[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, NEW YORK UNIVERSITY]

THE BASIS FOR THE PHYSIOLOGICAL ACTIVITY OF -ONIUM COMPOUNDS. VI. RATES OF HYDROLYSIS OF CERTAIN ESTERS OF CHOLINE AND ITS ANALOGS[1]

BY R. R. RENSHAW AND N. BACON[2]

RECEIVED MARCH 5, 1926 PUBLISHED JUNE 5, 1926

The great amount of work that has been done in attempting to correlate chemical and physical properties with physiological activity has shed almost no light on the fundamental processes involved in the action of nerve poisons. Of course the difficulties here are almost, if not quite, insuperable, and probably only negative evidence will be obtained until a very considerable advancement has been made in the theories and methods of fundamental sciences.

It has seemed to the writers that two important factors in this problem have not heretofore been considered. These have to do with the character of the substances and with the type of physiological activity studied. Most of the work has been carried on with substances of relatively intricate structures in which changes in stability, tautomerism, physical properties, etc., brought about by minor alterations in the complex might have been overlooked or difficult to evaluate. Secondly, the type of physiological activity that has been selected for study (as, for instance, narcosis) has usually been too gross, that is, shown by too great a variety of substances, to be of much value from the point of view of mechanism.

In order, then, that the investigation should not be so greatly handicapped by these two factors, we have selected for study the simple alkyl and substituted alkyl-onium compounds. These have a high product of simplicity of structure with specificity of physiological action, that is, while being very simple structurally they still have a highly specialized action on a restricted portion of the nervous system.

We have undertaken to compare the chemical activity and physical properties of a series of these compounds with their muscarine and nicotine effects. These effects are produced by the substances,[3] when circulating in the blood stream, by their acting upon definite mechanisms of a restricted portion of the nervous system. The action is highly selective.

[1] This problem is being carried on in coöperation with Dr. Reid Hunt of the Harvard Medical School. The physiological data are the basis of another series of papers published elsewhere by him.

[2] In part, the experimental data presented in this paper are from a thesis submitted by Nicholas Bacon, June, 1924, to the Faculty of the Graduate School of New York University for the degree of Master of Science.

[3] The criteria which Hunt has used for determining these effects have been described, *J. Pharm.*, **25**, 319 (1925). These compounds also give the curare action, but this effect is shown by too great a variety of substances to be of much value in this investigation.

It seemed at the outset that the effects of these substances could not be due to any ordinary type of chemical activity that they might show. The following considerations, it is believed, will make this clear. Tetramethylammonium salts give a moderately strong muscarine[4] action (stimulation of the inhibitory nerve endings to the heart and certain other organs), an intense stimulating nicotine action (stimulation of the nerve cells of the autonomic ganglia) and, in higher concentrations, the paralytic nicotine effect (paralysis of the same autonomic ganglion cells). Tetraethylammonium salts, on the other hand, do not give the muscarine action, nor do they exhibit appreciably the stimulating nicotine effect; but they do show the paralytic nicotine action almost as strongly as tetramethylammonium salts. Dale[4a] has emphasized these facts and called attention to the difficulty of finding similarities and dissimilarities of these two ions which would account for their similar and dissimilar actions.

Regardless of the weight of this reasoning, it seemed desirable to obtain experimental data on the chemical activity of a number of these -onium compounds, for it may be that the physiological activities are determined by a product of chemical and physical properties. In addition, Dale and others[5] have suggested that the varied evanescence of the action of the esters and ethers of choline may be associated with the rapidity with which they hydrolyze. Acetylcholine, which gave a very powerful but very evanescent depressor effect on the blood pressure, appeared from experiments of these investigators to be easily hydrolyzed by alkalies. The nitric ester and the ethyl ether, however, gave a less powerful but a more prolonged effect and they found that these substances have a much greater resistance to alkalies.

The present paper deals with the rates of hydrolysis of certain esters of choline and related compounds. The results indicate that the rapidity of hydrolysis is related neither to the degree nor to the evanescence of their muscarine effects.

Since these substances are carried to the tissues by the blood stream, it seemed necessary to study their hydrolysis under conditions as nearly like those of that fluid as possible. Due to the relatively very large volume of the blood as compared with the amount of material injected, as well as to its rapid flow and its high buffer action, there seemed no likelihood of appreciable local changes in its hydrogen-ion concentration on account of hydrolysis of the materials. The only practicable method which suggested itself of determining the rates of hydrolysis at a constant hydroxyl-ion concentration, and thus simulating the conditions in the circulation,

[4] (a) Burns and Dale, *J. Pharmacol.*, **6**, 417 (1915). (b) Hunt and Renshaw, *ibid.*, **25**, 320 (1925).

[5] (a) Dale, *J. Pharmacol.*, **6**, 183 (1914). (b) Ewins, *Biochem. J.*, **8**, 44 (1914). (c) Fourneau and Page, *Bull. soc. chim.*, **15**, 544 (1914).

was to determine the rate at which very dilute alkali needed to be added in order to keep the reaction mixture at a definite alkalinity.

Method and Apparatus.—The rates of hydrolysis were determined at body temperature (37°) and at an hydroxyl-ion concentration of P_H 7.8, slightly greater than that of blood (P_H 7.4). This concentration was determined by a distinct pink of cresol-red indicator. The color tint was checked against that of cresol red in a carefully prepared borate buffer of P_H 7.8. The reaction flask was immersed in an insulated glass thermostat so constructed that the color tint of the solution could be viewed through windows against the outside light. The weighed sample was added to the flask containing five or seven drops of cresol-red indicator solution and the designated amount of distilled water, previously heated to 37°. The flask was immediately connected with a stopper fitted with a mercury-sealed mechanical stirrer and a tube for admitting standard alkali. This inlet tube was sealed onto a narrow-stemmed, 25cc. buret, and its tip drawn out to a capillary in order to make possible the addition of small drops of alkali. The upper end of the buret was connected with a reservoir containing the standard barium hydroxide. With care in keeping the color tint approximately constant by the addition of the very dilute alkali, rather good duplicate values were obtained.

Since these determinations were made in the presence of a constant hydroxyl-ion concentration, pseudo monomolecular reactions were indicated. The rates were, therefore, compared in terms of constants for that type of reaction.

TABLE I

HYDROLYSIS OF ACETOXYMETHYL-DIMETHYLSULFONIUM BROMIDE, $CH_3COOCH_2S(CH_3)_2Br$[6]

1ST RUN. 0.0414 G. IN 100 CC. WATER PLUS 5 DROPS OF INDICATOR SOLUTION.

Time (min.)	6	9	14	18	25	32	40	50
Cc. of 0.083 *N* Ba(OH)$_2$	2.12	2.7	3.56	3.9	4.24	4.42	4.5	4.57
K	0.1033	0.0986	0.1068	0.1055	0.1030	0.1031	0.0997	0.1097

The calculated requirement for neutralizing two acid equivalents is 4.59 cc. of alkali.

2ND RUN (TWO OBSERVERS). 0.0501 G. IN 200 CC. OF WATER PLUS 7 DROPS OF INDICATOR SOLUTION.

Time (min.)	4	8	12	16	20	24	32	40	52
Cc. of 0.083 *N* Ba(OH)$_2$	2.28	3.25	3.94	4.38	4.69	4.95	5.29	5.44	5.55
K	0.1310	0.1089	0.1017	0.0956	0.0913	0.0903	0.0914	0.0904	0.0950

The calculated requirement for neutralizing two acid equivalents is 5.59 cc. of alkali.

The data in Table I show that this sulfonium compound undergoes a deep-seated decomposition, yielding two equivalents of acid. Due to the constancy of the values of *K*, and due to the greater stability of the ester derivatives of the formocholine type as compared with hydroxyl deriva-

[6] For the preparation of this sample, see paper IV of this series, THIS JOURNAL, **48**, 519 (1926).

tives with regard to -onium dissociation, the authors feel certain that the hydrolysis of the ester group is the first reaction taking place and that it is the one measured. The resultant formocholine analog then rapidly undergoes -onium dissociation, giving the second molecule of acid as follows: $HOCH_2S(CH_3)_2Br \longrightarrow (CH_3)_2S + BrCH_2OH \longrightarrow CH_2O + HBr$.

Acetylcholine Bromide, $CH_3COOC_2H_4N(CH_3)_3Br$.—Acetylcholine has frequently been used in physiological experimentation since Hunt and Taveau[7] discovered its extraordinary physiological activity and showed how an extremely delicate test for choline could be obtained by converting the choline into the acetyl derivative. In the past it seems to have been prepared by the acetylation of choline with subsequent isolation as the chloride or the platinum double salt. The chloride is so very hygroscopic that extreme precautions are required in keeping and weighing it, and the platinum double salt requires a hydrogen sulfide precipitation. We find that the preparation of the bromide by direct condensation of liquid trimethylamine with bromo-ethyl acetate has much to commend it, since the yield is good, and the product keeps well and is only slightly hygroscopic.

To a well-cooled (—15°) mixture of liquid trimethylamine and toluene contained in a pressure bottle was added 1 gram-molecular equivalent of freshly distilled, thoroughly cold (—15°) bromo-ethyl acetate. After the contents were mixed and the bottle sealed, the materials were allowed to come to room temperature slowly. At the end of 24 hours the reaction was practically complete. The product was purified by several reprecipitations from its solution in cold absolute ethyl or methyl alcohol by ether. Some alcoholysis occurs when it is recrystallized from hot alcohol solutions. The compound crystallizes from absolute alcohol in hexagonal prisms melting at 143° (corr.) when heated at the rate of 3° per minute.

Anal. Calcd. for $C_7H_{16}NO_2Br$: Br, 35.34. Found: 35.25, 35.29.

TABLE II

HYDROLYSIS OF ACETYLCHOLINE BROMIDE

0.1272 g. sample in 200 cc. of water plus 7 drops of indicator solution.

Time (min.)	15	30	48	72	90	119	146	182	200
Cc. of 0.083 *N* $Ba(OH)_2$	0.075	0.15	0.22	0.34	0.44	0.63	0.80	0.94	1.01
$K \times 10^{-3}$	.744	.749	.690	.717	.749	.824	.864	.824	0.810

3.75 cc. of the base would be required for complete hydrolysis of the ester group.

The rate is so slow that only about one-seventh of the compound hydrolizes in three and one-half hours. It is, therefore, about 125 times slower than the rate for acetoxymethyl-dimethylsulfonium bromide. The muscarine effects of these two substances are, however, of about equal evanescence.[8]

Acetoxymethyl-trimethylammonium Bromide, $CH_3COOCH_2N(CH_3)_3$-

[7] Hunt and Taveau, *Brit. Med. J.*, **2**, 1788 (1906).

[8] Reid Hunt, private communication.

Br.—This salt was prepared for study because it is much less hygroscopic than the chloride, and because we suspected that it would undergo -onium dissociation less readily than the iodide. The process used was similar to that for the other halogen salts (condensation of liquid trimethylamine with bromo-methyl acetate).[9] Upon the addition of ether to a cold alcohol solution it precipitates in the form of fine needles, melting at 159° (uncorr.) when heated at the rate of 3° per min.

Anal. Calcd. for $C_6H_{14}NO_2Br$: Br, 37.69. Found: 37.66, 37.80.

TABLE III

HYDROLYSIS OF ACETOXYMETHYL-TRIMETHYLAMMONIUM BROMIDE

0.0488 g. sample in 200 cc. of water plus 7 drops of indicator solution.

Time (min.)	10	20	31	45	60	75	90	124
Cc. of 0.062 *N* $Ba(OH)_2$	1.1	2.1	2.7	3.2	3.5	3.8	3.9	4.0
K	0.0341	0.0402	0.0400	0.0410	0.0423	..	..	...

The calculated requirement for complete hydrolysis of the ester group is 3.73 cc.

The rate for this substance is approximately 50 times that of acetylcholine, while the evanescence of the effects of the two substances is about equal.[8]

The excess of alkali consumed over the calculated amount may possibly be explained by a dissociation of the -onium structure of the formocholine, as follows: $HOCH_2N(CH_3)_3Br \longrightarrow (CH_3)_3N + BrCH_2OH \longrightarrow CH_2O + (CH_3)_3NHBr$. The formaldehyde produced may then take up, during the considerable time that this hydrolysis is in progress, a small amount of alkali by the Cannizzaro reaction.

The foregoing results were not appreciably affected by the presence of trimethylammonium acetate and bromide which may have been formed by a secondary dissociation of the -onium structure. We have found that these salts have no significant buffer action, at least over the range in which we were interested. Two hundredths cc. of 0.074 *N* sodium hydroxide changed the Sörensen values (P_H) of 10 cc. of 0.4% trimethylammonium bromide 1.4 units. The Sörensen value of 10 cc. of a stoichiometric mixture of accurately standardized approximately 0.1 *N* solutions of trimethylamine and acetic acid was changed 1.4 units by 0.15 cc. of 0.081 *N* acetic acid.

TABLE IV

HYDROLYSIS OF CHLORO-ACETYL CHOLINE BROMIDE

0.0612-g. sample in 200 cc. of water plus 7 drops of indicator solution.

Time (min.)	3	6	15	28	45
Cc. 0.037 *N* $Ba(OH)_2$	5.5	6.0	6.15	6.27	6.27
K	0.688	0.507	0.249	...	...

The calculated requirement for complete hydrolysis of the ester group was 6.3 cc.

The rate of hydrolysis was so rapid in this case that accurate results

[9] Renshaw and Ware, THIS JOURNAL, **47**, 2990 (1925).

could not be obtained.[10] It is clear, however, that the rate is from 800 to 1000 times as rapid as the unsubstituted acetylcholine. Hunt has found that in the evanescence of the muscarine action these substances are extraordinarily alike.

β-Nitro-oxyethyl-trimethylammonium Bromide, $O_2NOC_2H_4N(CH_3)_3Br$.—This compound was prepared by condensing β-bromo-ethyl nitrate with liquid trimethylamine. It was purified by several fractional precipitations of its solutions in methyl alcohol by ether. The bromide of this ester has a relatively low solubility in absolute ethyl alcohol; m. p., 187° (corr.).

Anal. Calcd. for $C_5H_{13}N_2O_3Br$: Br, 34.9. Found: 34.7.

A 0.0614g. sample in 200 cc. of water plus 7 drops of indicator solution had consumed 0.05 cc. of 0.061 *N* barium hydroxide solution in 30 minutes, and 0.09 cc. of the same alkali at the end of 150 minutes; 4.37 cc. of the alkali would have been required for complete hydrolysis of the ester groups.

This resistance to hydrolysis is in line with the results of Hofmann and Höbold[11] who were able to recover a considerable quantity of the perchlorate of this ester after having evaporated a sample of it with ammonium hydroxide.

In Table V, the constants for the rates of hydrolysis are compared with physiological data obtained by Hunt.

TABLE V

A COMPARISON OF THE RATES OF HYDROLYSIS OF CHOLINE ESTERS WITH THEIR MUSCARINE AND NICOTINE EFFECTS

			Muscarine action			Stimulating nicotine action		
		$K \times 10^3$	Amt., mg.	Blood pres., mm.	Duration of action	Amt., mg.	Blood pres., mm.	Duration of action, min.
1.	$ClCH_2COOC_2H_4N(CH_3)_3Br$	5–600.0	0.5	−37	Equal	2.0	+ 33	4
2.	$CH_3COOCH_2S(CH_3)_2Br$	100.0	.1	−32	and	10.0	none	...
3.	$CH_3COOCH_2N(CH_3)_3Br$	40.0	.1	−41	very	10.0	+ 33	1.5
4.	$CH_3COOC_2H_4N(CH_3)_3Br$	0.8	.0005	−52	evanescent	10.0	+ 16	7
5.	$O_2NOC_2H_4N(CH_3)_3Br$	immeasurably small	.1	−41	Very prolonged	10.0	+142	3

The data indicate that the rates of hydrolysis are not proportional to either the evanescence or the extent of the physiological activity of these esters. At most, the rates can only be involved in the evanescence of the chloro-acetyl and the nitrate esters. While it is true that the conditions under which these rates of hydrolysis were determined are similar to

[10] It was estimated from Senter's data [*J. Chem. Soc.*, **91**, 472 (1907)] on the hydrolysis of sodium chloro-acetate with water and with 0.1 *N* alkali at 102° that, at 37°, less than 1% of the chloro-acetate ion would be decomposed with water and less than 3% with 0.1 *N* alkali after 120 minutes. It was therefore believed that at the end of 45 minutes only a very small amount of this decomposition had taken place with 0.037 *N* alkali. This was confirmed by titrating the ionizable halogen in the solution after the rate of hydrolysis had been measured.

[11] Hofmann and Höbold, *Ber.*, **44**, 1767 (1911).

those in the blood only in regard to the effective concentration of hydroxyl ion and temperature, and while it is conceivable that the order of the rates of hydrolysis by an esterase on these derivatives might be different from the order of the rates of hydrolysis by the hydroxyl ion, still, so far as the author is aware, there is no case known where the rate of hydrolysis of an ester by an enzyme is appreciably greater than the rate by which the same will be hydrolyzed by hydroxyl ion. To explain, then, the evanescent action of acetyl choline by rapid hydrolysis due to an esterase, it would be necessary to assume an unheard-of rate for the esterase as compared with hydroxyl ion, as well as very greatly different rates for *similar* acetic acid esters.

The data in Table V do not indicate any relationship between the rates of hydrolysis and either the extent or the duration of the stimulating nicotine action of these esters.

The authors wish to express their appreciation to the Directors of the Bache Fund, National Academy of Sciences, for a grant made to support this investigation.

Summary

1. Improved methods for the preparation of acetylcholine bromide, acetylformocholine bromide and the nitric ester of choline bromide are described.

2. The rates of hydrolysis of five esters of the choline type have been determined at body temperature and at approximately the hydrogen-ion concentration of blood.

3. A comparison of the rates of hydrolysis of these compounds with their physiological properties, as determined by Hunt, shows that the rates cannot in all cases be the determining factor in either the extent or the evanescence of their action.

NEW YORK, N. Y.