mel. This gummy product was refluxed in 10 ml of 0.1N sodium methoxide for 10 min and the solution was neutralized with 1N hydrochloric acid. After evaporation of the solvent, the product was dissolved in 20 ml of water, and extracted twice with 10 ml of chloroform. The aqueous layer was concentrated to about 5 ml and was applied to the Dowex 1×4 (OH^-) resin column (2 cm × 7 cm). The column was washed with 300 ml of water and eluted with 50% methanol. Fractions which showed a UV λ_{max} at 272 mμ were collected. The combined solutions were concentrated to dryness *in vacuo* giving 184 mg of a crystalline residue which was recrystallized from 95% methanol, giving 157 mg of 1-(β-D-arabinofuranosyl)cytosine (VII). mp 212—213° (uncorr.). $[\alpha]_D^{20}$ +146° (c=1.04, H_2O). UV $\lambda_{max}^{0.1N\ HCl}$ mμ (ε): 280.5 (9910); $\lambda_{max}^{H_2O}$ mμ (ε): 271.3 (9290); $\lambda_{max}^{0.1N\ NaOH}$ mμ (ε): 273.9. (9920). NMR (in DMSO-d_6 TMS internal 60 Mc) (δ): 7.63 (1H, doublet, J=7.5 cps, C_5-H), 7.03 (2H, singlet, NH_2), 6.07 (1H, doublet, J=4.0 cps, C_1-H), 5.72 (1H, doublet, J=7.5 cps, C_6-H). IR no absorption band corresponding to the C_6 carbonyl group. *Anal.* Calcd. for $C_9H_{13}O_5N_3$: C, 44.44; H, 5.39; N, 17.27. Found: C, 44.87; H, 5.53; N, 17.00.

(b) VII From Tri-O-acetyl-AraU (IVb): Anhydrous diethylaniline hydrochloride (14.8 g) was added to a solution containing 74 ml of ethyl acetate and 74 ml of phosphorous oxychloride and the mixture was refluxed for 10 min. Ten grams of IVb was added to the solution. After refluxing the reaction mixture protected by a drying tube for 7 hr, the solvent was removed under reduced pressure in an oil bath adjusted to 70—80°. Further evaporation was continued for about 30 min. The residue was dissolved in 270 ml of chloroform and saturated with dry ammonia gas at 0° with vigorous stirring. The resulting inorganic salts were filtered off under dry conditions. The resulting solution was saturated again with dry ammonia gas at 0° and allowed to stand at room temperature. After 13 hr, the resulting inorganic salts were removed by filtration and the filtrate was evaporated to dryness *in vacuo*. The residual viscous solution was dissolved in 100 ml of methanolic ammonia (saturated at −10°) and allowed to stand at room temperature for 18 hr. The solution was evaporated to dryness *in vacuo*. The residue was extracted twice with 200 ml of hot benzene.

The powdery product was dissolved in small amount of water and applied to a Dowex 1×4 (OH^-) resin (200 ml) column. The column was washed with one liter of water and eluted with 5 liter of 50% methanol. Ara-C fractions were collected and concentrated to dryness. Crude Ara-C was recrystallized from 95% methanol using active charcoal to afford 4.6 g of Ara-C. All physical properties of this compound were identical with those described above.

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Mechanism of the Intestinal Absorption of Drugs from Oil in Water Emulsions. III.[1] Absorption and Biotransformation of Methyl Orange

Kiichiro Kakemi (the late), Hitoshi Sezaki, Hiroyasu Ogata, and Chieko Nagai

Faculty of Pharmaceutical Sciences, Kyoto University[2]

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The sulfonated water-soluble azo dyes are widely used as colorings for foods and pharmaceutical preparations. As many azo dyes are potent carcinogens, and some azo dyes are known to be toxic, much attention have recently been paid to their biological dispositions. Scheline and Longberg[3] reported on the absorption, metabolism, and excretion of a sulfonated

1) Part II: K. Kakemi, H. Sezaki, S. Muranishi, H. Ogata, and S. Isemura, *Chem. Pharm. Bull.* (Tokyo), **20**, 715 (1972).
2) Location: *Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*
3) R.R. Scheline and B. Longberg, *Acta Pharmacologica et Toxicol.*, **23**, 1 (1965).

azo dye, acid yellow, by rats. They found that only about 1% of the dose was found unchanged in the urine, and no dye was present in the feces after its oral administration. On the contrary, intraperitoneal administration of the same dye resulted in rapid urinary excretion of the dye, but none of its metabolite. They suggested that the dye was reduced by the intestinal flora and its reduction products were absorbed. Enzymatic reduction of azo dyes by the gut flora has been reported by many authors.[4] On the other hand, other possibilities of azo dye transport may be suggested. Kunze and Vogt[5] reported the intestinal absorption of phenol red in the rat and found that an active transport of very low capacity was mediating. Also, the possibility of biotransformation of azo dyes in the intestinal membrane seems to exist.

It has been found in our laboratory that absorption of methyl orange, a water-soluble azo dye, was enhanced when the dye was circulated through the intestine of the rat as oil in water emulsions.[6] This paper is a report of the results of the large intestinal absorption and biotransformation of methyl orange which is necessary for the elucidation of the mechanism of absorption of the dye from oil in water emulsions and from surfactant micellar solutions.

Experimental

Materials——Methyl orange and sulfanilic acid were commercial samples of reagent grade and were chromatographically pure. FMN, triphosphopyridine nucleotide (NADP), and glucose-6-phosphate were obtained from Sigma Chemical Co., Ltd. Polysorbate 80 was obtained from Tokyo Kasei Co., Ltd. Other chemicals used were of reagent grade quality.

Preparation of *in Situ* Large Intestinal Loop——Male Wistar rats weighing 170—220 g were used in all experiments. The rats were treated similarly as in *in situ* large intestinal absorption experiment described in our previous paper.[1] Two ml of sample solution was injected into the loop. After one hour, drug remaining in the lumen was collected as completely as possible by washing out with pH 7.4 phosphate buffer solution, and analyzed.

Preparation of *in Vitro* Large Intestinal Loop——Freshly excised loop of rat large intestine, 10—15 cm in whole length, was tied at one end and 2.0 ml of drug solution was placed within the loop. The other end of the loop was then tied and the entire loop was immersed in 3 ml of pH 7.4 phosphate buffer solution and incubated at 37°. In the case of an everted sac, same procedure was followed except that within the everted sac, 2 ml of pH 7.4 phosphate buffer solution was placed and the sac was immersed in 3 ml of drug solution at 37°. At the end of appropriate time interval, the loop or the sac, and the bathing solution were removed completely for analyses.

Stability of Methyl Orange in incubated Medium——*In vitro* loop or everted sac was prepared by the procedure as described elesewhere. Within the loop or the sac, 2 ml of pH 7.4 phosphate buffer solution was injected. The loop or the sac was immersed in 6 ml of pH 7.4 phosphate buffer solution and incubated for one hour at 37°. After incubation, the outer bathing medium was removed and methyl orange was dissolved in the aliquot of the medium. Overall volume of the methyl orange solution was adjusted to 10 ml and methyl orange concentration to 300 mcg/ml. The solution was incubated at 37° and 0.5 ml samples were taken at appropriate time intervals.

Stability of Methyl Orange in the intestinal Homogenate——Large intestines freshly removed from three rats were minced and homogenized with pH 7.4 phosphate buffer solution by Potter Elvehjum glass homogenizer. The homogenate was diluted to 50 ml with the buffer solution. Appropriate amount of methyl orange was added to 15 ml of the diluted homogenate and the volume of the homogenate was adjusted to a final volume of 30 ml. The incubation was carried out immediately at 37°.

Assay Methods——1) *In Situ* Loop Preparations: When the concentration of methyl orange was 10 to 30 mcg/ml, 25 ml of the collected solution from the lumen was removed and 5 ml of isoamyl alcohol and 10 g of sodium chloride were added to the solution. When the concentration of methyl orange was 60 mcg/ml or more, 6 ml of isoamyl alcohol and 2.5 g of sodium chloride were added to 5 ml of the collected solution. The mixture was shaken and centrifuged. Three ml of the separated organic layer was mixed with 1 ml of ethanol containing 4v/v% of hydrochloric acid. The mixture was centrifuged and the optical density of the clear supernatant was read at 525 mμ.

4) R.R. Scheline, *Acta Pharmacologica et Toxicol.*, **26**, 332 (1968); *idem*, *J. Pharm. Sci.*, **57**, 2021 (1968); A.J. Ryan, J.J. Roxon, and A. Sivayavirojana, *Nature*, London, **219**, 854 (1968).
5) H. Kunze and W. Vogt, *Naunyn-Schmiedbergs Arch. Pharmak. u. Exp. Path.*, **256**, 139 (1967).
6) H. Ogata, to be published.

2) *In Vitro* Loop: The loop or the sac, and outer solution were homogenized with Potter Elvehjem glass homogenizer. The homogenate was made to a volume of 30 ml with pH 7.4 phosphate buffer solution. When the concentration of methyl orange of test solution was 300 mcg/ml, 6 ml of isoamyl alcohol and 2.5 g of sodium chloride were added to 5 ml of the homogenate and shaken for 20 minutes. When methyl orange concentration was 50 mcg/ml, 25 ml of the homogenate was removed and 6 ml of isoamyl alcohol and 10 g of sodium chloride were added and shaken for 20 minutes. After centrifugation, 3 ml of the organic layer was removed and 1 ml of ethanol containing 4 v/v% of hydrochloric acid was added to the organic layer and centrifuged. The clear supernatant was read at 525 mμ. In this case, methyl orange was not given 100% recovery and standards were always run simultaneously to correct the incomplete recovery. As tissue blank could not be removed completely, average tissue blank obtained with the way as described above without methyl orange was substituted for the tissue blank at each run.

Paperchromatography——The inner and the outer solutions of zero and 4 hours sample in *in vitro* loop procedures were collected and freeze-dried. The remainder was dissolved in ethanol and filtered through a Büchner funnel. The filtrate was evaporated under reduced pressure. And again the remainder was dissolved in ethanol. This procedure was repeated until no precipitate remains in ethanol. The samples obtained were chromatographed on paper with *n*-butanol saturated with pH 3.5 citrate buffer solution, and concentrated hydrochloric acid–*n*-butanol (1:4) as the ascending solvents. The paper was air-dried and the amines were detected by spraying with Ehlrich reagent or nitrous acid followed by alpha naphthylamine. Metabolites obtained after repeated chromatography were eluted from paper with distilled water and the pH of the solution was made alkaline, and ultraviolet spectra were measured on Hitachi recording spectrophotometer.

Result and Discussion

Disappearance of Methyl Orange from in situ Large Intensinal Loop

As shown in Fig. 1, it seems that methyl orange disappears from the large intestinal perfusate with a concentration dependent and an independent processes. In Fig. 1 (b), at high concentrations of methyl orange, total amount of methyl orange disappeared from the loop was in proportion to the bulk concentration of methyl orange. The amount of methyl orange disappeared with concentration independent process at each methyl orange concentration was obtained by drawing a straight line parallel to the total amount curve at high methyl orange concentration region. The amount of methyl orange disappeared with concentration dependent process was obtained by subtracting the amount of disappeared with concentration independent process from the total amount disappeared. Michaelis-Menten kinetics applied to the amount of concentration dependent process is presented in Fig. 1

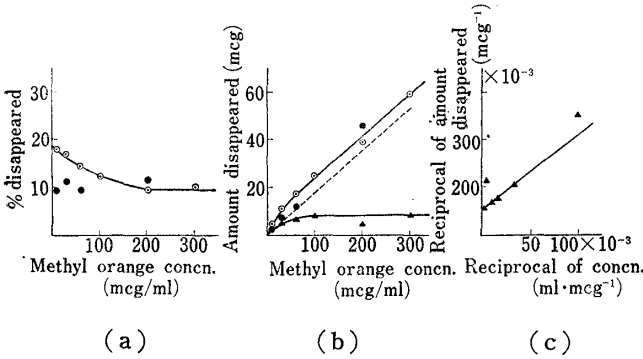

Fig. 1. Methyl Orange Disappearance from *in Situ* Large Intestinal Loop

(a) Concentration dependency of methyl orange disappearance in the presence (●) and absence (⊙) of 0.01% Polysorbate 80.
(b) Amount of methyl orange disappeared with concentration dependent process (▲) and with concentration independent process (------). ⊙, ● represent total amount of methyl orange disappeared in the absence and presence of Polysorbate 80, respectively.
(c) Lineweaver-Burk plot of methyl orange disapperance with concentration dependent process

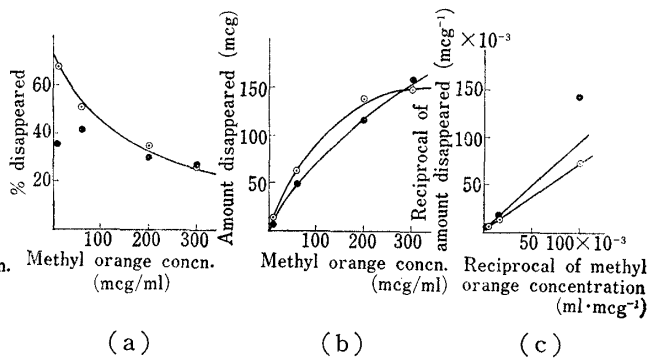

Fig. 2. Methyl Orange Disappearance from *in Vitro* Large Intestinal Loop Incubation System

(a) Concentration dependence of methyl orange disappearance in the presence (●) and absence (⊙) of 0.01% Polysorbate 80.
(b) Amount of methyl orange disappeared in the presence (●) and absence (⊙) of 0.01% Polysorbate 80 as a function of methyl orange concentration.
(c) Lineweaver-Burk plot of methyl orange disappearance in the presence (●) and absence (⊙) of 0.01% Polysorbate 80.

(c), in which values of 10 mcg/60 min and 0.3 mcg/ml were obtained for V_{max} and K_m, respectively. It is interesting to note that by the addition of a very low concentration, concentration close to critical micellar one, of polysorbate 80, the process seems to be inhibited completely and methyl orange disappears only by the concentration independent process. When methyl orange solution was injected into the loop and the drug was collected and assayed immediately, the recoveries were 98% or more. It was also confirmed that the amount of the drug adsorbed on the intestinal mucosal surface during absorption experiment and left intact in the intestinal membrane by the washing-out procedure with the buffer solution at the end of the experiment was negligible. Several mechanisms of the concentration dependent process, namely, the first, an active transport, the second, decomposition by the intestinal flora, and the third, biotransformation by mucosal enzymes, will be proposed.

Disappearance of Methyl Orange from in vitro Large Intestinal Loop

Fig. 2 represents total decrease of methyl orange from inside of the loop, the tissue itself, and the bathing buffer solution.

Fig. 2 (a) and (b) give concentration dependency of methyl orange disappearance for 135 min at 37°. Concentration independent process, shown in Fig. 1, was not detected in this case. Fig. 2 (c) represents Michaelis–Menten kinetics as applied to this type of disappearance. K_m in this experimental condition was about 100 mcg/135 min and the process is saturated at methyl orange concentration above 300 mcg/ml. From the results, it seems reasonable to conclude that concentration dependent process observed in *in situ* experiments is not an active transport mechanism. In this experiment, concentration dependent process was not inhibited completely by the addition of 0.01% polysorbate 80. Results of the analyses of data by Michaelis-Menten kinetics suggested that as if Polysorbate 80 had acted upon disappearance process competitively.

Fig. 3 shows the stability of methyl orange in the solution which was preincubated at the side of mucosa or serosa for one hour. Methyl orange was entirely stable in both solutions. This suggests that the disappearance of methyl orange would due neither to the decomposition by the intestinal flora nor to the enzymes which are soluble in the intestinal fluid as suggested by Mizuno, *et al.*[7]

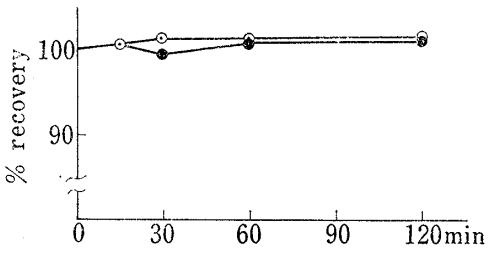

Fig. 3. Stability of Methyl Orange in the Solutions Pre-incubated with Intestinal Tissues

—⊙—: solution pre-incubated at the mucosal side
—●—: solution pre-incubated at the serosal side

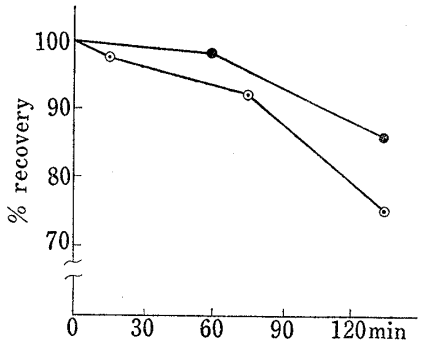

Fig. 4. Disappearance of Methyl Orange from Solutions placed in Different Side of the Intestine

—●—: drug placed in the serosal side
—⊙—: drug placed in the mucosal sde

When methyl orange was initially placed in the serosal side of an everted sac of the intestine, disappearance of the drug was delayed in comparison with the case in which the same drug was placed in the mucosal side of the non-everted sac of the intestine (Fig. 4).

7) N. Mizuno, M. Aoki, A. Kamada, and S. Sumimoto, *Vitamins* (Japan), **38**, 125 (1968).

It may be considered from the time course of the methyl orange disappearance that the drug disappears mainly from the mucosal side of the intestine and methyl orange initially placed in the serosal side is transported to the mucosal side and biotransformed there. Paperchromatographic analysis of the inner and outer solutions of *in vitro* loops showed two colored spots (Table I) in which (A) was unchanged dye and (B) was sulfanilic acid. Ultraviolet and visible spectra of (A) and (B) were just the same ones of authentic samples of methyl orange and sulfanilic acid, respectively. From these observations, the disappearance of methyl

Table I. Identification of Methyl Orange and its Metabolites

Solvent system		*Rf* values: Sample 0 hr	Sample 4 hr	Standard Methyl orange	Sulfanilic acid
n-BuOH saturated with pH 3.5 citrate buffer	A	0.25	0.24	0.29	0.02
	B	0.02	0.03		
conc. HCl–*n*-BuOH (1:4)	A	0.57	0.58	0.57	0.38
	B	···	0.41		

p-Dimethylaminoaniline, the other degradation product, does not interfere with the color development by Ehrlich reagent or by alpha naphthylamine.

orange from the perfusate seems to be due to the reduction following the manner shown in Chart 1. This reaction seems to be almost independent of the compositions of media. As shown in Table II, same biotransformation products were obtained in the following media: distilled water, ethyl laurate-water emulsions (oil/water volume ratio=1/3), pH 7.4 phosphate buffer solution, and Krebs-Ringer solution. The reaction shown in Chart 1 was inhibited

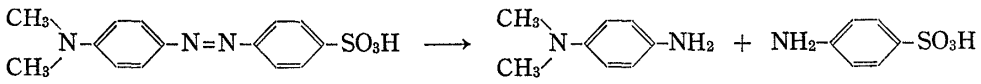

Chart 1. Reduction of Methyl Orange in the Rat Large Intestine

Table II. Effect of Incubation Media on Methyl Orange Stability in the Rat Large Intesine *in Vitro*

Media	M.O. conc. (mcg/ml)	Method	% remained
Distilled water	300	loop	75.0
pH 7.4 Phosphate buffer	300	loop	65.4
Emulsion[a)]	300	loop	83.4
pH 7.4 Phosphate buffer	50	everted sac	88.6
Krebs ringer	50	everted sac	86.3
Krebs ringer 95% O_2 5% CO_2	50	everted sac	104.7
Krebs ringer N_2	50	everted sac	78.2
pH 7.4 Phosphate buffer N_2 cofactor[b)]	20	homogenate (135 minutes incubation at 37°)	103.3
Distilled water	300	loop (135 minutes incubation at 37° after boiling for 5 minutes)	98.1

a) oil: ethyl laurate, emulsifier: 0.1% v/v Polysorbate 80
oil/water volume ratio $\phi=1/3$
b) FMN 10 mM, NADP 1×10^{-2} mM, Nicotinamide 1 mM, Glucose-6-phosphate 5×10^{-1} mM

completely by gassing with O_2+CO_2 (95:5) and, in opposition, accelerated by gassing with nitrogen. The reaction seems to be an enzymatic one since the reaction is inhibited completely by boiling the intestinal loop. An interesting point in Table II is that the reaction was inhibited in the homogenate medium even when the homogenate was incubated with nitrogen as the gas phase and with co-factors. Feldman and Gibaldi[8] also reported that the extent of recovery of phenol red from the homogenate of rat intestinal tissue differed from the one obtained from the intact intestinal sacs. They attributed this difference to a different binding manner of phenol red in each preparation. One of the reasons why extent of methyl orange reduction was different in the homogenate and in the intact tissue may be that in the former, specific binding manner related to methyl orange reduction was destroyed, and other pathways became predominant. Further investigation will be necessary to clarify these mechanisms.

Two possible mechanisms of methyl orange transport from the large intestinal lumen are proposed; one, passive transport, and the other, active-like process having enzymatic reduction of methyl orange on the mucosal side of the membrane. Polysorbate 80 inhibited only the former at its concentration close to the critical micellar one. Since the concentration of the surfactant is so low, influence of its interaction with methyl orange[9] seems to be negligible and inhibition by Polysorbate 80 may better be related to a direct or indirect action of the surface-active agent molecules to the site of methyl orange reduction. It has been reported that Polysorbate 80 molecules were adsorbed on the surface of the intestinal membrane of the rat non-specifically.[10] Specific binding site of methyl orange related to methyl orange reduction are covered by Polysorbate molecules, or are destroyed by change of the protein structure followed by the adsorption of Polysorbate 80 molecules. It is also worthy to note that the manner of inhibition *in situ* by the surface-active agent was different from the one observed *in vitro*.

8) S. Feldman and M. Gibaldi, *J. Pharm. Sci.*, **57**, 1234 (1968).
9) K. Kakemi, H. Sezaki, S. Muranishi, H. Ogata, A. Furuya, and M. Fujii, to be published.
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Formation of Indolizines *via* Pyridinium 3-Carbomethoxyallylides

Yasumitsu Tamura, Yoshio Sumida, and Masazumi Ikeda

Faculty of Pharmaceutical Sciences, Osaka University[1]

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In connection with our studies on the intramolecular 1,5-cyclization of ylides [1→2; X=CH, R=C_6H_5 and X=N, R=-$(CH_2)_3$-],[2,3] the cyclization reaction of pyridinium 3-carbomethoxyallyl bromide (**1a**) in the presence of base has been investigated. The product obtained, however, was not expected 1-carbomethoxyindolizine but methyl 3-(1′-carbomethoxy-3′-indolizinyl)acrylate (**5a**). In this report the structural assignment of the rather unusaual reaction product is described.

1) Location: *Toneyama, Toyonaka, Osaka.*
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