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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Received October 16, 1952. P.S.E.B.M., 1952, v81.

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

* This investigation was supported in part by a grant from Eli Lilly & Co. and in part by a grant from the Division of Research Grants and Fellowships of the National Institutes of Health, U. S. Public Health Service.

† 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.

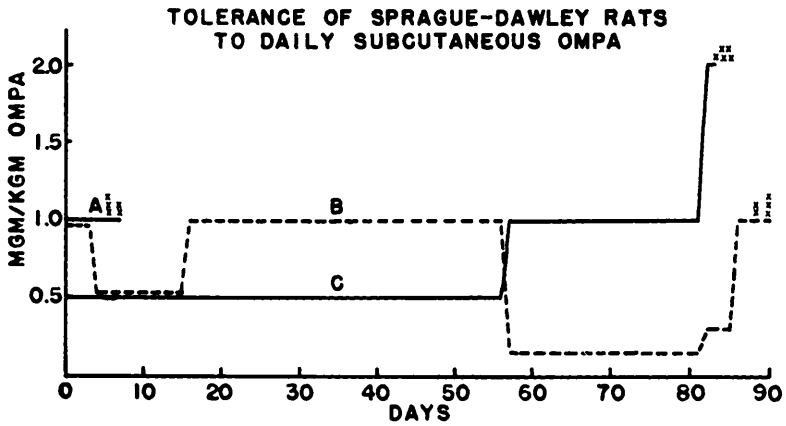

FIG. 1. Graphic representation of the OMPA dosage schedules in different groups of rats. The dose, as indicated on the ordinate, was given daily for the number of days indicated on the abscissa. Each × represents the death of 1 rat, and the number of ×'s on each line represents the total number of rats started in the corresponding group.

and Coon(4) have previously shown that the LD_{50} for parenterally administered OMPA in rats was about 8 mg‡ when given in one dose. They also showed that the daily administration of 2 mg caused death in 50% of the animals after the third dose, and the remainder of the animals died after the fourth dose. The failure of at least some of the animals to survive more than a total of one single-dose LD_{50} given at the rate of 2 mg/day was unexpected. Daily doses of 1 and 1.5 mg had a similar effect. Thus with these daily doses an efficient cumulation was observed and death of the animals occurred when the total quantity of OMPA administered was close to the LD_{50} value obtained for single doses. In this same experiment, however, it was reported that the animals tolerated daily doses of 0.25 and 0.50 mg/day for as long as 60 days, indicating a lack of efficient cumulation at these doses or suggesting the possibility that some degree of tolerance may develop.

The following experiments were undertaken in order to determine whether any tolerance would develop in rats that were treated daily with sublethal doses of OMPA.

Materials and methods. Adult male Sprague-Dawley or Maguran rats were employed as the test animals. OMPA was administered subcutaneously as a 0.1% aqueous solution. Cholinesterase measurements were performed manometrically essentially according to the method described by DuBois and Mangun(7). Blood was obtained for the assays from the tails of the rats in 0.2 cc amounts. After the Warburg flasks were gassed for 5 minutes with 95% N_2 and 5% CO_2 and equilibrated for 15 minutes, the acetylcholine was tipped in from the side arm. Readings were taken at 5-minute intervals for 30 minutes. The activity of the whole blood cholinesterase (representing a combination of the red cell and serum cholinesterase) was expressed in microliters of CO_2 liberated in an average 5 minute period.

Results. In Fig. 1 is illustrated the OMPA dosage schedules and mortality results (each x = 1 death) of three groups of five Sprague-Dawley rats each. Group A received 1 mg daily. Three of these animals died after the sixth dose and the remaining two died after the seventh dose. Group B received 1 mg daily for three days. The dose was then decreased to 0.5 mg for 12 days. Following this it was increased to 1 mg for 41 days. During this period, in contrast to the results in group A, there were no deaths, nor were there any toxic symptoms. Next, the daily dose of these animals was reduced to 0.15 mg for 25 days, increased to 0.3 mg for 4 days and finally to 1 mg. This latter dose caused death in two of the animals after the fourth dose and in the remaining three after the fifth dose. Thus the tolerance that had previously developed in this group was lost after a pro-

‡ All doses given in this paper are expressed in terms of body weight in kilograms.

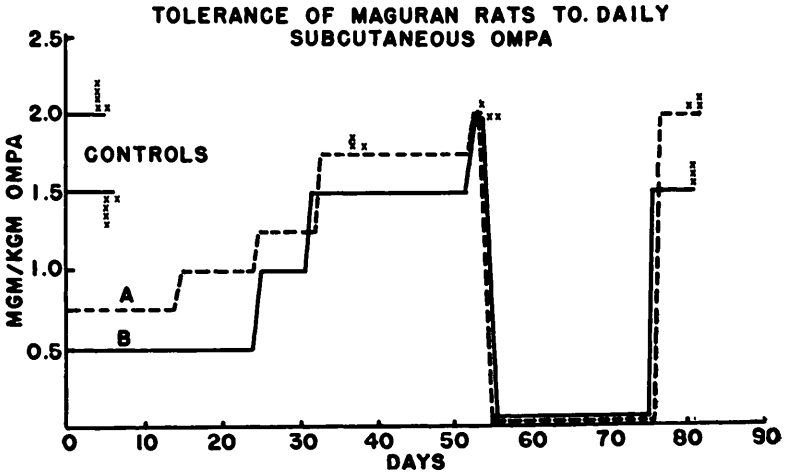

FIG. 2. Legend the same as in Fig. 1.

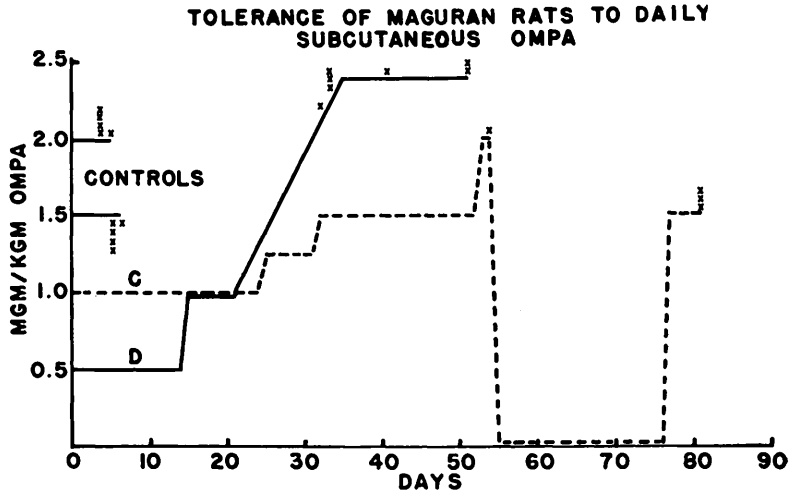

FIG. 3. Legend the same as in Fig. 1.

longed period of very low dosage followed by a sudden return to the previously tolerated dose. Group C was given 0.5 mg for 56 days. This was increased to 1 mg/day for 26 days without fatality. Two mg for 2 days, however, resulted in 100% mortality; thus Group C also demonstrated the ability to tolerate 1 mg for a long time. These experiments show that a tolerance to a 1 mg/day schedule can be induced by a daily dose of 0.5 mg given for 12 or more days.

Dosage schedules and mortality results for different groups of Maguran rats are graphed in Fig. 2 and 3. Although this strain seemed to be more resistant to the OMPA than the Sprague-Dawley, the results in general were in conformity. A daily dose of 2 mg caused 100% mortality in 5 days and a daily dose of 1.5 mg caused 100% mortality in 6 days. These groups of 5 rats each are considered to be the controls in both Fig. 2 and 3.

Group A (Fig. 2), consisting of 7 rats received 0.75 mg for 14 days, then the dose was gradually increased. Four of the rats tolerated 1.75 mg for 20 days but could not tolerate 2 mg. Group B was treated in a similar manner. Whereas they could tolerate 1.5 mg for 21 days they lost this ability after a prolonged rest period. Group C (Fig. 3) was begun at 1 mg for 24 days and the results were similar to those observed in Group B. After group D received 1 mg for 7 days the

TABLE I. Cholinesterase Inhibition by OMPA of Maguran Rat Whole Blood.
(See Fig. 2 and 3 for dosage schedules.)

Group	Control	22 days	36 days	21 days rest
A		7.1	8.8	
B		5.1	6	
	21.8*			21.7
C		4.4	6.8	
D		5.3	6	

* μl CO_2/5 min./.2 cc.
Control and 21 days rest values are from pooled blood samples from individuals of each group.

dose was increased by increments of 0.1 mg/day for 14 days. One rat died after it had received the dose of 2.2 mg and 3 died after they had received 2.3 mg. One rat tolerated 2.4 mg for 7 days and the remaining 2 rats did not die until the 17th day.

The results obtained from the Maguran rats indicate that whereas 1.5 mg of OMPA daily caused 100% mortality of the rats so treated for 6 days, it was possible, by means of slowly increasing the dose of OMPA, to build up a relative tolerance to a daily dose as high as 2.2 to 2.4 mg, although at this latter dose all rats were dead after 17 days.

Additional suggestive evidence for tolerance can be seen in Table I in which the cholinesterase inhibition by OMPA of Maguran rat whole blood is shown. It can be seen that the levels of cholinesterase activity in the various groups are about the same on the 22nd and 36th day of treatment, with a slight tendency to increase on the latter day. After 21 days without treatment the blood levels had returned to pre-treatment values.

Although all the rats before death had typical cholinergic symptoms, such as diarrhea, exophthalmos, muscular twitchings, salivation, and respiratory distress indicative of bronchial spasm, the gross and microscopic examinations of 14 of the rats shortly after death revealed no abnormalities in the liver, kidney, intestine, heart, spleen, skeletal muscles, lungs or brain.§ It was concluded that death was due to biochemical causes with no change in morphology.

§ Grateful acknowledgement is made to Dr. John B. Storer for the pathologic studies.

Discussion. The results presented here indicate that it is possible to increase the tolerance of rats to OMPA by gradually increasing the daily dose. The most striking characteristic of this tolerance is not the size of the dose of OMPA which could be tolerated, but rather the length of time that the animals could tolerate, without symptoms, a daily dose which normally killed in a few days. This tolerance was lost after a period of discontinued administration of the OMPA. Blood cholinesterase levels were in accord with the above observations since the levels did not continue to decrease after prolonged treatment and even tended to show a slight rise.

The mechanism by which this tolerance develops is unknown. It is possible that a) production of cholinesterase has been stimulated, b) detoxification mechanisms are more rapid, or c) the conversion of OMPA to an active cholinesterase inhibitor(4) by the liver is slower. Since the mechanism of this increased tolerance is unknown we have little information upon which to base the importance of these findings in connection with the daily use of OMPA in myasthenia gravis patients or in chronic exposure in those who are using this agent as an insecticide. If man also develops a tolerance, however, it would appear wise to increase a patient's dose of OMPA gradually in order to induce a tolerance which would be expected to minimize the incidence of toxic reactions. Furthermore, after the administration has been stopped for any length of time it might be hazardous to restart it at the same dose level as previously tolerated. One might also expect less toxic effects after prolonged administration of the same dose. Although myasthenia gravis is a variable disease we have seen some clinical evidence of a tolerance developing to OMPA. For example, we have seen patients who, while on the same daily dose of OMPA will no longer need atropine to counteract undesirable cholinergic symptoms after several weeks of therapy.

Summary. The cumulative effect of OMPA in rats has been confirmed. It was possible to induce a tolerance to OMPA by gradually increasing the daily dose or by treating the animals daily with a sublethal dose for a pro-

longed period. Animals prepared in this manner were able to tolerate for several weeks, without symptoms, a daily dose which would normally kill all rats in a few days. No gross or microscopic pathological changes were seen in the organs of the rats examined in this study.

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Received October 20, 1952. P.S.E.B.M., 1952, v81.

In vivo Inhibition of Liver and Brain Monoamine Oxidase by 1-Isonicotinyl-2-Isopropyl Hydrazine.* (19910)

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It has been shown that 1-isonicotinyl-2-isopropyl hydrazine (IIH) inhibits *in vitro* the monoamine oxidase of liver and brain of 6 different species(1,2). The inhibition is marked at concentrations as low as 0.2×10^{-5} molar. The question thus arose as to the possible occurrence of such a reaction *in vivo,* with the consequent production of symptoms as observed in the therapeutic application of IIH.

Studies on the cellular localization of monoamine oxidase reveal the enzyme activity to be largely present in the mitochondria(3-5). The addition *in vitro* of IIH to a mitochondrial suspension results in the disappearance of the monoamine oxidase activity. There is no restoration of activity by dialysis for 3 hours. It seemed then, that the administration of IIH to intact animals, followed by the isolation of mitochondria from tissues for the determination of monoamine oxidase activity would provide an indication as to whether the enzyme is inhibited *in vivo.*

Procedure. 1-Isonicotinyl-2-isopropyl hydrazine phosphate (IIH)† and isonicotinic acid hydrazide (INH)† dissolved in M/15 phosphate buffer pH 7.2 were injected subcutaneously into adult, male and female rats (Sprague-Dawley) and guinea pigs. Two hours later the animals were sacrificed by decapitation or by intravenous injection of air. The brain and liver were immediately removed, washed and chilled. Mitochondria were isolated by centrifugal fractionation of 1:10 tissue homogenates in 0.88 M sucrose solution according to the method of Hogeboom *et al.*(6). The characteristic morphology of the mitochondria was confirmed by phase microscopy. The determination of monoamine oxidase activity was carried out by the conventional manometric procedure for the estimation of oxygen uptake, and by microdiffusion analysis for the estimation of ammonia production. The incubation system contained, in a final volume of 2 ml, homogenate or washed mitochondria suspended in M/15 phosphate buffer pH 7.2 and 0.01 M tyramine hydrochloride (F. Hoffmann-La Roche); oxygen was employed in the gas phase. The enzyme suspensions per vessel were equivalent to 0.1 g fresh tissue for liver homogenate, 0.2 g original tissue for liver mitochondria and 0.33 g grey cortical tissue for brain mitochondria. The incubation was

* This work has been aided by a grant from the Multiple Sclerosis Foundation of Illinois.

† The compounds used were obtained from Hoffmann-La Roche, Inc., Nutley, N. J. IIH = Marsilid, INH = Rimifon, and from Bristol Laboratories, Syracuse, N. Y. (INH).