Inst., 1947, v8, 103.
6. Hanks, J. H., and Wallace, R. E., PROC. SOC. EXP. BIOL. AND MED., 1949, v71, 196.
7. Golub, O. J., *J. Immunol.*, 1948, v59, 71.
8. Commoner, B., and Mercer, F., *Nature*, 1951, v168, 113.

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Metabolic Demethylation of 3,5,5-Trimethyl-2,4-Oxazolidinedione (Trimethadione, Tridione).* (19907)

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Methyl groups are now known to be removed metabolically from nitrogen in a wide variety of chemical types of N-methyl compounds. As some of these compounds find extensive use as drugs and as the metabolic products are in some cases themselves pharmacologically active, the reaction of demethylation has important practical implications in therapeutics. From analogy with the closely related derivatives of barbituric acid and hydantoin, it could be expected that the antiepileptic drug, trimethadione, would undergo demethylation in the mammalian body. The product of the reaction would be 5,5-dimethyl-2,4-oxazolidinedione (henceforth to be designated "DMO"). The present report concerns the isolation of this substance from the urine of dogs receiving trimethadione.

Materials. Samples of DMO were supplied through the kindness of Dr. Roger W. Stoughton of Mallinckrodt Chemical Works and Dr. M. A. Spielman of Abbott Laboratories. The compound melts at 76-78°C. The ionic form absorbs strongly in the ultraviolet, the absorption peak lying at wavelengths below 210 mμ. The molar extinction coefficient is 1.1×10^4 at 215 mμ and diminishes sharply with increasing wavelength. In this spectral region the absorption of the undissociated molecule in solution in negligible. At 37°C and at an ionic strength of 0.1 the pK′ of DMO as calculated from spectrophotometric data is 6.2.†

Trimethadione was isolated from commercial capsules and recrystallized from water and from petroleum ether. It melted at 44-46°C. At wavelengths longer than 215 mμ the absorption of trimethadione in aqueous solution is negligible in comparison with that of the ionic form of DMO. A test was made for the possibility of contamination with significant amounts of DMO by examination of the ultraviolet absorption of a buffer of pH 8 shaken with an ether solution of trimethadione. In this way it was demonstrated that the sample of trimethadione could not contain as much as 0.2 per cent DMO.

Administration of drugs. Both dogs of Table I were females. The drugs were given in aqueous solution intraperitoneally, the total dose being divided into two portions, which were administered several hours apart. The solution of trimethadione contained 5 g and that of DMO 10 g per 100 ml. With each dog the trimethadione experiment preceded the DMO experiment by at least 3 weeks.

Isolation of DMO from urine. Urine was collected from the dogs for seven days after the drug injection. The urine was acidified to pH about 2 and extracted with ethyl ether. A batch of urine would be divided into four portions, which would be successively shaken with two portions of ether, each five times the volume of each portion of urine. The ether extract was distilled to a volume of 100 ml and was then extracted with four 25 ml portions of a saturated aqueous solution of $NaHCO_3$. The bicarbonate extract was acidified with hydrochloric acid to pH about 2 and shaken with two 50 ml portions of benzene, which were discarded. The aqueous phase was then

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† The pK′ as determined by potentiometric titration has been reported by Erlenmeyer *et al.*(1) as 6.1.

TABLE I. Recovery of 5,5-dimethyl-2,4-oxazolidinedione (DMO) from the Urine of 2 Dogs Receiving Trimethadione and DMO.

		Dose		Material recovered from urine			
Wt, kg	Drug administered	mM/kg	Total wt, g	Wt, g	m.p., °C	Mixed with authentic DMO, m.p., °C	Yield, %
10.9	T*	7	10.9	1.20	75–78	76–78	12.2†
	DMO	7	9.8	1.27	74–78	74–78	13
11.9	T	7	11.9	.27	74–77	74–78	2.5
	DMO	7	10.7	.90	75–78	75–78	8.4

* T = Trimethadione.

† In another exp. this animal was given the same dose of trimethadione; the recovered yield of demethylated product was only 5.4%.

extracted with four successive 75 ml portions of ether. The ether extract was evaporated in a dish at room temperature. The residue was dissolved in water, and by means of systematic "countercurrent" distribution between water and ethylene chloride it was possible to eliminate considerable amounts of colored materials having partition coefficients higher and lower than that of DMO. The distribution was carried out in such a way that nearly all of the DMO accumulated in the final aqueous fractions. Those aqueous fractions containing the greater part of the desired material were treated with sufficient Norit to remove any remaining color and were then extracted with ether. After evaporation of the ether, the residue was crystallized from benzene. Some fractions were freed of tenacious impurities by sublimation at reduced pressure. No search was made for unchanged trimethadione or for other possible metabolic products.

Results. As is shown in Table I, the demethylated product was isolated from the urine of dogs that had received trimethadione and was identified by melting point and mixed melting point. Further evidence of identity was furnished by a study of the ultraviolet absorption spectrum of the material from the first dog after trimethadione administration. Within the limits of experimental error the spectrum of the urinary product coincided with that of authentic DMO at different values of pH. The pK′ of the urinary product as calculated from spectrophotometric data did not differ significantly from the value found for synthetic DMO.

On later occasions the same dogs were given the same molar dose of DMO itself. The yields recovered from the urine are shown in Table I.

Discussion. Isolation of relatively pure DMO from urine by the present procedure undoubtedly entails large losses. The yields shown in Table I are accordingly of very limited quantitative significance. Nevertheless, comparison of the isolated yields when the N-methyl compound and the non-methylated compound are administered would indicate that the extent of demethylation of trimethadione is considerable. The present experiments do not suffice to permit an evaluation of the role played by DMO in the effects seen after administration of trimethadione. There is reason, however, to believe that DMO is retained in the body for long periods of time. The neurological effects of the doses of DMO given to the dogs in these experiments were evident for many hours and the drug continued to appear in the urine for several days. The very transient effect reported(2) for intravenous doses of DMO in mice is probably attributable to physical redistribution of the drug rather than to chemical destruction, as intraperitoneal doses of 1.5 g per kg will maintain deep anesthesia in mice for about 2 hours. It would appear not unlikely that the rate of elimination of DMO may be slow relative to the rate of its production from trimethadione. In this circumstance the chronic oral administration of trimethadione would lead to more extensive accumulation of the product of demethylation than of the original drug, as has been demonstrated in the analogous case of mephobarbital (N-methyl phenobarbital) (3). Knowledge of the actual extent of accumulation of DMO resulting from trimethadi-

one administration will be possible only when an analytical method is available for the determination of DMO in blood. In the treatment of epilepsy with trimethadione it may well be that DMO plays a significant part in the therapeutic effect.

Summary. 3,5,5-Trimethyl-2,4-oxazolidinedione (trimethadione, Tridione) is demethylated by the dog to yield 5,5-dimethyl-2,4-oxazolidinedione. This metabolic product has been isolated from urine and identified.

1. Erlenmeyer, H., Kleiber, A., and Loebenstein, A., *Helv. Chim. Acta,* 1938, v21, 1010.

2. Stoughton, R. W., and Baxter, J. H., Jr., *J. Pharm. and Exp. Therap.,* 1941, v73, 45.

3. Butler, T. C., *J. Pharm. and Exp. Therap.,* 1952, v106, 235.

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Application of Histochemical Technic for Cholinesterase to Paraffin Sections.* (19908)

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(Introduced by J. S. Nicholas.)

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The application of histochemical technics for enzyme localization in tissue sections offers one a promising approach to the problem of biochemical differentiation. In embryological work, however, it is often necessary to examine the material not on random and isolated sections, but on serial sections from which spatial relationships of the various structural elements can be reconstructed. For the preparation of serial sections, the paraffin method is at present the only convenient and reliable technic. Unfortunately not all enzymes survive the harsh treatment usually involved. The enzyme cholinesterase, in which we have been particularly interested in relation to neural differentiation, is one which is relatively susceptible to inactivation. Gomori's method(1) for cholinesterase localization was devised for use with paraffin sections, but the extremely low rate of reaction and the non-specificity of the substrates (long-chain fatty acid esters of choline) restrict its usefulness. In the histochemical technic for cholinesterase recently developed by Koelle and Friedenwald(2) and Koelle(3), the substrate used was acetylthiocholine which was found to be hydrolyzed by the enzyme at a rate comparable to that of the natural substrate, acetylcholine. Furthermore, by the use of appropriate concentrations of DFP, this technic is capable of differentiating the 2 types of cholinesterase. The method was used only for fresh teased preparations or frozen sections. To increase the usefulness of this technic, particularly for embryological work, we attempted to explore the possibility of preparing the specimen for paraffin sectioning with a minimum loss of enzyme activity prior to histochemical treatment.

The enzyme content of normal frog brain has shown a high degree of bilateral symmetry. It is, therefore, ideally suited for the present work of testing the effect of various histological treatments on the enzyme activity of the tissue. The brain was divided into left and right halves, one serving as control. Since the frog central nervous system has been shown to contain exclusively "specific" cholinesterase, all enzyme activities were measured manometrically using acetylcholine as substrate.

Effect of fixatives and lyophilization on cholinesterase activity. It is obvious that for histochemical purposes, the fixatives usually employed in histological procedures should be avoided. Although acetone has been most commonly used as a fixative in histochemical applications, prolonged treatment of the fresh tissue with cold acetone was found to inactivate most of the cholinesterase. Ethyl al-

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