negative entropy change resulting from complexation supports the previously suggested involvement of a charge-transfer interactive mechanism with caffeine acting as a donor and riboflavin as the acceptor (6, 9). Such an interaction would possibly reduce the electrophilicity of riboflavin and might, therefore, reduce its reactivity by discouraging the nucleophilic attack of hydroxide ion, an attack undoubtedly involved in the hydrolytic cleavage of the isoalloxazine ring.

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Effect of Deuterium Oxide on Local Anesthetic Activity of Procaine

By S. V. SUSINA, F. D. HITER, F. P. SIEGEL, and M. I. BLAKE

Deuterium oxide was used as a solvent for procaine and the effect on the stability and local anesthetic activity was noted. The ED_{50} was determined by the method of Chance and Lobstein and comparison was made with aqueous procaine solutions. In water the ED_{50} was 1.8% while in deuterium oxide it was 1.0%. The LD_{50} was determined by intraperitoneal injection in mice. There appears to be no significant difference in the toxicity of procaine in either solvent. Stability studies indicate that procaine is more stable in deuterium oxide at pH and "apparent" pH values of 8.0, 8.5, and 9.0.

THE THERAPEUTIC efficacy of drugs instilled into the eye depends, among other factors, on the pH of the solution in which the drug is administered and on the stability of the drug under such conditions. Procaine is a typical example. These relationships may be adduced from kinetic data and pharmacological studies. Higuchi, *et al.* (1), have studied the influence of pH and temperature on the rate of procaine decomposition in aqueous solutions. It is apparent from their work that procaine deteriorates rapidly in the alkaline pH range. Inhibition of procaine decomposition by complex formation has also been studied (2).

Topical anesthetic activity of procaine depends primarily on the extent of availability of free base. At pH values below 7.0 procaine is relatively inactive topically. Hind and Goyan (3) have shown that at this pH the per cent of procaine free base is less than 1, and even at pH 7.4 it increases only to 3.4%, but at pH 9.0 it becomes 58.5%. The lipoid nature of the corneal epithelium controls the rate at which absorption of procaine will occur in the cornea. The greater the concentation of procaine free base in the lacrimal fluid, the greater should be the extent of procaine penetration and absorption from the cornea which should result in greater and more prolonged local anesthetic activity.

The present study was undertaken to determine the effect of deuterium oxide on the stability of procaine solutions at "apparent" pH values comparable to those in protium oxide (distilled water). In addition, comparison was made of the local anesthetic activity in both systems over the pH range 5.0 to 9.0.

The toxicity of deuterium oxide in biological systems was described by Morowitz and Brown (4). Katz (5) has reviewed the literature dealing with the chemistry and biology of deuterium oxide. Algae and other microorganisms have been cultured in 99.6% deuterium oxide (6). It has been demonstrated (5) that mice and rats can survive when up to about one-third of their body water is replaced by deuterium oxide. When the blood serum approaches about 20% deuterium oxide content, toxic manifestations become evident. Since it appears that deuterium oxide is toxic in animals only when the concentration

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in the body fluids reaches high levels, and since deuterium oxide resembles the common solvent protium oxide more closely than any other, it seems appropriate to explore possible pharmaceutical applications of this solvent.

EXPERIMENTAL

Toxicity Study.—The effect of deuterium oxide on the toxicity of procaine was studied by determining the LD_{50} in this solvent. This was conducted on BDF-1[1] strain mice weighing about 25 Gm. Two per cent procaine hydrochloride solutions were prepared in water and deuterium oxide. The doses administered varied from 165 mg./Kg. to 200 mg./Kg. The route of administration was by intraperitoneal injection and each of the four dose levels was administered to a group of 10 mice. See Table I. The LD_{50} was determined by use of the method of Miller and Tainter (7).

TABLE I.—TOXICITY OF PROCAINE IN WATER AND IN DEUTERIUM OXIDE

Procaine in Water		Procaine in Deuterium Oxide	
Dose, mg./Kg.	Death, %	Dose, mg./Kg.	Death, %
165	20	170	10
175	30	180	40
190	50	190	50
200	60	200	60

Determination of ED_{50}.—The cornea provides an ideal site for testing surface anesthesia. It is free of special sensory cells and the free, unsheathed nerve endings are imbedded in the corneal layers. Therefore, the drug has a single uniform membrane to penetrate after which it is in direct contact with sensory nerve fibers. The cornea is also free of fluid channels and blood vessels. It has been shown (8) that the guinea pig elicits a more regular reflex response than the rabbit. In this investigation a modification of the method of Chance and Lobstein (8) was employed.

The local anesthetic potency of procaine in water and deuterium oxide was determined on the cornea of guinea pigs weighing 225–250 Gm. Each animal was held immobile in plastic restraining cages which allowed easy access to the eyes for the instillation of solution. Aqueous procaine solutions were freshly prepared prior to use by dissolving procaine hydrochloride in 0.1 *M* phosphate buffer (pH 7.0) in concentrations ranging from 0.5 to 2.0%. The concentrations of procaine in deuterium oxide ranged from 0.25 to 1.0% and were prepared in a deuterated phosphate buffer solution (pD 7.4). Two drops of the solution were placed on the cornea filling the space between the eyelids. The resulting clearly visible film of solution was allowed to remain in contact with the cornea for 15 seconds. After this period the excess fluid was removed by initiating a blink reflex. At the end of 45 seconds, a stimulus was applied to the cornea of the eye by means of a single thick horsehair which was pressed against the center of the cornea. The same amount of pressure was applied with each stimulation. This was repeated at 1-minute intervals for a period of 1 hour. A failure to respond to the stimulus (the blink reflex) indicated effective anesthesia. The anesthetic effect, recorded as per cent protection, was calculated from the number of minutes in which the blink reflex did not occur out of the 60-minute study period. A minimum of 12 animals or 24 test sites was used for each test solution and the reported value, expressed as a percentage, is the average value for that number of determinations. The logarithm of the concentration of procaine was plotted *vs.* the probit of the per cent protection (Fig. 1). The concentration of procaine solution which produced 50% protection was then determined from the curve. This value is the effective $dose_{50}$ (ED_{50}).

Effect of pH and pD on Anesthetic Activity.—One per cent procaine solutions were buffered at pH 5.0, 6.0, 7.0, 8.0, and 9.0. One-tenth molar buffer solutions were prepared with KH_2PO_4 and sodium hydroxide for pH values 5.0 to 8.0. A sodium borate and hydrochloric acid buffer system was used for pH 9.0. The concentration of procaine in deuterium oxide was 1/2%. The deuterium oxide buffer systems were prepared from the same reagents except that all replaceable hydrogen was exchanged with deuterium. All buffers in deuterium oxide were adjusted to an "apparent" pH corresponding to the pH of the aqueous buffer solutions. The "apparent" pH was measured with a Beckman pH-meter (model H-2) and an appropriate correction (9) to the reading was made to obtain pD.

The corneal reflex test described earlier was used to determine the per cent protection at the different pH and pD values. The data are shown graphically in Fig. 2.

Procaine Stability Study.—The decomposition of procaine in protium oxide and deuterium oxide solutions was studied by the method described by Higuchi, *et al.* (1). The time required for procaine to decompose to 50% of the original concentration (half-life) was determined at pH 8.0, 8.5, and 9.0

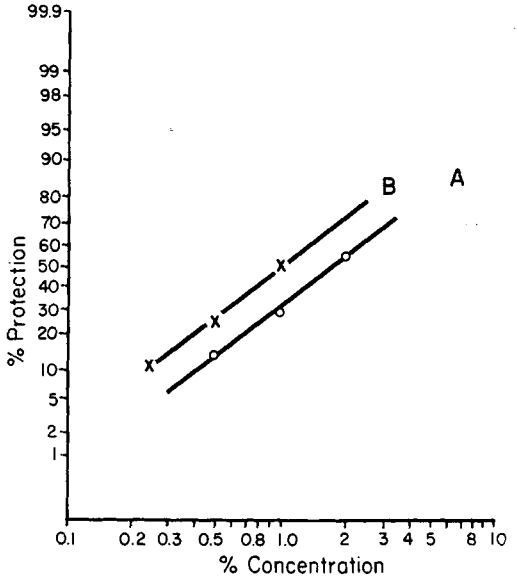

Fig. 1.—Per cent protection plotted in probits; concentration of procaine plotted as logarithm. Curve *A*, procaine in water; curve *B*, procaine in deuterium oxide.

[1] Simonsen Laboratories, White Bear, Minn.

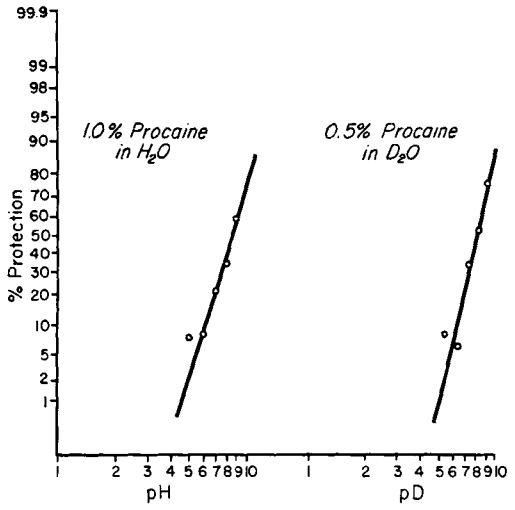

Fig. 2.—Effect of pH and pD on local anesthetic effect of procaine. Per cent protection plotted in probits; pH and pD plotted as logarithm.

in aqueous buffer solutions and at corresponding pD values for the deuterated system. The replaceable hydrogens in procaine hydrochloride were not exchanged with deuterium prior to the study in the deuterium oxide solutions. The hydrogen introduced in this way did not appreciably affect the hydrogen content of the deuterium oxide. This was verified by analyzing the reaction mixture for deuterium content. The data for these studies are recorded in Table II.

TABLE II.—COMPARISON OF THE RATE OF HYDROLYSIS OF PROCAINE IN PROTIUM OXIDE AND DEUTERIUM OXIDE AT 40°C.

Protium Oxide System		Deuterium Oxide System		
pH	Half-life, hr.	pD	Half-life, hr.	$T^1/_2\ D_2O^b / T^1/_2\ H_2O$
8.0	38	8.4 (8.0)[a]	115	3.0
8.5	13	8.9 (8.5)	38	2.9
9.0	6.5	9.4 (9.0)	13	2.0

[a] "Apparent" pH. [b] Ratio of half-lives at "apparent" pH in D_2O and pH in H_2O.

RESULTS AND DISCUSSION

The LD_{50} for procaine hydrochloride in water was 190 ± 12 mg./Kg. and for procaine in deuterium oxide was 190 ± 8 mg./Kg. The data for the LD_{50} studies are shown in Table I. There does not appear to be any significant difference in the toxicity of procaine in either solvent system as determined by intraperitoneal injection in mice.

An appropriate dose of procaine which produced a measurable local anesthetic effect was obtained from a dose response curve (Fig. 1) for procaine in both protium and deuterium oxide solutions. The ED_{50} for procaine in water (curve *A*) is 1.8% ± 0.8%. For procaine in deuterium oxide (curve *B*) the ED_{50} is 1.0% ± 0.5%. The pH of the aqueous procaine solution was 7.0 and that of the procaine in deuterium oxide solution was an "apparent" pH of 7.0 or a pD of 7.4. It appears that the ED_{50} of procaine in deuterium oxide is about one-half of the ED_{50} for procaine in water.

In the study dealing with the effect of pH and pD on the local anesthetic activity concentrations of 1.0% procaine in water and 0.5% procaine in deuterium oxide were used. Per cent protection as a function of pH and pD is shown graphically in Fig. 2. At pH values below 7.0 the per cent protection was low. This in agreement with the findings of Hind and Goyan (3) and Swan (10) who showed that at a pH below 7.0 the per cent of free base is less than 1%. Poor anesthetic activity is expected since it is only the free base which is capable of producing this effect. As the pH is increased to 7.5 the per cent protection increased to 30% and at pH values of 8.0 and 9.0 it increased to 35% and 60%, respectively. At pD 7.4 the per cent protection was 32% and at pD 8.4 and 9.4 the protection increased to 51% and 75%, respectively. At comparable pH and "apparent" pH values the deuterium oxide system offers greater protection than an aqueous system. While per cent protection was approximately the same, the concentration of procaine in deuterium oxide was one-half of that in the aqueous system.

The stability studies indicate that procaine is more stable in deuterium oxide solutions as compared to aqueous solutions at the same pH and "apparent" pH. Increased local anesthetic activity of procaine in deuterium oxide may be accounted for by the fact that procaine free base is more stable in deuterium oxide solutions or that at the same pH and "apparent" pH, a deuterium oxide solution of procaine will contain more free base than in the corresponding aqueous solution.

A simple pH effect is ruled out by the fact that much higher ratios of half-lives are obtained when pH and pD are compared. For example, when aqueous procaine at pH 8.0 is compared with procaine in deuterium oxide at "apparent" pH 8.0, the ratio of half-lives is 3.0. See Table II. However, when an aqueous solution of procaine at pH 8.5 is compared to procaine in deuterium oxide at pD 8.4, the ratio $T^1/_2\ D_2O / T^1/_2\ H_2O$ becomes 8.8.

The availability of deuterium oxide at a reasonable cost has stimulated much research with this solvent in recent years. However, a review of the literature indicated that pharmaceutical applications of this solvent have not been explored. It was the purpose of this investigation to observe the effect of deuterium oxide on the stability of procaine in alkaline solutions and to note the influence on local anesthetic activity. Further studies are currently in progress in which other labile drugs are being investigated. In addition to the effect of this solvent on the stability and activity of certain drugs, it is hoped that information may be obtained that will better explain the mechanisms of drug action and decomposition.

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Chromatography and Electrophoresis of Phenothiazine Drugs

By THEODORE J. MELLINGER and CLYDE E. KEELER

Three separation techniques for various phenothiazine tranquilizers are reported in this paper. Electrophoresis with suitable buffer systems and proper voltage permitted fast and distinctive migration of these phenothiazine bases, but tailing interfered with the usefulness of this technique. Paper chromatography with salt solutions as solvents proved to be a valuable and easy procedure for the separation of this group of drugs. Thin-layer chromatography with silica gel showed the most accurate separation of phenothiazine compounds. Combination of chromatography with color reaction or fluorescence permitted good differentiation of the various phenothiazine tranquilizers.

INCREASING INTEREST in the therapy of mental disorders has led to widespread use of phenothiazine derivatives, that are on the market under various names and chemical modifications. There is an immense clinical literature concerning these drugs but little is known about their separation or identification by means of chromatographic procedures or electrophoresis. Three kinds of techniques have been explored and are reported in this paper: electrophoresis, paper chromatography, and thin-layer chromatography. They were tested upon 23 drugs in the search for well reproducible separation methods with variation in R_f values as well as distinctive spots or zones devoid of tailing.

METHODS

Electrophoresis was carried out with buffer systems proposed by Werum, *et al.* (1). The 23 drugs were applied side by side along the starting line on a strip of Whatman 3 MM paper 30 cm. in width. Several strips were run with each buffer system. A Gordon-Misco apparatus for horizontal paper electrophoresis was used. The average potential difference between the electrodes was 500 to 800 v.

Paper chromatography was carried out with Whatman 3 MM filter paper. The dissolved substances were applied in amounts of 5 to 10 mg. in thin streaks along the starting line and were developed in the ascending direction.

Thin-layer chromatography was run on glass plates 20 × 20 cm. coated with a layer of 500 μ of silica gel G Merck. The coated plates were dried overnight in an incubator at 38°. After spotting the drugs, the chromatograms were developed in the ascending direction.

To locate the drugs, their fluorescence was examined under an ultraviolet lamp with a peak emission at 253 mμ and one at 360 mμ. To produce color reactions, 40% sulfuric acid was sprayed upon the developed chromatograms.

RESULTS

Electrophoresis.—Because all phenothiazine drugs in this study are bases, and therefore positively charged molecules, they migrate toward the negative end of the paper. With appropriate buffer systems and a proper potential difference between the two poles, migration of the drugs was accomplished in 40 to 60 minutes. With the new organic buffers proposed by Werum, *et al.*, the migrating phenothiazine compounds could be followed easily by their fluorescence under ultraviolet light. These new buffers do not quench the fluorescence of the drugs as barbiturate buffers do. When the buffer solutions were modified by leaving out the formamide, the same migration was obtained and the color reactions were more distinctive.

Table I presents the electrophoretic values of 19 phenothiazine compounds and of four other substances closely related to the phenothiazine tranquilizers. The migration values of the drugs are shown in the vertical columns, as they were run on the same paper. However, the values for each substance cannot be compared exactly on the horizontal lines, since they were not run together. In spite of this, there can be seen a general trend in most of the substances to decrease the speed of migration with increasing pH, possibly due to decreasing ionization at higher pH. This is less apparent for the three sulfoxidized phenothiazines, imipramine, and the two thypendyl compounds. It cannot be ascertained from these experiments whether the deviations of the latter compounds are merely due to physico-chemical differences, or are

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