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.

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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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TABLE I. Effect of Fixing and Dehydration on Cholinesterase Activity of Fresh Tissue.

Treatment	Cholinesterase activity		% inactivation
	Untreated	Treated	
Ethyl alcohol (½ hr 0°C)	336	0	100
Acetone (½ hr 0°C)	392	107	73
Freeze-drying	385	387	0

cohol completely destroyed the enzyme (Table I). As an alternative to the use of fixative and dehydrating reagents, fresh tissue was lyophilized in preparation for paraffin embedding. For practical purposes we found Gersh's technic for freeze-drying too cumbersome, and the time required for complete dehydration excessively long. A greatly simplified version of the technic was therefore adopted. The tissue was frozen in acetone previously cooled in a stainless-steel vessel immersed in a freezing mixture of acetone and solid CO_2. The frozen tissue was transferred with a pre-chilled spatula into the lyophilizing vessel kept at low temperature. Once high vacuum was established, no attempt was made to regulate the temperature of the drying tissue. No thawing occurred throughout the process of lyophilizing under these conditions. The average time for complete dehydration of tissue the size of the adult frog brain was less than one hour. Smaller fragments of tissue were dehydrated in considerably less time. The cholinesterase activities were determined on the homogenates of the treated and untreated brain halves. For comparison, the activities are expressed in arbitrary units per half-brain. It is clear that the enzyme activity of fresh tissue is completely unaffected by the present procedure of lyophilizing.

Effect of embedding, sectioning and hydrating. During embedding, temperature inactivation of the enzyme was minimized by the use of low melting-point paraffin (Bioloid, 43-45°C) and brief period of infiltration. The lyophilized tissue was introduced directly into a vessel of melted paraffin kept at a constant temperature of 45°C with immediate evacuation of the vessel continued for one minute. Upon breaking the vacuum the tissue sank to the bottom and was then embedded in the usual manner. Thus the total time in which the tissue was exposed to 45°C was less than 3 minutes. Subsequent sectioning showed that infiltration was complete. Sections were usually cut at 20-40 μ. For thinner sections, it would be necessary to employ some means of chilling the paraffin block as well as the knife. Since the tissue had not been fixed, the use of water as a flotant had to be avoided in mounting the sections. The sections were affixed directly upon albuminized slides and flattened by pressing with a camel's hair brush while warming the slide by the heat of the hand. Since no drying was necessary, the slides could be deparaffinized immediately in xylol which does not affect the enzyme activity. Experiments were then carried out to ascertain the most suitable means of hydrating the deparaffinized sections. The effect of the commonly used hydrating agents on enzyme activity was determined in the following manner. The mounted sections of an entire half-brain were deparaffinized in xylol, and treated with various hydrating agents. After passing into aqueous medium, they were care-

TABLE II. Effect of Hydrating Agents on Cholinesterase Activity of Tissue Sections.

Material	Hydrating agent	Cholinesterase activity		% inactivation
		Untreated	Treated	
Frog brain	None (sections dried in air)	356	348	2
" "	Dioxane	375	0	100
" "	Alcohol	324	0	100
" "	Acetone	432	333	22
Rat ventricle*	"	158	116	26
" " †	"	51	35	31

* Acetylcholine-hydrolyzing activity—μl CO_2/mg N/hr.
† Benzoylcholine- " " " .

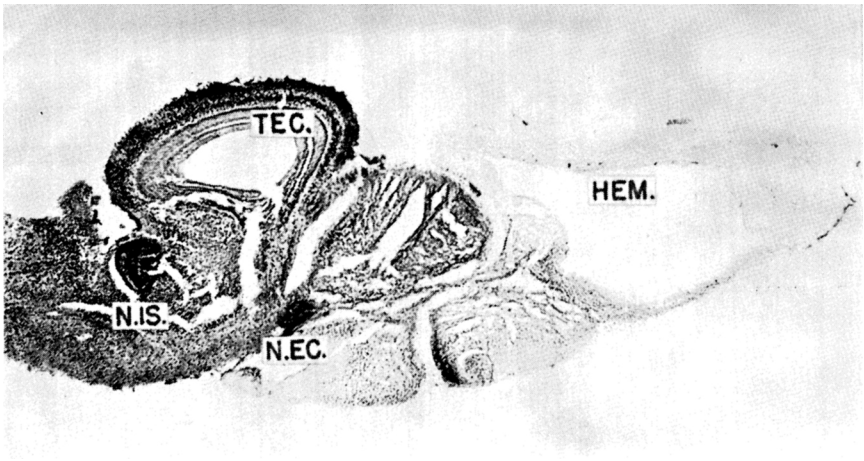

FIG. 1. Histochemical demonstration of cholinesterase in frog brain. Sagittal section. CH., optic chiasma; HEM., hemisphere; N.EC., nucleus ectomamillaris; N.IS., nucleus isthmus; TEC., tectum.

fully removed by scraping from the slide, avoiding any loss of material, and then homogenized in Ringer-bicarbonate solution. The activity of the treated half was measured and compared with that of the untreated lyophilized half of the brain. Cholinesterase activities are expressed in arbitrary units per half-brain. Average values from a series of experiments are given below.

As shown above, the use of alcohol or dioxane quickly and completely inactivates the enzyme. On the other hand, relatively little damage to the enzyme was caused by similarly brief treatment with acetone, although prolonged exposure to acetone resulted in extensive inactivation of the enzyme (Table I). Similar results were obtained with rat ventricles which contain predominantly non-specific cholinesterase. The degrees of inactivation of the 2 cholinesterase types are comparable.

Histochemical results. The histochemical technic employed in the present investigation is that of Koelle(3). The deparaffinized sections, after 2 brief rinsings in cold acetone and in Ringer's solution, were transferred directly to the incubation solution. Half an hour of incubation at 37° C was found to be adequate for frog brain. After the development of color in H_2S, the sections were fixed in 10% formalin saturated with CuS. They were then dehydrated through CuS-saturated alcohols, cleared in xylol and mounted in balsam. A typical picture of cholinesterase distribution in adult fog brain is shown in Fig. 1. The almost complete absence of staining of the hemisphere will be noted, as well as the concentration of the enzyme in the tectal layer of the optic lobe and some of the neurological centers, notably the nucleus isthmus. Detailed description of the enzyme distribution in frog brain will be given elsewhere. Brief incubation of the sections in 10^{-5} M DFP prior to the addition of substrate completely inhibited the histochemical staining. Cytologically, the enzyme activity seems to be concentrated in or on the nuclei. This may, however, be a diffusion artefact as suggested by Koelle(4) in his later modification of his technic, in which it was shown that if intracellular diffusion of the enzyme were controlled by means of high salt concentration at a lower pH, the site of enzyme activity was localized in the cytoplasm. Unfortunately, under these conditions, the enzyme activity was found to be greatly depressed, especially that of specific cholinesterase which was reduced by as much as 80%. When this modified histochemical procedure was applied to paraffin embedded and sectioned material, the staining was found to be feeble and poorly differentiated even after prolonged incubation due presumably to an excessively high overall inactivation of the enzyme. The use of unphysiologically high concentrations of salt in

the reaction mixture as a means of "fixing" the enzyme raises the question of the *normal* state of diffusibility of the enzyme within the cellular structure. In any case, the exact cytological localization of cholinesterase in nerve cells cannot at present be considered unequivocally established. For the demonstration of the site of enzyme activity on a histological level, the present adaptation of the original histochemical procedure in which intracellular diffusion of the enzyme is not "controlled," seems entirely adequate and satisfactory.

It should be admitted that the very much simplified and abbreviated procedure of tissue lyophilization used here does result in some minor imperfections in the tissue sections, especially when the tissue is excessively bulky, *e.g.*, whole brain of an adult frog. Improved quality of the sections could, however, be obtained by the employment of more elaborate apparatus for critical temperature regulation in the process of lyophilization.

Summary. The histochemical technic of Koelle for cholinesterase demonstration in fresh tissue was adapted to paraffin sections. The effect of certain histological treatments on enzyme activity was determined. A procedure is described for the preparation of the specimen for paraffin sectioning prior to histochemical treatment with maximum retention of enzyme activity.

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Production of Tolerance in the Rat to Octamethyl Pyrophosphoramide (OMPA).* (19909)

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Octamethyl pyrophosphoramide, first synthesized by Schrader(1) in 1947, has become of increasing interest as a systemic insecticide† as indicated by the reports from England of Ripper, *et al.*(2) and Heath, *et al.*(3). Following an extensive pharmacological study by DuBois, Doull and Coon(4), in which it was demonstrated that this compound is a potent *in vivo* anticholinesterase agent with a selective action on the cholinesterase of peripheral tissues and that its toxicity could be effectively reduced with atropine (increasing the LD_{50} for mice and dogs fourfold), it aroused considerable interest as a possible therapeutic agent in the treatment of myasthenia gravis. For this purpose it has been used with success by at least two different groups of investigators in the United States, namely, Rider, *et al.*(5) and Gregory, Futch and Stone(6). In addition to this use it is also now available as an insecticide (Monsanto Chemical Co.). Since the therapeutic use of OMPA in myasthenia gravis involves chronic administration, and since its manufacture and use as an insecticide involve probable repeated exposures it is highly desirable to obtain as much information as possible about the toxicity of this compound. Of special concern is knowledge of the cumulative effect of daily administration of the drug, tolerance to the drug, and possible long range pathologic effects. DuBois, Doull

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† A systemic insecticide is defined as one that is absorbed by the plant and translocated in the sap so that parts of the plant other than those treated become toxic to (sucking) insects.