosphate, Ba, .0025 M; ATP, .0007 M; DPN, .0005 M; nicotinamide, .04 M; K_2HPO_4-KH_2PO_4 buffer, pH 7.4; .01 M; $NaHCO_3$, .048 M; 5 cc 1:4 mouse brain homogenate. (d) Succinate oxidation—sodium succinate, .05 M; cytochrome C, .00017 M; $CaCl_2$, .0004 M; $AlCl_3$, .0004 M; Na_2HPO_4-HCl buffer, pH 7.4; .03 M; 1 cc 1:4 mouse brain homogenate; final vol. 3 cc. (e) Cytochrome oxidase-sodium ascorbate, .0114 M, in place of succinate, otherwise the same as (d).

nate preparations were also used to study glucose oxidation. These were prepared by homogenizing the brain in Ringer's phosphate buffer solution and using 0.028 M glucose as substrate. The method of Utter, Wood, and Reiner(6) was used for the study of anaerobic glycolysis; and that of Schneider and Potter(7) for the study of succinate dehydrogenase and cytochrome oxidase systems. The rate of oxygen uptake or carbon dioxide evolution was followed in a Warburg manometer at 36.3°C. After a 15-minute period for gassing and temperature equilibration the stopcocks were closed and readings were taken at 10-minute intervals for one hour. All determinations were made in duplicate, and each experiment was repeated several times on different groups of control and experimental animals. The values in Table I represent the average of 3 or more separate experiments.

In a second study, 10 control and 10 tolerant mice were given thiopental sodium, 85 mg per kg intraperitoneally (approximately an $L.D._{50}$ dose), the mice in each group sacrificed after 5 minutes, and the brains prepared for *in vitro* respiration studies as above.

Results. Table I summarizes the results of the experiments. Thiopental in concentrations of from 167 mg per cc to 667 mg per cc produced from 30 to 80% inhibition of the oxidation of glucose, pyruvate, and lactate. However, the control and tolerant mouse brains showed no difference in the rate of oxidation of these substrates, nor in the degree of inhibition caused by thiopental on the oxidation of these substrates. There was no difference in the anaerobic glycolysis of glucose, the succinic dehydrogenase or the cytochrome oxidase activity. None of

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7. Schneider, W. C., and Potter, V. R., *J. Biol. Chem.*, 1943, v149, 217.

these systems in either control or tolerant brain preparations were inhibited by the thiopental concentration used. The results obtained with the oxidation of glucose using the isotonic homogenate preparations were essentially the same as those shown in Table I. In studies in which an L.D.$_{50}$ dose of thiopental was injected into the animals, rather than added to the flasks, no inhibition of glucose oxidation was observed in control or tolerant brain preparations.

Discussion. If the sole mechanism of action of barbiturates in producing hypnosis is a depression of the carbohydrate metabolism of the brain, then it should follow that tolerant animals, requiring a higher brain concentration of the barbiturate to maintain hypnosis, should show less inhibitory effects of the barbiturate on brain respiration than normals. This was not found to be true. If such differences had been present, it should have been possible to demonstrate them by the technics used, since the brains of control animals are almost twice as sensitive to the hypnotic effects of thiopental as those of tolerant animals. Further, the minimum *in vitro* concentration of thiopental required to inhibit glucose oxidation by brain homogenates is equivalent to an *in vivo* brain concentration which we have found to be uniformly lethal(8). Our observation that the injection of an L.D.$_{50}$ dose of the barbiturate in mice failed to affect the *in vitro* carbohydrate metabolism of the brain substantiates similar observations of others(2). However, since slices were used by these workers, failure to observe inhibition was attributed to diffusion and dilution of the drug in the surrounding medium. The use of the homogenate technic circumvents this difficulty since the enzyme and drug are equally diluted. Several reports in the literature comparing hypnotic doses of barbiturates *in vivo* and drug concentrations required for inhibition of brain respiration *in vitro*(9,10) are based on the contention (as reported by Tatum *et al.* for amytal in rabbits)(11) that the brain contains four times as much of the barbiturate as other tissues. Accurate spectrophotometric methods for the determination of barbiturates in tissue have shown that most of the barbiturates do not reach higher concentrations in the brain than in most of the other parenchymatous tissues(12). It appears unlikely that the concentrations of barbiturate equivalent to those found in the brain during hypnosis would inhibit *in vitro* glucose metabolism by brain. It should also be noted that pyribenzamine(13) and some of the local anesthetics(2,14) similarly depress the *in vitro* metabolism of glucose, but are convulsant when administered in equivalent concentrations *in vivo*. These observations do not rule out the possibility of selective activity of barbiturates on some local area of the brain so sensitive that the effects could not be demonstrated on whole brain preparations, though *in vitro* studies have shown no difference in the sensitivity of various areas of the brain to pentobarbital(15).

Summary. 1. No difference was demonstrable in the rate of *in vitro* oxidation of glucose, lactate, pyruvate and in the succinic dehydrogenase and cytochrome oxidase systems by brain homogenates from control and thiopental-tolerant mice.

2. Thiopental was found to inhibit the *in vitro* oxidation of glucose, lactate, and pyruvate but there was no difference in the degree of inhibition between control and tolerant mouse brain homogenates. The anaerobic glycolysis of glucose and the activity of succinic dehydrogenase and cytochrome oxidase were unaffected by the thiopental.

3. No inhibition of the *in vitro* oxidation of glucose was observed in the brain homog-

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14. Watts, D. T., *J. Pharm. and Exp. Therap.*, 1949, v96, 325.

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enates from normal or tolerant mice after receiving an L.D.$_{50}$ dose of thiopental.

4. It is concluded that the observed *in vitro* depression of brain respiration by barbiturates cannot be the sole mechanism responsible for barbiturate hypnosis.

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A Method for Quantitative Estimation of Small Amounts of D-Tubocurarine Chloride in Plasma.* (17909)

GERTRUDE P. QUINN† AND SIEGFRIED WOISLAWSKI
(Introduced by H. C. Hodge)
From the Division of Pharmacology and Toxicology, School of Medicine and Dentistry, University of Rochester, Rochester, N. Y.

To date no chemical method for the determination of D-tubocurarine in plasma has been reported. A method by which two micrograms of D-tubocurarine chloride per ml of plasma can be estimated has been developed and is presented along with data on the plasma levels associated with paralysis in the rabbit. The principle of the procedure is that employed by Brodie and Udenfriend (1) for many organic bases, that is, extraction of the alkaloid into an organic solvent and subsequent complex formation with methyl orange. D-tubocurarine cannot be extracted from alkaline solution into organic solvents. It may, however, be extracted from a potassium iodide solution at pH 10. Other salts were unsatisfactory because they caused the formation of emulsions between the plasma and solvent, gave high blanks, or failed to result in complete recovery.

Reagents. Ethylene dichloride. Because this solvent usually contains impurities which react with methyl orange, it was purified in one of 2 ways: (a) by treating with activated charcoal, Norite, or (b) by washing once with one-fifth volume of N hydrochloric acid and then several times with water. The purified solvent was stored in a glass-stoppered bottle. *Methyl Orange.* One gram of the sodium salt of methyl orange was dissolved in 500 ml of water and the resulting solution was shaken several times with equal volumes of purified ethylene dichloride to remove soluble organic impurities. The free acid of methyl orange was then precipitated by the addition of 2 ml of concentrated sulfuric acid, filtered on a Buchner funnel, washed several times with water and dried *in vacuo*. *Acid-alcohol.* Two ml of concentrated sulfuric acid were dissolved in 100 ml of absolute ethanol. *Potassium-iodide-glycine buffer.* A buffer solution of about pH 10 was prepared by mixing 6.0 ml of 0.1 M glycine solution containing sodium chloride (7.505 g glycine and 5.85 g sodium chloride per liter) with 4 ml of 0.1 N sodium hydroxide. To 10 ml of this buffer, 12.8 g of reagent grade potassium iodide were added and the resulting solution was kept in a brown bottle to protect it from the light. The buffer is very stable but the buffer-potassium-iodide solution is not and the mixture should be prepared daily. *Citric acid buffer.* A citric acid buffer solution, pH 5.0 was prepared by mixing 10.3 ml of 0.2 M dibasic sodium phosphate with 9.7 ml of 0.1 M citric acid.

Procedure. Three ml of plasma containing 5 to 60 micrograms of D-tubocurarine chloride are pipetted into a 60 ml glass-stoppered bottle containing 10 ml of ethylene dichloride. One-half ml of the potassium iodide-glycine buffer solution is added and the mixture is shaken for 5 minutes, decanted into a test tube, centrifuged and the aqueous

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† Present address: Research Service, Third (New York University) Medical Division, Goldwater Memorial Hospital, New York City.

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