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Studies on Decomposition and Stabilization of Drugs in Solution. XX.[1] Hydrolysis of Acylcholine[2]

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Hydrolysis of acylcholines in 0.1N hydrochloric acid solution and that in borate buffer solution [$Na_2B_4O_7$(0.01M)+H_3BO_3(0.16M)+NaCl(0.04M)] at 60° were studied.

Hydrolysis in Acidic Solution——Octanoylcholine iodide, decanoylcholine iodide, dodecanoylcholine iodide and hexadecanoylcholine iodide (micellar acylcholines) were less stable below their critical micelle concentrations (CMC) and more stable above their CMC. Acylcholine with longer hydrocarbon chain was more stable than shorter chain.

Hydrolysis in Basic Solution——Linear relationship was found between concentration (log scale) of acylcholine and time on hydrolysis at its low concentration (below CMC) but was not found at its high concentration (above CMC). But it might be considered that initial stage was linear. The linear portion at initial stage and the gradually curved portion with course of time were discussed.

(1) The linear portion at initial stage

Hydrolysis was accelerated by formation of micelle and it was observed that the longer the hydrocarbon chain was, the less the stability became.

(2) The gradually curved portion with course of time

It was considered that fatty acid produced by hydrolysis of acylcholine in borate buffer solution was ionic type and it formed complex with cationic acylcholine, and repression of hydrolysis was ascribed to solubilization of this complex into micelle of acylcholine.

Hydrolysis of acylcholine in acidic solution and that in basic solution at 60° were studied.

Relationship between stability and formation of micelle on acylcholine was discussed.

Hydrolysis of acetylcholine,[4] acetyl-β-methylcholine, propionylcholine, butyrylcholine,[5] and succinylcholine[6] have already been reported. But these acylcholines were lower compounds. The effect of sodium lauryl sulfate on the stability of acetylcholine chloride, propionylcholine iodide, butyrylcholine iodide, hexanoylcholine iodide, octanoylcholine iodide and dodecanoylcholine iodide was studied in this series of study XIV and XVII.[7] There has been no report which was studied on the stability of higher acylcholines in the viewpoint of this series of study XIII and XV.[8] In this study, hydrolysis of acylcholines in 0.1N hydrochloric acid solution and that in borate buffer solution [$Na_2B_4O_7$(0.01M)+H_3BO_3(0.16M)+NaCl(0.04M)] at 60° were studied.

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[1] Part XIX: H. Nogami and Y. Kanakubo, *Chem. Pharm. Bull.* (Tokyo), **17**, 2008 (1969).

[2] This work was presented at Meeting of Kanto Branch, Pharmaceutical Society of Japan, Tokyo, January 1965, and at the 85th Annual Meeting of Pharmaceutical Society of Japan, Fukuoka, April 1965.

[3] Location: *a) Hongo, Tokyo; b) Asahi-machi, Niigata.*

[4] E. Hofmann, *Helv. Chim. Acta*, **8**, 138 (1930); J. Butterworth, D.D. Eley and G.S. Stone, *Biochem. J.*, **53**, 30 (1953).

[5] L. Larsson, *Acta Chem. Scand.*, **8**, 1017 (1954).

[6] L.E. Tammelin, *Acta Chem. Scand.*, **7**, 185 (1953); T. Suzuki, *Chem. Pharm. Bull.* (Tokyo), **10**, 912 (1962); *idem, ibid.*, **10**, 1017 (1962).

[7] H. Nogami, S. Awazu and M. Iwatsuru, *Chem. Pharm. Bull.* (Tokyo), **11**, 1251 (1963); H. Nogami, J. Hasegawa and M. Iwatsuru, *ibid.*, in press.

[8] H. Nogami, S. Awazu and Y. Kanakubo, *Chem. Pharm. Bull.* (Tokyo), **11**, 13 (1963); H. Nogami and Y. Kanakubo, *ibid.*, **11**, 943 (1963).

Experimental

Materials——1) Acylcholines, Sky Blue FF, Sudan III and Purified Water: They were as same as the previous report.[1]

2) Orange II [Sodium *p*-(2-Hydroxy-1-naphthylazo)benzene Sulfonate]: This was a Merck's Präparat.

3) Fatty Acids: Butyric acid was a guaranteed reagent. Caproic acid, caprylic acid, capric acid and lauric acid were extra pure grades, respectively.

4) Methantheline Bromide: This was a product of Dainippon Pharmaceutical Co., Ltd.

5) Ethylene Dichloride: This was washed with dil. NaOH and water, and distilled after drying.

6) Buffer Solution used for Hydrolysis:

$$Na_2B_4O_7(0.01\text{M})+H_3BO_3(0.16\text{M})+NaCl(0.04\text{M})$$

Buffer solutions used for formation of methantheline laurate were borate buffer solutions and phosphate buffer solutions of various pH values. A glass electrode pH meter HM-5A (Toa Eletronics Ltd.) was used for measurement of pH values.

Other reagents were guaranteed reagents.

Determination of Acylcholines——1) Hestrin's Method:[9] Acetylcholine chloride (ACC), propionylcholine iodide (PCI), butyrylcholine iodide (BCI) and hexanoylcholine iodide (HCI) were determined by this method. Alkaline hydroxylamine reagent was prepared freshly before use by mixing equal volumes of 3.5M NaOH and 2M hydroxylamine. The mixture was kept for about 3 hours at room temperature. Four ml of alkaline hydroxylamine reagent was added to 2 ml of sample. After at least 1 minute, pH was brought to 1.2±0.2 with 2 ml of HCl solution and 2 ml of 0.37M $FeCl_3$ in 0.1N HCl was added. The absorbance was measured spectrophotometrically at 5 minutes after the addition of Fe^{3+} to keep the error from fading constant. The wave length was 520 mμ.

2) Orange II–Chloroform Method:[10,11] Octanoylcholine iodide (OCI), decanoylcholine iodide (DeCI), dodecanoylcholine iodide (DoCI) and hexadecanoylcholine iodide (HdCI) were determined by this method. Two ml of 3.2 mM Orange II in 0.1 M NaCl, 8 ml of 0.1 M NaCl, 2 ml of sample and 20 ml of $CHCl_3$ were pipetted into an about 50 ml glass–stoppered centrifugal bottle. It was shaken for 15 minutes by a shaking apparatus and then centrifuged for 5 minutes. Shaking time of HdCl was necessary over 30 minutes. The $CHCl_3$ layer was measured spectrophotometrically at 485 mμ. OCI was able to determine by both Hestrin's method and Orange II–Chloroform method. Results of both methods were in good agreement.

Kinetic Procedure——1) Hydrolysis in Acidic Solution: Accurately weighed amount of acylcholine and 5 ml 1N of HCl were placed in a 50 ml volumetric flask and made to 50 ml with purified water. The flask was immersed in a thermostatically controlled water bath maintained at 60±0.1°. Two ml of sample was taken from the flask at given intervals, diluted with purified water and determined by previous methods. Fatty acid and choline produced by hydrolysis of acylcholine did not interfere the colorimetry.

2) Hydrolysis in Basic Solution: Borate buffer solution was used instead of 0.1N HCl.

Determination of CMC——1) Colorimetry of Sky Blue FF: Absorption of Sky blue FF was measured as same as the previous report[1] on 0.1M NaCl and 0.1N HCl of various concentrations of DoCI, respectively. The CMC of DoCI in 0.1 N HCl was approximately coincident with that in 0.1M NaCl. When DoCI was solubilized only in water, it was necessary to warm the solution at about 45°, but DoCI was solubilized in 0.1M NaCl or 0.1N HCl at room temperature. It was considered that this phenomenon was ascribed to exchange of gegen–ion.

Ionic strength of borate buffer solution used for hydrolysis was about 0.07. Absorption of Sky blue FF was measured as same as the previous report[1] on 0.07M NaCl and borate buffer solution of various concentrations of DoCI, respectively. The CMC of DoCI in borate buffer solution was approximately coincident with that in 0.07M NaCl.

2) Solubilization of Sudan III: As the CMC of DoCI in 0.1N HCl was approximately coincident with that in 0.1M NaCl, 0.1M NaCl was used instead of 0.1M HCl, and as the CMC of DoCI in borate buffer solution was approximately coincident with that in 0.07M NaCl, 0.07M NaCl was used instead of borate buffer solution, to prevent hydrolysis of acylcholines. The procedure was as same as the previous report[1] but temperature was 60°.

Formation of Methantheline Laurate——Methantheline bromide solutions (0.2 mM) of various pH values were prepared by mixing 0.2M H_3BO_3–0.05M NaCl (0.2 mM methantheline bromide) and 0.05M $Na_2B_4O_7$ (0.2 mM methantheline bromide). Ten ml of sample and 10 ml of ethylene dichloride (3 mM lauric acid) were pipetted into an about 50 ml glass–stoppered centrifugal bottle, which was shaken for 30 minutes by a shaking apparatus and then centrifuged for 5 minutes. Ethylene dichloride layer was measured spectro-

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[9] S. Hestrin, *J. Biol. Chem.*, **180**, 249 (1949).

[10] A.V. Few and R.H. Ottewill, *J. Colloid Sci.*, **11**, 34 (1956).

[11] G. Zografi, P.R. Patel and N.D. Weiner, *J. Pharm. Sci.*, **53**, 544 (1964).

photometrically at 283 mμ.[12] Methantheline bromide in phosphate buffer solution was studied as same as borate buffer solution.

Effect of Fatty Acids on Stability of DoCI——Effect of fatty acids on stability of DoCI was studied. On hydrolysis of DoCI (5 mM), butyryic acid, caproic acid, caprylic acid, capric acid and lauric acid (0.5 mM) were added previously, respectively. In this experiment, change of pH value of solution by addition of fatty acid was not found. The spectrophotometer used in above experiments was a Shimazu QB-50 spectrophotometer.

Results and Discussion

Hydrolysis in Acidic Solution

Linear relationship was found between concentration (log scale) of acylcholine and time on hydrolysis in 0.1N hydrochloric acid solution within the time of this experiment. The example of DoCI was shown in Fig. 1.

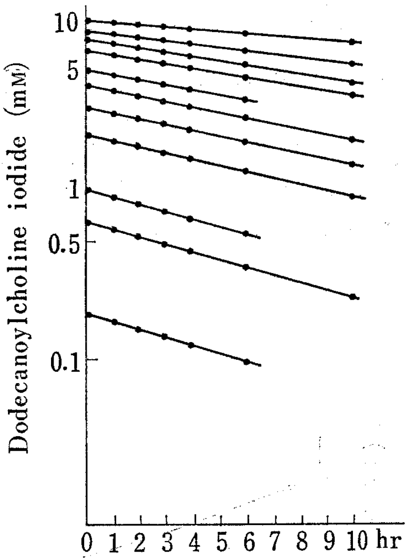

Fig. 1. Hydrolysis of Dodecanoylcholine Iodide in 0.1N HCl Solution at 60°

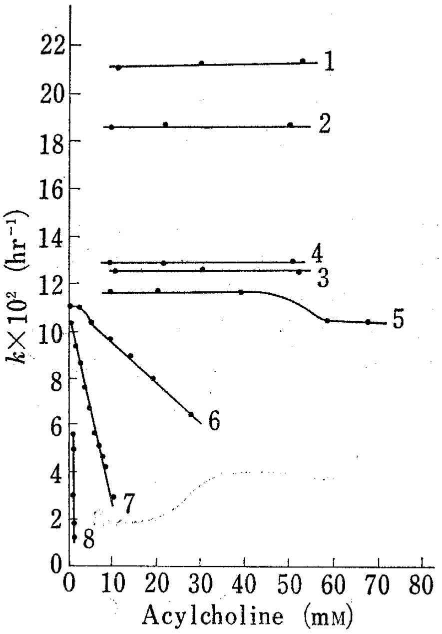

Fig. 2. Rate Constants of Acylcholines in 0.1N HCl Solution at 60°

1: acetylcholine chloride
2: propionylcholine iodide
3: butyrylcholine iodide
4: hexanoylcholine iodide
5: octanoylcholine iodide
6: decanoylcholine iodide
7: dodecanoylcholine iodide
8: hexadecanoylcholine iodide

Rate constants of various concentrations of acylcholines from ACC to HdCI were shown in Fig. 2.

In the concentration range of this experiment, little change of rate constants was observed with increase of concentration on ACC, PCI, BCI and HCI, but decrease of rate constants was observed on OCI, DeCI, DoCI and HdCI. Relationships between rate constants and solubilization of Sudan III on various concentrations of OCI, DeCI, DoCI and HdCI were shown in Fig. 3, 4.

CMC obtained by solubilization method agreed approximately with flection point where rate constant decreased. It was observed that the longer the hydrocarbon chain was, the more the stability became in Fig. 2. This phenomenon was contrary to that of sodium alkyl sulfate

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[12] H. Nogami, S. Awazu, K. Watanabe and K. Sato, *Chem. Pharm. Bull.* (Tokyo), **8**, 1136 (1960).

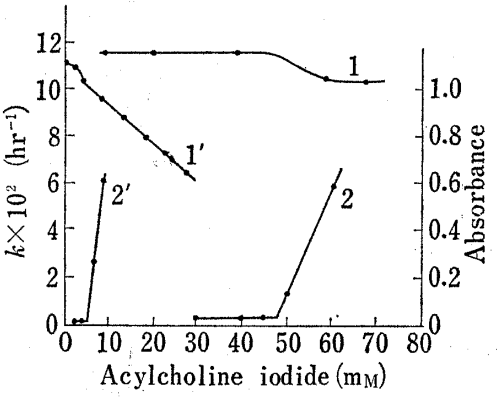


Fig. 3. Relationship between Rate Constant [0.1N HCl Solution(1, 1′)] and Solubilization of Sudan III [0.1M NaCl Solution (2, 2′)] in Acylcholine Iodide Solution at 60°

1, 2 : octanoylcholine iodide
1′, 2′: decanoylcholine iodide

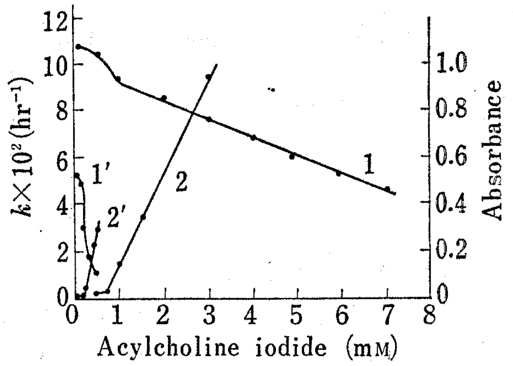


Fig. 4. Relationship between Rate Constant [0.1N HCl Solution(1, 1′)] and Solubilization of Sudan III [0.1M NaCl Solution(2,2′)] in Acylcholine Iodide Solution at 60°

1, 2 : dodecanoylcholine iodide
1′, 2′: hexadecanoylcholine iodide

which the longer the hydrocarbon chain was, the less the stability became.[8] In this case, it was considered that more repulsion of hydrogen ion occurred on micelle of acylcholine with longer hydrocarbon chain than that with shorter chain, and micelle of acylcholine with longer chain was less subjected to catalytic effect by hydrogen ion than that with shorter chain. Rate constants of sodium octyl sulfate and sodium lauryl sulfate at 70° and those of OCI and DoCI at 60° were shown in Fig. 5.

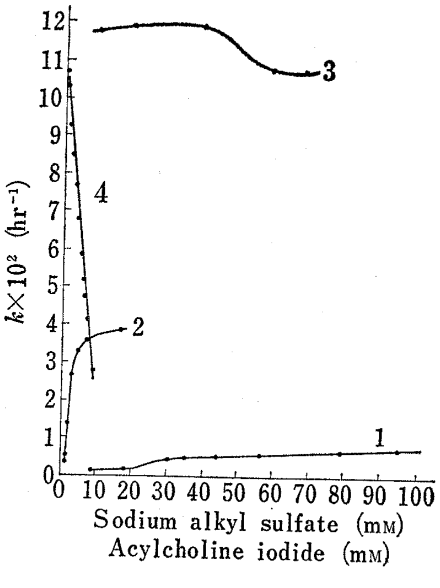


Fig. 5. Rate Constants of Sodium Octyl Sulfate(1) and Sodium Lauryl Sulfate(2) in 0.1N HCl Solution at 70° and Rate Constants of Octanoylcholine Iodide(3) and Dodecanoylcholine Iodide(4) in 0.1N HCl Solution at 60°

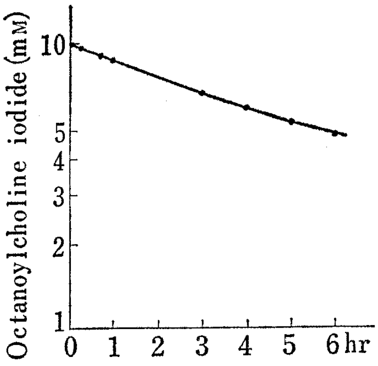


Fig. 6. Hydrolysis of Octanoylcholine Iodide in Borate Buffer Solution at 60°

change of pH

hr	0	1	2	3	4	5	6
pH	7.79	7.74	7.70	7.68	7.64	7.56	7.52

Hydrolysis in Basic Solution

On hydrolysis of acylcholine in borate buffer solution, pH value shifted to low value at high concentration of acylcholine with course of time, and effect of change of pH value could not be neglected. Hydrolysis and pH values of each 1 hour on OCI (10 mM) were shown in

Fig. 6. When pH value shifted to low value, rate of hydrolysis decreased. The concentration (10 mM) of OCI was below its CMC.

Hydrolysis and change of pH values on PCI, BCI and HCI (about 5 mM) were shown in Fig. 7. Linear relationship was found between concentration (log scale) and time.

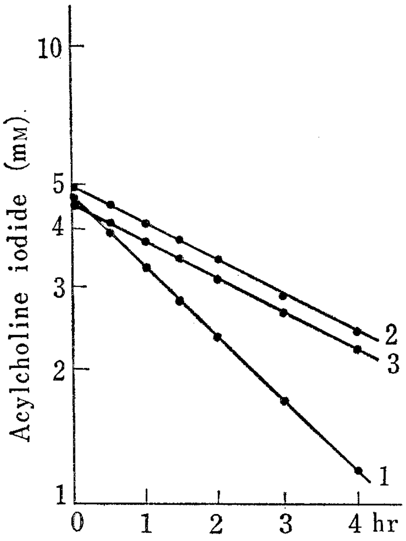


Fig. 7. Hydrolysis of Propionylcholine Iodide(1), Butyrylcholine Iodide(2) and Hexanoylcholine Iodide(3) in Borate Buffer Solution at 60°

	initial concn. (mω)	initial pH	final pH	
1)	4.71	7.80	7.75	(after 4 hr)
2)	4.99	7.77	7.70	(after 4 hr)
3)	4.56	7.75	7.70	(after 4 hr)

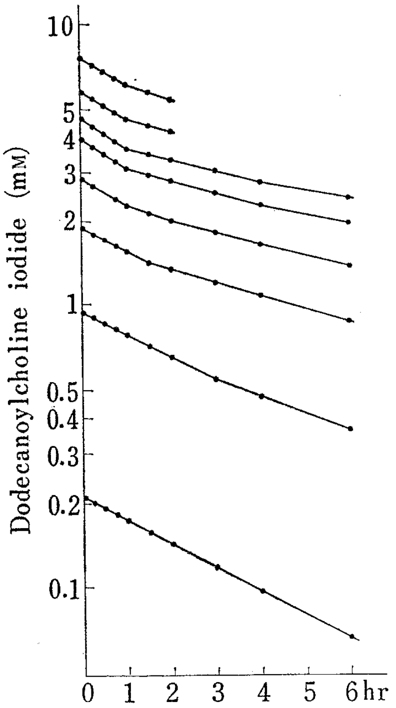


Fig. 8. Hydrolysis of Dodecanoylcholine Iodide in Borate Buffer Solution at 60°

initial concn. (mM)	initial pH	final pH
7.53	7.75	7.69 (after 2 hr)
5.70	7.74	7.70 (after 2 hr)
4.65	7.73	7.69 (after 6 hr)
3.81	7.72	7.71 (after 6 hr)
2.79	7.72	7.72 (after 6 hr)
1.90	7.74	7.73 (after 6 hr)
0.94	7.73	7.73 (after 6 hr)
0.21	7.75	7.75 (after 6 hr)

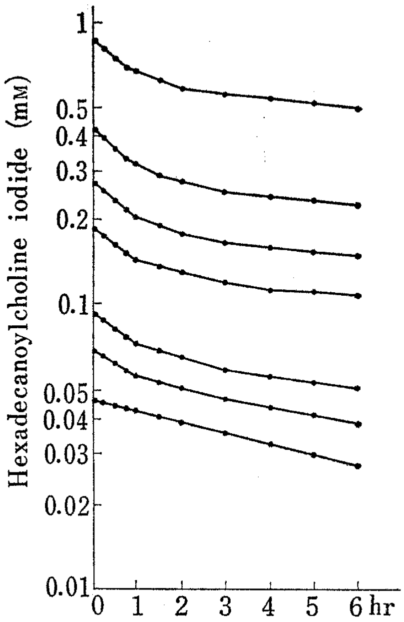


Fig. 9. Hydrolysis of Hexadecanoylcholine Iodide in Borate Buffer Solution at 60°

initial concn. (mM)	initial pH	final pH	
0.86	7.75	7.70	(after 6 hr)
0.42	7.75	7.74	(after 6 hr)
0.27	7.77	7.74	(after 6 hr)
0.18	7.77	7.74	(after 6 hr)
0.09	7.77	7.74	(after 6 hr)
0.07	7.75	7.74	(after 6 hr)
0.05	7.77	7.75	(after 6 hr)

Hydrolysis and change of pH values on OCI, DeCI, DoCI and HdCI were studied. On about 5 mM concentration, linear relationship was found between concentration (log scale) and time on OCI and slight curve was found after 4 hours on DeCI. Curve was found after 1 hour on DoCI and this phenomenon was found already below 1 mM on HdCI. It could not be considered that this phenomenon was ascribed to change of pH values. Examples of DoCI and HdCI were shown in Fig. 8, 9. Reaction time was shortened at high concentration of acylcholine to reduce effect of pH as possible. For example, reaction time was 2 hours above 5 mM concentration on DoCI.

The linear portion at initial stage and the gradually curved portion with course of time were discussed.

1) The Linear Portion at Initial Stage

Relationships between rate constants and solubilization of Sudan III on various concentrations of OCI, DeCI, DoCI and HdCI were shown in Fig. 10, 11.

As hydroxyl ion was attracted on micelle of acylcholine, hydrolysis was accelerated by formation of micelle, and it was observed that the longer the hydrocarbon chain was, the less the stability became as same as sodium, alkyl sulfate in acidic solution.[8] In this case, it was

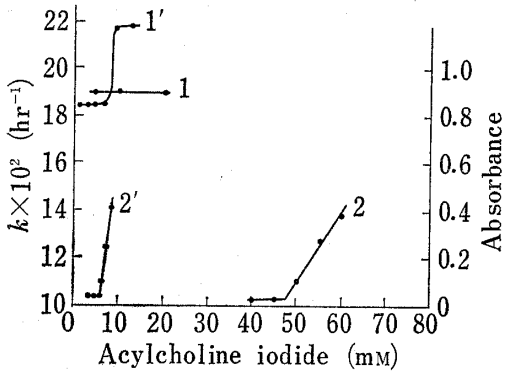


Fig. 10. Relationship between Rate Constant [Borate Buffer Solution(1, 1')] and Solubilization of Sudan III [0.07 M NaCl Solution (2, 2')] in Acylcholine Iodide Solution at 60°

1, 2 : octanoylcholine iodide
1', 2': decanoylcholine iodide

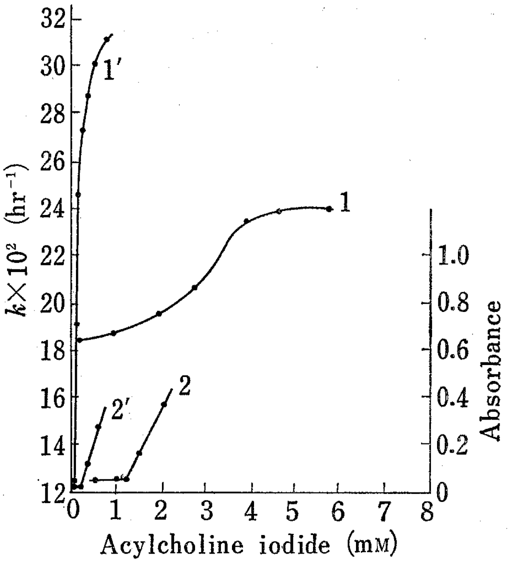


Fig. 11. Relationship between Rate Constant [Borate Buffer Solution(1, 1')] and Solubilization of Sudan III [0.07 M NaCl Solution(2, 2')] in Acylcholine Iodide Solution at 60°

1, 2 : dodecanoylcholine iodide
1', 2': hexadecanoylcholine iodide

considered that more attraction of hydroxyl ion occurred on micelle of acylcholine with longer hydrocarbon chain than that with shorter chain.

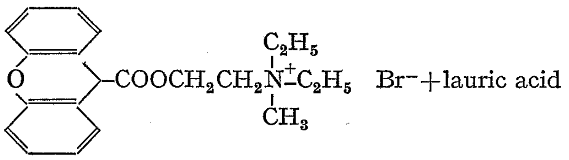
Form results in this study, more discussion was difficult. To more discuss these phenomena, more investigation was necessary.

2) The gradually Curved Portion with Course of Time

It was considered that fatty acid produced by hydrolysis of acylcholine in borate buffer solution was ionic type and it formed complex with cationic acylcholine, and repression of hydrolysis with course of time was ascribed to solubilization of this complex into micelle of acylcholine. For example, pK_a of lauric acid was 5.3 (20°)[13] and it was considered that complex was not formed in acidic solution.

To discuss this phenomenon, analogical experiment was carried out and formation of complex was presumed. Namely, methantheline bromide, quaternary ammonium compound which has absorption in ultra violet area, was used and effect of pH on formation of complex with lauric acid was studied. In this case, following chart was considered.

methantheline bromide



$\xrightarrow{\text{acidic solution}}$ no complex (insoluble in ethylene dichloride)

$\xrightarrow{\text{basic solution}}$ complex (soluble in ethylene dichloride)

Effect of pH on formation of methantheline laurate was shown in Fig. 12. The higher the pH value was, the more the amount of methantheline laurate which was extracted by

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[13] V. Nyren and E. Back, *Acta. Chem. Scand.*, 12, 1305 (1958).

ethylene dichloride became. When ethylene dichloride, in which lauric acid was not contained, was used, there was little amount of methantheline which was extracted by ethylene dichloride. Accordingly, it was presumed that complex was formed between DoCI and lauric acid produced by hydrolysis of DoCI in basic solution, but was not formed in acidic solution.

It was expected that if fatty acid participated with stabilization of acylcholine, more stabilization effect might be observed by addition of fatty acid. This result was shown in Fig. 13.

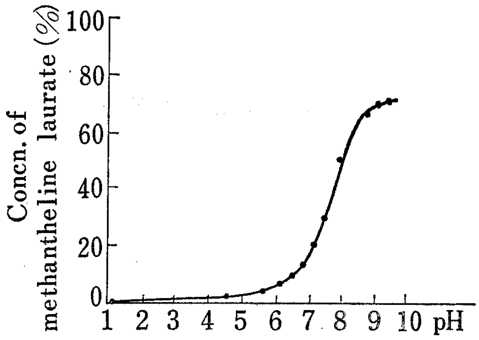

Fig. 12. Effect of pH on Formation of Methantheline Laurate

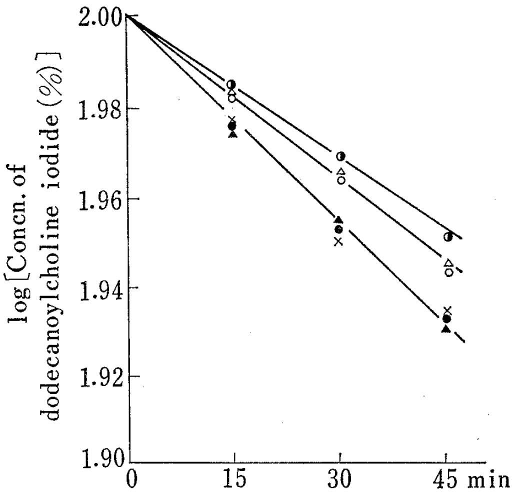

Fig. 13. Effect of Fatty Acid on Hydrolysis of Dodecanoylcholine Iodide in Borate Buffer Solution at 60°

●: no acid
▲: butyric acid
×: caproic acid
○: caprylic acid
△: capric acid
◐: lauric acid

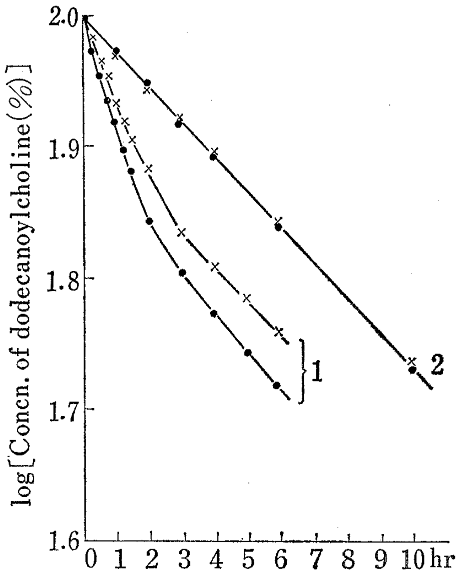

Fig. 14. Effect of Lauric Acid on Hydrolysis of Dodecanoylcholine Iodide in Borate Buffer Solution (1) and in 0.1N HCl Solution (2) at 60°

●: no acid
×: lauric acid

Little difference was observed among addition of butyric acid, that of caproic acid and no addition of acid, but apparent difference was observed between addition of lauric acid and no addition of acid.

Hydrolysis of DoCI in borate buffer solution and that in 0.1N hydrochloric acid solution were shown in Fig. 14. The apparent difference was observed between addition of lauric acid and no addition of acid in borate buffer solution, but the difference was not observed in 0.1N hydrochloric acid solution.

It was considered that the longer the hydrocarbon chain of fatty acid which formed complex was, the less the solubility of complex in water became and the more the solubility in micelle became, and fatty acid protected DoCI from hydroxyl ion. Nogami, Hasegawa and Iwatsuru[7] reported that hydrolysis of acylcholine was repressed by addition of sodium lauryl sulfate, and the longer the hydrocarbon chain of acylcholine was, the more the amount of complex distributed to micelle was, and the extent of stabilization effect was decided by the step of solubilization of complex into micelle.

Studies on stabilization of drugs by additives have already been reported, but in this study, product by hydrolysis participated with stabilization, so to speak autostabilization.

Acknowledgement The authors express their thanks to Dr. J. Hasegawa and Dr. S. Awazu for their valuable suggestions and discussions.