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Effect of Ascorbic Acid on the Intestinal Absorption of Bile Salts and Metabolism of Cholesterol in Guinea Pigs[1]

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Effect of ascorbic acid on *in situ* small intestinal absorption and hepato-biliary system of bile salts was investigated, by employing guinea pigs, for the purpose of explaining the physiological role of it to lower serum cholesterol. Firstly, the major bile acid and its salt were identified as chenodeoxycholic acid and its taurine conjugate, respectively. The critical micelle concentration (cmc) value of sodium chenodeoxycholate was estimated to be about 0.9 mM, suggesting approximately the same order as reported on others with di-hydroxy groups. Added ascorbic acid did not exert any effect on the cmc value. Sodium taurochenodeoxycholate was favorably absorbed from the ileum portion of guinea pigs, and the absorption there appeared to obey such a type of saturation kinetics as has been demonstrated with other bile salts. Ascorbic acid was observed to inhibit the absorption significantly and especially in the lower concentration region of sodium taurochenodeoxycholate than its cmc, where the percentage of inhibition was approximately 30 to 35. Double reciprocal treatment did not indicate any feasibility of competitive inhibition for the system of taurochenodeoxycholate and ascorbic acid. This effect of ascorbic acid should be explained neither by a change in physicochemical properties of the bile salt nor the direct action on intestinal mucosa, based on the results of cmc and additional pretreatment tests, but may be rather discussed by some participation in the hydrolysis and/or dehydroxylation of that salt occurring in the intestinal tissues other than mucosal membrane. Intraperitoneal administration of this vitamin and its dehydro form for 4 days enhanced the biliary amount of total chenodeoxycholate by about 60% of the control. Both *in situ* and *in vitro* experiments supported the *in vivo* results, so this metabolic aspect could be also recognized as one of the physiological functions of ascorbic acid which has been reported to lower serum cholesterol, in addition to suppressive effect on the ileal absorption of taurochenodeoxycholate.

The biological roles of ascorbic acid other than antiscorbutic activity have been extensively investigated in man, monkey, and guinea pig which are unable to synthesize it. Several reports on its physiological role have attended especially to the effect of lowering serum cholesterol. In guinea pigs, enhancement of cholesterol level in serum and whole body,[3] facilitation of its synthesis in adrenal and liver,[4] and suppression of its metabolism in liver[5] have been demonstrated in the condition of scurvy. Myasnikov has observed that ascorbic acid reduces the development of hypercholesterolemia and retards that of experimental lipoidosis of the aorta in rabbits,[6] and Samuel has suggested a similar effect in a part of patients with hypercholesterolemia and atherosclerosis.[7] On the other hand, Spittle has concluded that the rise in serum cholesterol noted in patients with atherosclerosis ingesting excessively large doses

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2) Location: *3-1, Tanabe-dori, Mizuho-ku, Nagoya, 467, Japan.*
3) *a*) S. Banerjee and H.D. Singth, *J. Biol. Chem.*, **233**, 336 (1958); *b*) R.E. Bolker, S. Fishman, R.D.H. Heard, V.J. O'Donnell, J.L. Webb, and G.C. Willis, *J. Exp. Med.*, **103**, 199 (1956).
4) *a*) A.R. Becker, H.B. Burch, L.L. Salomon, and C.G. King, *J. Am. Chem. Soc.*, **75**, 2020 (1953); *b*) R. Guchhait and N.C. Ganguli, *Biochim. Biophys. Acta.*, **51**, 607 (1961).
5) R. Guchhait, B.C. Guha, and N.C. Ganguli, *Biochem. J.*, **86**, 193 (1963).
6) A.L. Myasnikov, *Circulation*, **17**, 99 (1958).
7) P. Samuel and O.B. Shalchi, *Circulation*, **24**, 24 (1964).

of ascorbic acid is entirely due to mobilization of arterial cholesterol.[8] In spite of numerous works concerning with the activity of lowering serum cholesterol by this vitamin, the exact mechanism involved is not known yet.

Small intestinal absorption of ascorbic acid in guinea pigs has been revealed to obey a type of saturation kinetics which is analogous to that of active transport mechanism.[9] Fate of cholesterol synthesized or accumulated in liver is well known to circulate enterohepatically following a stepwise conversion to conjugated bile salts. Although several works have been extensively illustrating the enterohepatic circulation of bile salts,[10] little is established with respect to the effect of this vitamin on that.

The purpose of this work is to evaluate the effect of ascorbic acid on small intestinal absorption of bile salts, metabolism of cholesterol in liver, and biliary excretion of bile salts in guinea pigs with a view to finding an access to the mechanism involved in lowering serum level of cholesterol.

Experimental

Materials——L-Dehydroascorbic acid (DAA) was prepared as described previously.[9] Sodium taurochenodeoxycholate (STCDC) and sodium glycochenodeoxycholate (SGCDC) were synthesized from chenodeoxycholic acid (CDCA) by the method of Norman[11] and used after recrystalization. Cholesterol (Ch) and cholic acid (CA) were used also after recrystallization. Ascorbic acid (AA) and all other chemicals used were of analytical grade.

Chromatographic Identification of Bile Acids and Their Conjugates in Guniea Pigs——Bile, Plasma, and intestinal mucosa samples were obtained by bile duct cannulation with polyethylene tubing (size No. 4) for about 5 hr, centrifugation (3000 rpm, 10 min) of cardiac blood, and homogenization of the epithelia following the method of Oda,[12] respectively. Each sample of 2 ml was treated in the following two ways: 1) extraction into 3 ml of ethylether after saponification with 1 ml of 2 N NaOH at 100° for 4 hr and acidification with 1 ml of 4N HCl, 2) extraction into 2 ml of *n*-butylalcohol after acidification with 2 ml of 4 N HCl. Extracted bile acids and their conjugates were analyzed by means of thin-layer chromatography (TLC) conducted in almost the same manner as described by Hofmann.[13] Authentic samples were used in the solution of ethylalcohol. Chromatographic conditions are given in the section of results.

Determination of Critical Micelle Concentration (cmc) for STCDC——In order to estimate the cmc value of STCDC in the same condition as the small intestinal recirculation tests, the reaction mixture was prepared in isotonic phosphate buffer solution (pH 7.4) containing 0.2% macrogol 4000 (MG). The cmc value was determined by the measurement of maximum absorbance of rhodamine 6G with or without 10 mM of AA at 37° according to the method of Carey.[14]

Absorption Experiments——After treatments of male Hartley guinea pigs weighing 300 to 350 g with fasting, anesthetization, small intestinal cannulation, and pre-recirculation in a similar manner to that reported previously,[9] the isotonic drug solution (pH 7.4) was continuously circulated through the tract for 2 hr. In order to evaluate a segmental specificity, jejunum and ileum portions were, for convenience, regarded as upper two-fifth and lower three-fifth parts of the small intestine which is the tract between Treitz's ligament and ileocecal valve. The duodenum-jejunum or ileum portion was employed for the experiments. Bile fistulation was applied to all animals tested. Drug solutions were prepared to be 0.5 to 5 mM by dissolving appropriate amounts of STCDC in the phosphate buffer solution which contained 0.2% MG as an indicator for water transport and were recirculated through the tracts. Effect of added AA (10 mM) on the ileal absorption of STCDC was examined in the same way as above. One hr pretreatment of ileum with 10 mM of AA was also made and then followed by 2 hr recirculation of drug solution immediately after washing with 50 ml of 0.9% NaCl solution. Two ml of the sample solution was withdrawn periodically and the concentrations of STCDC and MG were determined.

8) C.R. Spittle, *Lancet*, **1**, 798 (1972).
9) K. Iwamoto, N. Ozawa, Y. Hayashi, T. Tsukamoto, and J. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **24**, 2021 (1976).
10) *a*) S. Lindstedt, *Acta Physiol. Scand.*, **40**, 1 (1957); *b*) S. Bergström, *Fed. Proc.*, **21**, 28 (1962); *c*) B. Borgström, G. Lundh, and A. Hofman, *Gastroenterology*, **45**, 229 (1963); *d*) R.H. Dowling, *ibid.*, **62**, 122 (1972); *e*) D.M. Small, R.H. Dowling, and R.N. Redinger, *Arch. Intern. Med.*, **130**, 552 (1972).
11) A. Norman, *Arkiv fiir Kemi*, **8**, 331 (1955).
12) T. Oda, S. Seki, and S. Watanabe, *Acta Med. Okayama*, **23**, 357 (1969).
13) A.F. Hofmann, *J. Lipid Res.*, **2**, 127 (1962).
14) M.C. Carey and D.M. Small, *J. Colloid & Interface Sci.*, **31**, 382 (1969).

Measurements for Hepatic Metabolism of Ch and Biliary Excretion of Total CDCA——These experiments were carried out in the following three ways.

1) *In Vivo* Tests: Drug solutions prepared in 0.9% NaCl solution were injected intraperitoneally to each animal as 88.1 and 87.1 mg per kg of AA and DAA, respectively, every 12 hr for 4 days. Bile was collected for 5 hr on the fifth day and 1 ml of that was used for analysis. Control animals were treated in the same as above except injection of 0.9% NaCl solution instead of drug one. Preliminary identification of bile acids manner and their salts were conducted by means of the TLC method.

2) *In Situ* Tests: Bile collections were made for 1, 2, and 2.5 hr before, during, and after the *in situ* ileal recirculation tests that were performed as described above, respectively. In this experiment, three types of drug solutions of STCDC (5 mM), AA (10 mM), and STCDC (5 mM) with AA (10 mM) were employed for the recirculation. Control recirculation was done with the phosphate buffer solution. One ml of the bile was used for analysis.

3) *In Vitro* Tests: Liver homogenates (10 w/v%) of guinea pigs were prepared customarily in Tris buffer solution (pH 7.4). The suspension of Ch and solution of AA and co-factors were also prepared in Tris buffer solutions as described by Guchhait.[5] The reaction mixtures that are shown in Table I were incubated at 37° for 2 hr. Five ml of the reaction mixture was withdrawn periodically and used for analysis.

Table I. Composition of Incubation Media

Component[a]	ml contained in			
	Control run		AA run	
	Sample	Reference	Sample	Reference
Liver homogenate (10 w/v %)	15	15	15	15
NAD soln. (0.5—50 mM)	5	5	5	5
AMP soln. (20 mM)	5	5	5	5
Nicotinamide soln. (200 mM)	5	5	5	5
L-Cysteine soln. (40 mM)	5	5	5	5
Ch suspension (1 or 5 mM)	10	—	10	—
AA soln. (1—100 mM)	—	—	5	5
BSA soln. (2.5 or 5 w/v%)	—	10	—	10
Tris buffer soln. (pH 7.4)	5	5	—	—

a) All were prepared in Tris buffer solution (pH 7.4)
NAD: nicotineamide adenine dinucleotide
AMP: adenosine monophosphate
BSA: bovine serum albumin (Fraction A)

Analytical Procedures——The concentrations of STCDC in the absorption experiments and total CDCA in bile were determined following the method of Eriksson.[15] One ml of bile or supernatant of the perfusion sample treated with 1 ml of 6% trichloroacetic acid were added to 3 ml of 65% H_2SO_4 and spectrophotometric measurements for these mixtures heated at 60° for 30 min were made at 390 nm. Another 2 ml of the perfusion sample was used for the analysis of MG according to the method of Hyden.[16] The concentration of Ch in the incubation mixture was determined by the method of Pearson.[17] The mixture of the sample and 1 ml of 2 N HCl was extracted with 4 ml of ethylether, and then spectrophotometric measurements for Ch extracted and treated in several steps[17] were made at 616 nm.

Results and Discussion

Identification and cmc Value of Bile Salts

Results of TLC analyses are shown in Fig. 1. Although CA was found in all biological materials tested in guinea pigs, CDCA was recognized as major component of free bile acids in both of bile and intestinal mucosa. Furthermore, it was confirmed that the taurine conjugate of CDCA could be the major one of bile salts encountered in the animal. Synthesized STCDC was utilized as a bile salt in all the experiments reported hereafter unless otherwise specified. The *Rf* values almost agreed with those reported by Hofmann[13] and one unidentified

15) S. Eriksson and J. Sjöval, *Arkiv für Kemi*, **8**, 303 (1955).
16) S. Hyden, *Kgl. Lantbruks-Hogskol. Annlr.*, **22**, 139 (1956).
17) S. Pearson, S. Stern, and T.H. McGavack, *Anal. Chem.*, **25**, 813 (1953).

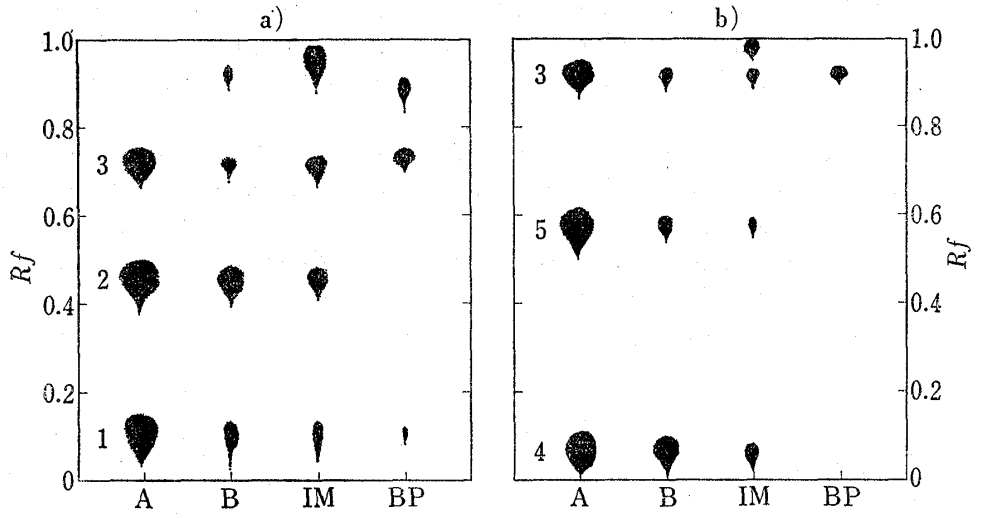

Fig. 1. Thin-Layer Chromatography of Bile Acid and Their Related Components in Various Biological Materials

A) authentic sample: 1) CA, 2) CDCA, 3) Ch, 3) STCDC, 5) SGCDC
B) bile, IM) intestinal mucosa, BP) blood plasma
plate: Silica gel G (Merck) coated in 250 μ and activated at 120° for 1 hr
solvents: a) acetic acid: carbontetrachloride: di-isopropylether: isoamylacetate: *n*-propanol: benzene (1: 4: 6: 8: 2: 2)
b) propionic acid: isoamylacetate: water: *n*-propanol (3: 4: 1: 2)
spray reagent: 10 w/v percent solution of phosphomolybdic acid (in ethanol)
treatment: heating (120°, 5 min)

component moved at the nearest to front should be considered to be Ch acetate, from its *Rf* value.

From metachromatic shift of rhodamin 6G, cmc values for STCDC in the phosphate buffer solution were estimated to be 0.90 mM and 0.93 mM with and without 10 mM of AA, respectively. Hence it is well demonstrated that AA has little effect on the cmc, a typical one of the physicochemical properties peculiar to bile salts. The cmc value obtained for STCDC is rather similar to those reported on some taurine conjugates of other dihydroxy bile acids.[18]

Effect of AA on Small Intestinal Absorption of STCDC

Segmental specificity for the absorption of STCDC was first tested. Fig. 2 indicates that the ileal absorption is much faster than the absorption from duodenum and jejunum. Although the absorption from both sites obeyed apparently first order kinetics, absorption ratios were approximately 30% and 90% for 2 hr perfusion through duodenum-jejunum and ileum, respectively. Among numerous investigations on the small intestinal absorption of bile salts, the specific absorption from and/or transport across ileum has been extensively demonstrated in rat, guinea pig, hamster, and man.[19] These works are, however, carried out mainly by *in vitro* tests, except a part. Lack,[19a] Holt,[19c] Playoust,[19d] and Tyor[19f] have suggested active transport mechanism of some bile salts other than STCDC from the findings that the system has a transport maximum and is inhibited by anoxia and some active transport inhibitors such as 2,4-dinitrophenol, sodium azide, KCN, and sodium iodoacetate. On the other hand, little but significant absorption found in duodenum-jejunum portion is considered

18) *a*) A.F. Hofmann and D.M. Small, *Ann. Rev. Med.*, **18**, 333 (1967); *b*) K. Kakemi, H. Sezaki, R. Konishi, T. Kimura, and M. Murakami, *Chem. Pharm. Bull.* (Tokyo), **18**, 275 (1970).

19) *a*) L. Lack and I.M. Weiner, *Am. J. Physiol.*, **200**, 313 (1961); *b*) I.M. Weiner and L. Lack, *ibid.*, **202**, 155 (1962); *c*) P.P. Holt, *ibid.*, **207**, 1 (1964); *d*) M.R. Playoust and K.J. Isselbacher, *J. Clin. Invest.*, **43**, 467 (1964); *e*) J.M. Dietschy, H.S. Salomon, and M.D. Siperstein, *ibid.*, **45**, 832 (1966); *f*) M.P. Tyor, J.T. Garbutt, and L. Lack, *Am. J. Med.*, **51**, 614 (1971).

to be compatible with the results by Hislop, Gordon and Krag[20] who have suggested that passive ionic or micellar diffusion involved in the jejunal part might be another contributory factor in the small intestinal absorption of bile salts responsible to their enterohepatic circulation.

In order to know *in situ* absorption mechanism of STCDC from the ileal portion and effect of AA on it in guinea pigs, its dependence on initial concentration was examined with and without 10 mM of AA. Absorption ratio for 2 hr recirculation ($(C_o-C_t)/C_o$) which were appropriately corrected by the change of MG concentration are given, in percentage, in Table II. Absorption rate (v) is calculated by Eq. 1:

$$v = (\text{absorption ratio})(C_o)/(L)(t) = (C_o-C_t)/(L)(t) \quad (1)$$

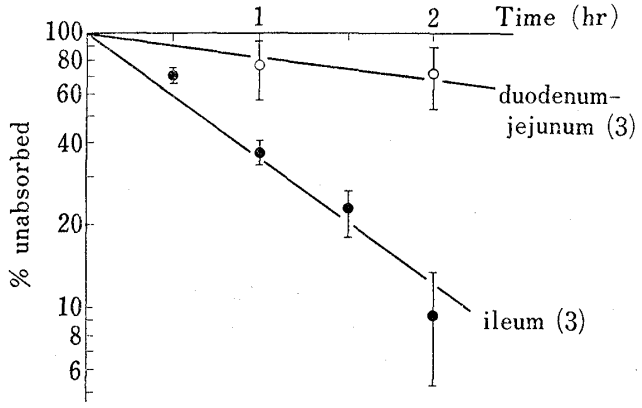

Fig. 2. Segmental Specificity for Small Intestinal Absorption of STCDC

initial concentration of STCDC: 1 mM
vertical bar: standard deviation
number in parentheses: number of runs

Table II. Absorption Percentage of STCDC with or without 10 mM of AA

$100(C_o-C_t)/C_o$	
Control (C_o, mM)	with 10 mM of AA (C_o, mM)
80.0±18.9[a] (0.47)	56.1±21.6[a] (0.41)
79.4±10.7 (0.72)	39.0± 7.5 (0.57)
75.3±15.8 (0.80)	56.2±14.1 (0.67)
69.9±10.6 (2.02)	50.8±12.7 (1.84)
47.7± 6.9 (4.15)	50.6± 3.7 (3.16)
32.6±10.9 (5.67)	37.6± 6.9 (5.14)

a) standard deviation

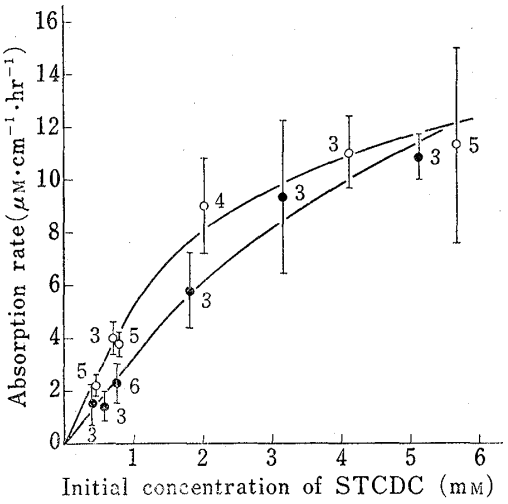

Fig. 3. Effect of Initial Concentration on Ileal Absorption of STCDC with or without AA

vertial bar: standard deviation
number in parentheses: number of runs
—○—: without AA
—●—: with 10 mM of AA

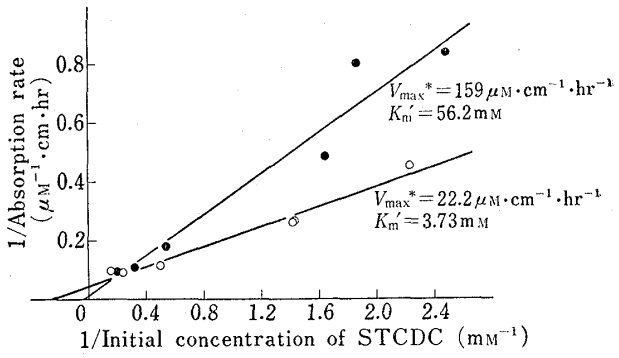

Fig. 4. Lineweaver-Burk Type Plots for Ileal Absorption Rate of STCDC with or without AA

○: without AA ●: with 10 mM of AA

20) *a*) I.G. Hislop, A.F. Hofmann, and L.J. Shoenfield, *J. Clin. Invest.*, **46**, 1070 (1967); *b*) S.G. Gordon, P.B. Miner, Jr., and F. Kern, Jr., *Biochim. Biophys. Acta.*, **248**, 333 (1971); *c*) E. Krag and S.F. Phillips, *J. Clin. Invest.*, **53**, 1686 (1974).

where C_0 and C_t are concentrations of initial (at $t=0$) and remaining (at $t=t$) drug respectively and L is whole length of ileum used for recirculation. Absorption rates were plotted against initial concentration of STCDC, as illustrated in Fig. 3. Table II and Fig. 3 indicate two points to be discussed: 1) The ileal transport of STCDC approaches a maximum rate with increasing load of it; 2) The ileal ability to absorb STCDC is apparently suppressed by AA. The first indication is reasonably explained by considering passively diffusive and actively saturative processes which have been proposed in *in vitro* and *in vivo* absorption experiments of taurocholate and cholate.[19e]. Other indication is confirmed by Lineweaver-Burk's type plots for the absorption rates, as shown in Fig. 4. These straight lines satisfied the general equation derived from enzyme reaction,[21] but they did not apparently indicate any involvement of competitive inhibition by AA. The apparent Michaelis constant, K_m', was calculated

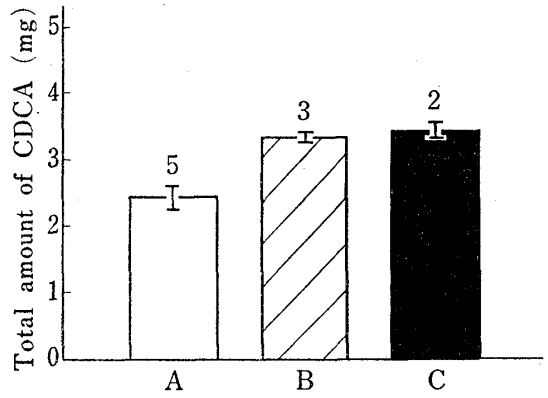

Fig. 5. Effect of AA and DAA on Total Amount of CDCA Excreted in Bile

A: control bile, B: bile collected after AA administration,
C: bile collected after DAA administration
vertical bar: standard deviation
number in parentheses: number of runs

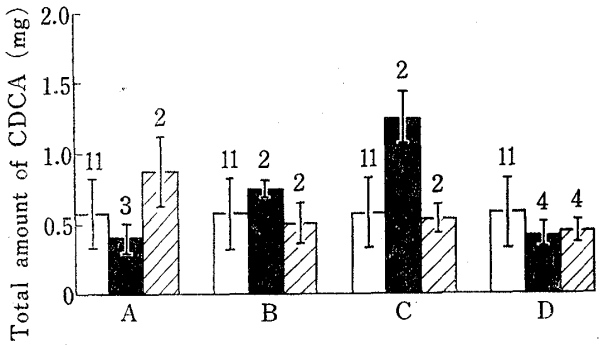

Fig. 6. Biliary Excretion Profile of CDCA under Various Conditions of Ileal Recirculation

ileal perfusion with (A) pH 7.4 phosphate buffer solution
(B) 5 mM of STCDC in buffer solution
(C) 5 mM of STCDC and 10 mM of AA in buffer solution
(D) 10 mM of AA in buffer solution
vertical bar: standard deviation
number in parentheses: number of runs
□: 1 hr-Bile collected before perfusion
■: 2 hr-Bile collected during perfution
▨: 2.5 hr-Bile collected after perfusion

TABLE III. Effect of Pretreatment with AA on Ileal Absorption of STCDC

Pretreatment	% absorbed in 2 hr[a)]
without AA	74.3±5.5[b)] (2)[c)]
with 10 mM of AA	78.5±4.2 (3)

a) initial concentration of STCDC=1 mM
b) standard deviation
c) number of runs

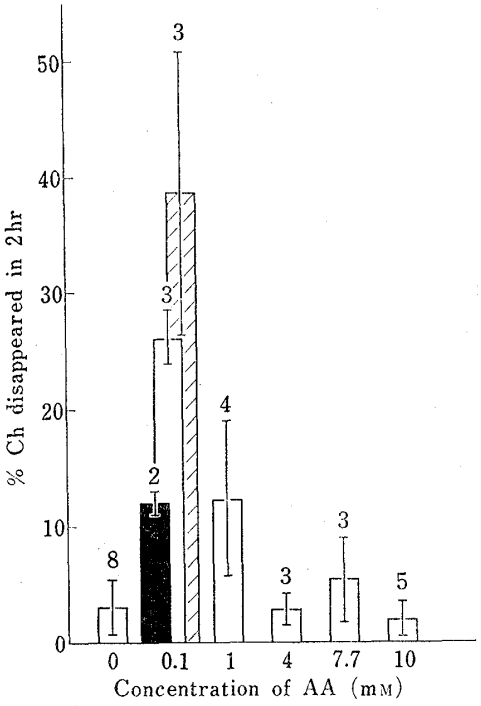

Fig. 7. Effect of AA and NAD on Disappearance of Ch from Liver Homogenate Incubation Medium

incubation condition: at pH 7.4 and 37°
vertical bar: standard deviation
number in parentheses: number of runs
concn. of added NAD:
■: 0.5 mM □: 5 mM ▨: 50 mM

21) $1/v=1/V_{max}^*+(K_m'/V_{max}^*)1/C_o$, where V_{max}^* and K_m' are apparent values for maximum rate and Michaelis constant, respectively.

to be 56 mM and 3.7 mM of STCDC with and without 10 mM of AA, respectively. A saturation kinetics demonstrated here for the ileal absorption of STCDC is extremely compatible with those reported on taurocholate[19a,e,22] and taurodeoxycholate.[22] Lack and Schiff[23] have suggested that a nuclear hydroxyl group is essential for active transport and, further, that the maximum rate of such transport is directly related to the number of hydroxyl group on the bile acid molecule.

Inhibitory effect by AA is considered to be limited especially to relatively lower concentration range of STCDC than cmc, and was significant there ($p<0.02$—0.2). Since pretreatment of ileum with 10 mM of AA did not have any significant effect as indicated in Table III, AA seems to have no direct action to harm an intact ability of the ileal mucosa to absorb STCDC. Gustaffson and Hill have reported that the intestinal flora is capable both to hydrolyze and to dehydroxylate some bile salts.[24] Further inspection would be necessary to know the exact mechanism of an inhibitory effect by AA, including a consideration of its nutritive effect on the intestinal flora. However, the inhibitory effect of AA on the ileal absorption of STCDC should be accounted to explain a part of the physiological roles of AA which has been reported to act against hypercholesterolemia, based on ileo-hepatic circulation of bile salts which are derived from Ch.

Effect of AA on Hepatic Metabolism of Ch and Biliary Excretion of Total CDCA

Preliminary TLC analyses of bile acids and their salts excreted in bile following *i.p.* administration of AA or DAA resulted in almost the same identification as shown in Fig. 1, except that SGCDC appeared to be excreted a little more than it is shown in Fig. 1. Fig. 5 indicates the total amount of CDCA excreted in the bile following the administration of AA and DAA. Both drugs displayed a significant effect in enhancing the biliary CDCA and/or its conjugates which have been proved to be derived from the stepwise biotransformation of Ch in liver.[25] Approximately the same extent of the effect by AA and DAA on equimolar base implies that either form of them, forming a readily reversible oxidation-reduction system, may act on the hepato-biliary system for Ch and/or STCDC.

In order to obtain further details of the effect by AA or DAA that is shown in Fig. 5, biliary excretion of total CDCA and metabolic disappearance of Ch were tested under *in situ* perfusion and *in vitro* incubation experiment, respectively. Fig. 6 indicates slight, remarkable, and no increases in the excretion of total CDCA with respect to the ileal perfusion of STCDC, STCDC plus AA, and AA. The slight increase caused by the loading of STCDC is suggested to be a similar result to that of Daniellsson and Elliott,[25] and this biliary excretion of total CDCA was much facilitated by adding AA to the recirculation solution of STCDC. The disappearance of Ch in *in vitro* incubation tests is shown in Fig. 7. Relatively lower concentration (0.1—1 mM) of AA was revealed to enhance the disappearance from the medium. The disappearance of Ch was, furthermore, enchanced by increasing the concentration of NAD used as a co-factor. Since metabolic pathway of Ch is reported to localize in the microsomal fraction of the liver,[25b] the results cited above are well recognized as a part of *in vivo* metabolic pathway.

Acknowledgement The authors wish to express deep gratitude to emeritus Prof. T. Tsukamoto for his useful suggestion and discussion on this work.

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23) *a*) L. Lack and I.M. Weiner, *Fed. Proc.*, **22**, 1334 (1963); *b*) E.R. Schiff and J.M. Dietschy, *J. Clin Invest.*, **47**, 87a (1968).
24) *a*) B.E. Gustaffson, T. Midvedt, and A. Norman, *J. Exp. Med.*, **123**, 413 (1966); *b*) M. J. Hill and B.S. Drasar, *Gut*, **9**, 22 (1968).
25) *a*) H. Daniellsson and T. Kazuno, *Acta Chem. Scand.*, **13**, 1141 (1959); *b*) W.H. Elliott and P.M. Hyde, *Am. J. Med.*, **51**, 568 (1971).