litters, or a total of 254 rats, which were first implanted with tumor tissue and later treated intratumorally with vaccine, proved to be 100% immune—subsequent implantations of viable tumor tissue failed to grow in every one of the 254 T rats treated in this manner.

In the T rats of the later generations, the rate of growth of the tumor grafts implanted previous to treatment was slower than in the rats of the earlier generations. Furthermore, they responded more favorably to intratumoral injections, in that, whereas in the earlier generations to obtain successful results it was necessary to treat small tumors 4–6 days after implantation, in the later generations the intratumoral treatments could be initiated on the seventh day with successful results. At the same time, fewer injections were required to bring about oncolysis. In some instances, oncolysis took place after only three injections.

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Histochemical Demonstration of Esterases by Production of Indigo[1]

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Esterolytic enzymes found in animal tissues are capable of splitting a wide variety of carboxylic acid esters (*1*). Among the aliesterases, specificity of action is provided by the size of the fatty acid moiety rather than the hydroxylic residue (*2, 3*). However, there is considerable overlapping in the hydrolytic properties of the carboxylic esterases. A simple ester such as β-naphthyl acetate is hydrolyzed by serum esterase, pancreatic lipase (*3*), serum cholinesterase, and acetylcholinesterase of brain and erythrocytes (*4*). A method for demonstrating esterase histochemically in acetone-fixed tissues was developed using β-naphthyl acetate as a substrate. By coupling the naphthol with a diazonium salt, an azo dye was produced which was deposited at the sites of enzymatic action (*5*). The process of fixation in acetone and embedding in paraffin removed most of the lipoids and inactivated nearly all the cholinesterase, so that the method served well to demonstrate the nonspecific esterases. However, the lipoid-soluble azo dye diffused in the lipoids of fresh-frozen sections, particularly in nervous tissue which contained acetylcholinesterase (*4, 6*).

Because of the poor solubility of indigo blue in both aqueous media and lipoids, it seemed worth while to examine the properties of indoxyl acetate as a possible substrate for esterases. At the pH optimum for esterase activity, indoxyl is rapidly oxidized to indigo blue and precipitated from solution (Fig. 1). Indigo

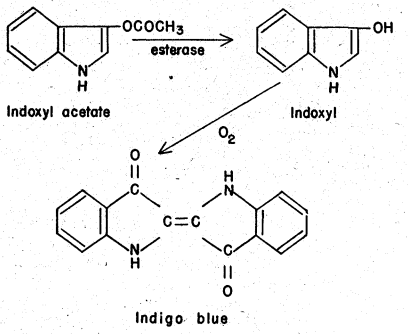

FIG. 1.

blue is a light-fast, stable, brilliant pigment. Indoxyl butyrate was also prepared and used in experiments to test the localization of acetylcholinesterase *vs* other esterases, particularly in nervous tissue.

Indoxyl acetate was prepared in the following manner: A 2-lb tin of sodium indoxyl in a mixed alkali flux, prepared as an intermediate in the industrial production of indigo, was obtained from a commercial source.[3] A portion of the flux was chipped from the solid mass and ground fine. The powder (64 g) was placed in a liter of water and ice in a 2-liter Erlenmeyer flask into which illuminating gas was constantly passed to prevent extensive oxidation. As soon as the melt dissolved, 40 ml 50% (by vol) sulfuric acid was rapidly added and stirred. This was followed by the addition of 25 ml acetic anhydride. The flask was stoppered and shaken. Illuminating gas was bubbled through the mixture vigorously for 10 min. A green-blue precipitate which had formed was collected with the aid of suction and washed with water. The filter cake was extracted six times by suspension in 300 ml boiling water, followed by rapid filtration by the suction pump. A little active charcoal was added during the last few extractions. The filtrates were combined, cooled, and 6–10 g of fine grayish needles was collected. The product was recrystallized from hot water or dilute alcohol in nearly white needles, mp, 126°–128°. The melting point has been reported (*7*) as being 126°–127°.

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[2] Acknowledgment for technical assistance is due Ralph Gofstein, Robert M. Rubin, Lorraine Lamy, and Shirley Golden.

[3] Provided through the courtesy of J. D. Nantz, Pharmaceutical Laboratories, National Aniline Division, Allied Chemical & Dye Corporation, 40 Rector St., New York. It may now be purchased from this firm.

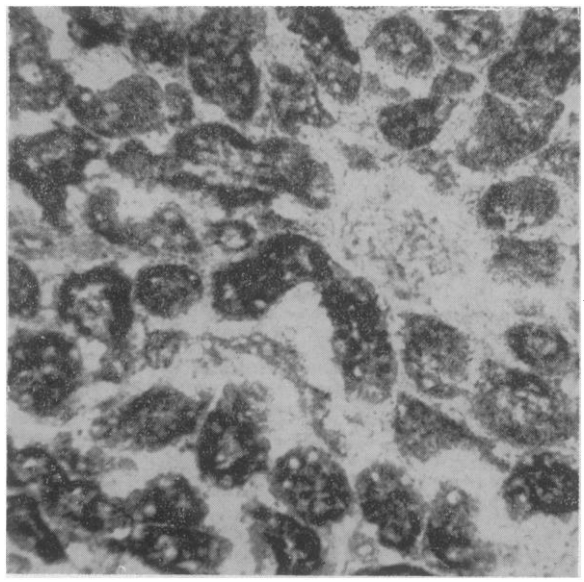

FIG. 2. Kidney cortex of a rat, 5 μ fresh, frozen-dried section. Marked esterolytic activity is indicated by granular deposit of indigo in the cytoplasm of the tubular epithelium. A glomerulus visible in the upper part of the photomicrograph shows sparse granulation. × 200.

The butyrate was prepared in a similar manner. Instead of acetic anhydride, 50 ml butyric anhydride (Eastman) was added. Crystals did not form readily because of the presence of the oily anhydride. The reaction mixture was allowed to stand at room temperature for several days until all the anhydride had decomposed and indigo-stained crystals had formed. The crystals were then collected and washed free of butyric acid. The product was crystallized by solution in methanol, treated with charcoal, and diluted, when heavy tan prisms separated, mp, 86°–87°. Infrared absorption spectroscopy revealed the presence of an ester linkage and no amide band.

Anal calcd for $C_{12}H_{13}NO_2$: C, 70.91 H, 6.45.
Found: C, 70.70 H, 6.60.

For the demonstration of esterases in tissues, fresh pieces of liver, pancreas, kidney, duodenum, fat, striated muscle, brain, and spinal cord were removed from rats killed by a blow on the head, and were frozen and stored at −30° C. Frozen sections, 5 μ thick, were prepared according to the Coons modification of the Linderstrøm-Lang technique (*8*) and dried. The slides carrying the mounted frozen-dried sections were incubated at room temperature in a fresh solution containing the following ingredients: 25 ml 2 *M* sodium chloride; 10 ml 0.1 *M* Michaelis barbital buffer, pH 8.5; 0.25 g calcium chloride in 14 ml distilled H_2O; 20 mg indoxyl acetate; and 1 ml acetone. The indoxyl acetate was dissolved in 1 ml acetone and mixed thoroughly with the 49-ml solution of salts and buffer just before use. This mixture was filtered into a Coplin jar containing the sections. After 10 min, the supernatant fluid was decanted, and the slides were washed in running tap water for 5 min. Cover slips were mounted with glycerogel. The procedure with indoxyl butyrate was the same except that the tissues were incubated for 15–20 min.

In the experiments in which esterase inhibitors were utilized, the tissue sections were incubated at room temperature with the inhibitor for 1 hr before the substrate was added.

Within 2 min the most reactive tissues, liver, kidney, and duodenum, hydrolyzed indoxyl acetate and were colored a perceptible blue. In 10 min, all the tissues studied were grossly colored, the central nervous system the least of all. The supernatant incubating medium remained clear and colorless during the 10-min period, indicating that spontaneous hydrolysis of the substrate did not occur in this time. In all the organs, the granular precipitate of indigo blue was limited to the cytoplasm; none occurred in the nuclei. No color developed in the sections if the tissues were first immersed in water at 98° C for 1 min.

The size and number of the granules varied from numerous small particles in liver and kidney (Fig. 2), few large particles in nervous tissue (Fig. 3), to a single large crystal which grew as a cluster from a single point in each fat cell (Fig. 4).

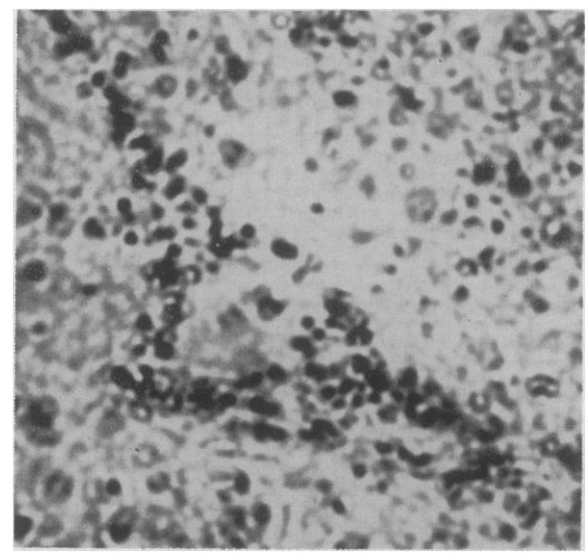

FIG. 3. Anterior horn cell from cervical region of a rat's spinal cord, 5 μ fresh, frozen-dried section. Granules of indigo are present in the perikaryon and in the cell processes. × 2250.

The cause of the granularity was not altogether clear. A slight delay in oxidation of indigo could have allowed sufficient diffusion to foci more favorable for oxidation and subsequent precipitation of indigo. A tendency to form a supersaturated colloidal solution of indigo at the sites of esterolytic activity in fresh tissue could have favored a certain degree of selective precipitation of the pigment on these or near-by foci. In tissues with few favorable foci, the granules would possibly grow to a larger size than in those with many foci. Therefore, within these limits, localization of enzymatic activity could not be assured.

The cytoplasm of liver cells stained intensely with a uniform punctate distribution of blue granules.

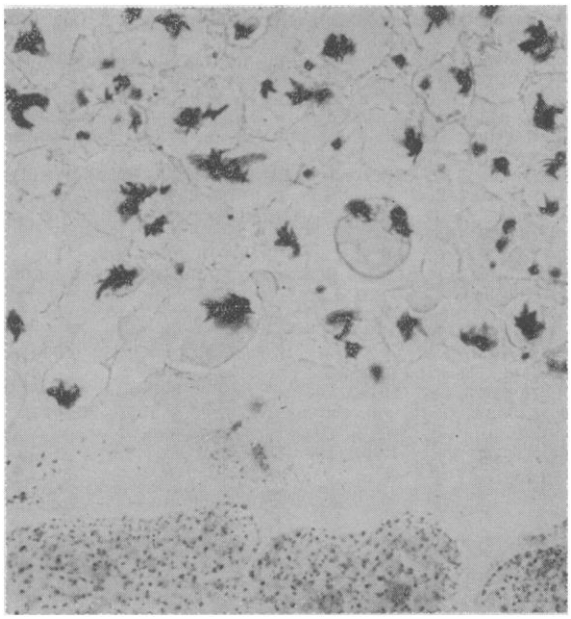

FIG. 4. Interscapular brown fat of a rat, 5 μ fresh, frozen-dried section. Note large crystalline deposits of indigo in each fat cell. Contrasting small granules at the base of the photomicrograph demonstrate the esterolytic activity in striated muscle. × 500.

There was no difference in concentration in either the portal or hepatic areas of the liver lobule.

In the pancreas, the acinar cells were darkly stained, as was the epithelium of the ducts. The islets of Langerhans were moderately positive.

All the elements of the nephron were positive. The cytoplasm of the epithelium of the proximal and distal convoluted tubules showed marked activity (Fig. 2), and the other tubular segments were moderately active. The glomeruli and all medullary elements were only weakly stained.

In the duodenum, the epithelium of the villi and the crypts of Lieberkühn reacted intensely, and Brunner's glands were moderately stained. The smooth muscle fibers and the neurones of the myenteric plexuses also displayed moderate activity.

Small punctate granules in the cytoplasm and at the surface of fibers characterized the staining of striated muscle.

The gray matter of the spinal cord and brain had a higher concentration of esterase than the white matter. In the anterior horns of the cord, the large stellate motor cells showed a moderate deposition of dark blue granules in the cytoplasm and in the axons and dendrites (Fig. 3). It was difficult to discern whether the indigo granules occurred on the cell surface as well as in the cytoplasm.

In the blood vessels in all organs studied, the smooth muscle layers were moderately positive, and the elastic tissue and endothelium were negative. Among the blood cells, intensely stained cytoplasmic granules occurred in the polymorphonuclear leucocytes.

Sodium taurocholate[4] (5×10^{-3} *M*) completely inhibited enzymatic activity in all tissues except pancreas. Enzymatic activity in this circumstance was considered to be due to lipase (*3*). Esterolytic action on β-naphthyl acetate was not as effectively inhibited by taurocholate. Eserine salicylate in concentration up to 8 μg/ml partially inhibited the degree of staining of central nervous system sections without any marked change in the distribution.

When indoxyl butyrate was used as substrate, the distribution of granular precipitate in all the tissues was similar to that of indoxyl acetate, except that the reaction was less intense despite a longer time of incubation. Spinal cord sections displayed weak esterolysis of the butyrate with and without eserine. However, the washed cell membranes of hemolyzed erythrocytes which were positively stained with the acetate were unstained with the butyrate. Since acetylcholinesterase of erythrocytes did not hydrolyze the butyrate, hydrolysis by nervous tissue was due to another esterase.

The carboxylic acid esterases are of three types: lipase, nonspecific or aliesterase, and cholinesterases. The first is an enzyme which has a predilection for esters of long chain fatty acids and is stimulated by sodium taurocholate. The second has a predilection for esters of short chain fatty acids and is inhibited by sodium taurocholate. The third has predilection for esters containing choline (*3*). Lipase occurs in large amounts only in the pancreas (*3*), whereas nonspecific esterase is widespread in its distribution in the tissues of the body (*9, 10*). Of the cholinesterases, serum cholinesterase is present in plasma, some organs, and in certain parts of the entire nervous system (*4, 11*); acetylcholinesterase is present in the central nervous system and membranes of erythrocytes (*11*). The latter enzymes may be distinguished from one another by their behavior toward acetyl β-methyl choline, benzoyl choline, butyryl choline (*11*), and carbonaphthoxy choline (*4*). Both enzymes are also able to hydrolyze certain acetic acid esters (*4*), β-naphthyl acetate, and indoxyl acetate.

The results of the present preliminary survey of esterase activity in fresh tissues of the rat indicate that indoxyl acetate is hydrolyzable by lipase, nonspecific esterase, and the cholinesterases. Lipase activity was demonstrable only in the pancreas in the presence of sodium taurocholate. Tissues known to be rich in nonspecific esterase were completely inhibited by taurocholate and not by eserine. A highly purified preparation of human serum cholinesterase[5] *in vitro* hydrolyzed both indoxyl acetate and butyrate. On the other hand, washed membranes of erythrocytes hydrolyzed only indoxyl acetate.

The staining of the central nervous system is presumably due in major part to acetylcholinesterase. The fact that eserine did not completely inhibit the reaction in sections of the brain and spinal cord, and that indoxyl butyrate was hydrolyzed to a slight extent by these sections, suggests that a small amount of

[4] Obtained from Elmer and Amend, New York.

[5] Provided through the courtesy of E. J. Cohn, University Laboratory for Physical Chemistry, Related to Medicine and Public Health, Harvard University.

another esterase is present in addition to acetylcholinesterase, in spite of a claim to the contrary (*6*). Since serum cholinesterase was not demonstrable in rat brain with the specific substrate, carbonaphthoxy choline (*4*), a small amount of the staining of the central nervous system with indoxyl acetate and butyrate must be attributed to the presence of a nonspecific esterase.

Other methods for the histochemical demonstration of esterases have been described (*4, 5, 12–15*). A comparison of the distribution of enzymatic activity with these methods, with and without enzymatic inhibitors, will be reported elsewhere.

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Comments and Communications

Statement on Peyote

IN CONNECTION with the current national campaign against narcotics, there has been some propaganda to declare illegal the peyote used by many Indian tribes. We are professional anthropologists who have made extensive studies of Peyotism in various tribes. We have participated in the rites and partaken of the sacramental peyote. We therefore feel it our duty to protest against a campaign which only reveals the ignorance of the propagandists concerned.

Briefly put, the propagandists argue that Peyotists are simply addicted to a narcotic and intoxicant, which they use orgiastically.

Peyote is a small, carrot-shaped, spineless cactus which, in the U. S., grows in the Rio Grande Valley. The top of the plant is usually cut off and sun-dried, forming the peyote button. When taken internally, it appears to have remarkable mental and physical effects, although these have not been thoroughly studied.

According to Webster's *Dictionary,* a narcotic is a drug that "allays sensibility, relieves pain, and produces profound sleep;" an intoxicant "excites or stupefies." According to Merck's *Manual,* the symptoms of drug addiction are increased tolerance and dependence. On the basis of our experience, we would say that peyote seems to have none of these effects. It does not excite, stupefy, or produce muscular incoordination; there is no hangover; and the habitual user does not develop any increased tolerance or dependence. As for the immorality that is supposed to accompany its use, since no orgies are known among any Indian tribes of North America, the charge has as much validity as the ancient Roman accusation of a similar nature against the early Christians.

Actually Peyotism is a religion, with a national intertribal organization incorporated under the name of "The Native American Church of the United States." Its modern form, developed about 1870, is Christianity adapted to traditional Indian beliefs and practices. The basic tenets of the Native American Church are given in its articles of incorporation:

> The purpose for which this corporation is formed is to foster and promote religious believers in Almighty God and the customs of the several Tribes of Indians throughout the United States in the worship of a Heavenly Father and to promote morality, sobriety, industry, charity, and right living and cultivate a spirit of self-respect and brotherly love and union among the members of the several Tribes of Indians throughout the United States . . . with and through the sacramental use of peyote.

The belief is that God put some of his Holy Spirit into peyote, which he gave to the Indians. And by eating the sacramental peyote the Indian absorbs God's Spirit, in the same way that the white Christian absorbs that Spirit by means of the sacramental bread and wine. Peyote is used by Peyotists in two ways: spiritually and medically.

The traditional practice of many Indian tribes was to go off in isolation to contemplate and fast until a supernatural vision was achieved. This is now replaced by a collective all-night vigil in which, through prayer, contemplation, and eating peyote, the Peyotist receives a divine revelation. For the Peyotist, this occurs because he has put himself in a receptive spiritual mood and has absorbed enough of God's power from the peyote to make him able to reach God. A scientific interpretation might be that the chemicals in peyote diminish extraneous internal and external sensations, thus permitting the individual to concentrate his attention on his ideas of God and, at the same time, affecting vision and hearing so that these ideas are easily projected into visions.