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Further Investigations of the Effect of Riboflavine Photolysates on the Intestinal Absorption of Certain Drugs in Rats and Their Mechanism of Action[1]

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Studies of the influence of riboflavine (RF) and flavine mononucleotide (FMN) photolysates on the intestinal membrane and its constituents have been pursued. The absorption experiments were carried out using *in situ* recirculation technique. It was found that the uptake of bromphenol blue (BPB) by the isolated intestinal epithelial cells was increased. Pretreatment experiments showed that, a period of 15 min was required for the hyperabsorptive state of the intestine to return back to its normal function. Histological studies of small intestinal mucosa could not demonstrate any detectable change. D-glucose absorption was not affected by the photolysates of riboflavine. NMR studies and liposome experiments revealed an interaction between the intermediate products of photolysis and egg phosphatidylcholine (PC). TLC and thiobarbituric acid test disclosed formation of lipid peroxides. A decrease in the surface tension was also occurred. From these data it was assumed that the intermediate products of photolyses attacked the double bonds of the unsaturated fatty acid side chain of PC molecules followed by cleavage and splitting of short chain fatty acids, consequently, change in permeability of the intestinal membrane due to disorganization of phospholipid molecules could be expected.

In recent publication[3] we have demonstrated the influence of riboflavine (RF) photolysates on the intestinal absorption of certain drugs in rats. It has been found that the absorption of phenol red (PR), bromphenol blue (BPB) and lactose isonicotinoylhydrazone (lactose-INH) was significantly increased when the experiments were carried out in the presence of RF and light. The remarkable enhancement in their absorption was attributed to an alteration in the permeability of the intestinal membrane to these drugs.

Our studies were extended for more clarification and to investigate the photosensitizing effect of RF, and the mechanism of action of its photolysates on the intestinal membrane and its constituents.

Experimental

Reagents——All the chemicals were reagent grade unless otherwise specified. Egg yolk phosphatidylcholine (PC) was prepared and purified according to the method of Rhodes and Lea[4]; commercial grade egg lecithin (Merck Company); β, γ-dimyristoyl L-α-lecithin (DML, Calbiochem, San Diego, Calif, U.S.A.); dicetyl phosphate (Sigma Chemical Company, St. Louis, Missouri, U.S.A.); Sephadex G-50 (Pharmacia, Uppsala, Sweden).

Animal Experiments——Male Wistar strain rats 150—200 g were used. The sourse of light, perfusion solutions and the intestinal absorption procedures have been previously mentioned[3] unless otherwise indicated.

Preparation of Epithelial Cells——Suspensions of rat intestinal epithelial cells were prepared as described by Reiser and Christiansen,[5] except that centrifugation was done at 3000 rev.min^{-1} and the cells were dispersed in pH 6.5 isotonic phosphate buffer.

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3) F.S. Ghazy, T. Kimura, S. Muranishi, and H. Sezaki, *J. Pharm. Pharmacol.*, **27**, 268 (1975).
4) D. N. Rhodes and C. H. Lea, *Biochem. J.*, **65**, 526 (1957).
5) S. Reiser and P.A. Christiansen, *Biochim. Biophys. Acta*, **225**, 123 (1971).

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Measurement of the Recovery Period——A 40.0 ml of 0.1 mM RF solution in pH 6.5 phosphate buffer was recirculated through the rat small intestine for one hour in the presence of light, then washed out (within 1 min) with the same buffer and the intestine was allowed to recover for the specified time. After the ellapse of the required period, a solution of 0.14 mM PR in phosphate buffer (40 ml) was recirculated for one more hour and the amount absorbed was determined.

Histological Studies——Pieces of intestine of control and treated rats were fixed in 20% formalin solution, then, 5μ thick histological sections were prepared by the freeze drying method, and stained with haematoxylin and eosin.

Nuclear Magnetic Resonance (NMR) Measurements——NMR spectra were obtained in deuterochloroform using a Varian HA 100 spectrometer. Tetramethylsilane (TMS) was used as an internal reference.

Thin-Layer Chromatography (TLC)——Procedures of Oette[6] for preparation of plates, their development and detection of spots were employed.

Preparation of Liposomes——These were prepared according to the method of Kinsky, Haxby, and others.[7] Lactose-INH was used as marker. Untrapped marker was then removed from the liposome preparation by chromatography on columns of Sephadex G-50 after its equilibration with pH 6.5 phosphate buffer and liposomes were eluted with the same buffer. The fraction that contained liposomes was indicated by visual turbidity.

Surface Tension Measurements——These were performed at 24° by means of a Du Noüy tensiometer.

Analytical Methods——Glucose determination was carried out according to the method described by Sasaki and others.[8]

Phenol Red was estimated by appropriate dilution of the samples with 1N NaOH and their extinction was measured at 550 mμ.

Lactose-INH and BPB were analysed by the previously described methods.[3] All the values were corrected for the control.

Results and Discussion

It is clear in Table I that, in the presence of flavine mononucleotide (FMN) and light the uptake of BPB by the isolated intestinal epithelial cells was significantly increased upon increasing the time of exposure, in contrast to those made in dark where it was remained constant. Since it has been known that FMN is one of the photodynamic compounds, the observed enhancement could be attributed to the photodynamic action of FMN on the isolated cells which led to an alteration in their permeability.

In our previous work[3] we have illustrated a transient effect of RF photolysates on the permeability of the intestinal membrane, therefore, in the present study we attempted to investigate the period required for the reversibility of the increase in PR absorption. It was found that (Fig. 1) the hyperabsorptive state of the intestine which caused by pretreatment

TABLE I. Effect of FMN Photolysates on Uptake of BPB[a] by the Isolated Intestinal Epithelial Cells[b]

Time in min	Uptake (μg/mg protein)	
	Protected	Exposed
15	7.98	9.58
30	7.98	12.24
60	7.98	14.90

a) The final concentration of BPB and FMN after addition of the epithelial cell suspension in pH 6.5 phosphate buffer was 0.075 and 1.0 mM respectively.
b) The yield of the epithelial cells in this preparation was equivalent to 1.42 mg protein/ml. A volume of 20 ml of the suspension was used.

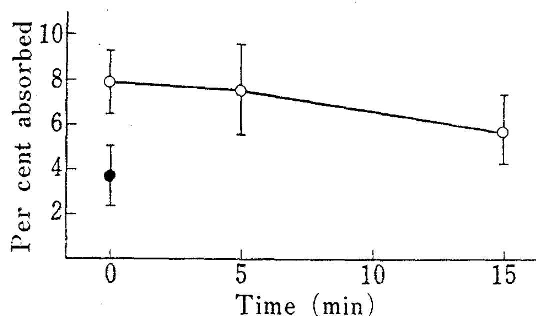

Fig. 1. Effect of the Period of Recovery after Pretreatment with Riboflavine Photolysates on the Intestinal Absorption of Phenol Red ● Control, ○ Pretreated

6) K. Oette, *J. Lipid Res.*, **6**, 449 (1965).
7) S.C. Kinsky, J. Haxby, C.B. Kinsky, R.A. Demel, and L.L.M. Van Deenen, *Biochim. Biophys. Acta*, **152**, 174 (1968).
8) M. Sasaki, Y. Ohba, and N. Ito, *Japan. J. Clin. Pathol.*, **16**, 55 (1968).

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with RF photolysates was returned to its normal absorptive state after 15 min where the difference in absorption compared to the control experiments was not significant. At the same time, the histological studies could not demonstrate any detectable change in the small intestinal mucosa of the treated rats.

Then we intended to investigate the influence of RF photolysates on the intestinal absorption of actively transported drugs. Table II shows that increasing the concentration of

Table II. Effect of Riboflavine Photolysates on the Intestinal Absorption of D-Glucose[a]

Concn. (mM)	% absorbed in one hour[b]		
	D-Glucose in pH 6.5 phosphate buffer	D-Glucose with riboflavine in pH 6.5 phosphate buffer	
		Protected	Exposed
1.0	84.81±8.25	89.86±6.26	85.90±8.93
10.0	67.72±3.68	69.42±7.66	67.67±5.77
100.0	38.45±8.45	33.68±9.44	38.64±5.48

a) D-Glucose was dissolved in either pH 6.5 phosphate buffer or 0.1 mM solution of riboflavine in the same buffer.
b) The per cent absorbed in one hour expressed as the mean of at least four determinations ± S.D.

D-glucose was accompanied by a decrease in its absorption. This is well established phenomenon and in accordance with the recent publication of Younoszai and Lynch.[9] It also demonstrates that, no significant difference between the percentage absorbed of D-glucose in the absence or presence of RF whether the experiments were carried out exposed to or kept away from light. This is an interesting finding, although RF photolysates have a striking effect on the absorption of certain passively transported drugs, it could not exert any significant influence on the absorption of those drugs transported actively. Moreover, it indicates to a certain extent that the photolysates of RF was not harmful to the enzymic and carrier mechanisms involved in the active processes of transport. Further experiments are needed for more clarification.

In the next phase of study we were interested to clarify the mechanism of action of RF photolysates on the constituents of the intestinal membrane. As it has been known that, phospholipids constitute a major component of biological membranes and possesses a definite role in their permeability, also, several previous works[10,11] showed that lecithin class of phospholipids is one of the most numerous phospholipids present in most mammalian tissues, therefore, our studies were designed to illustrate whether the photolysates of RF are capable of attacking them. Fig. 2A demonstrates NMR spectrum of egg PC in the absence of FMN and presence of light which is in agreement with that observed by Chapman and Morrison.[12] In contrast, irradiation of egg PC dispersion in the presence of FMN caused a marked change in assignments (Fig. 2B). The peak at 7.2 ppm corresponding to an allylic protons in the fatty acid side chain was disappeared, and that corresponding to an olefinic protons at 4.6 ppm was significantly reduced. The striking difference in NMR spectra could be ascribed to occurrence of cleavage in the unsaturated fatty acid side chain of egg PC molecules and splitting of short chain fatty acids due to the effect of FMN photolysates. Consequently, change in the permeability of the intestinal membrane caused by an alteration of the chain length of the unsaturated fatty acid side chain could be expected.

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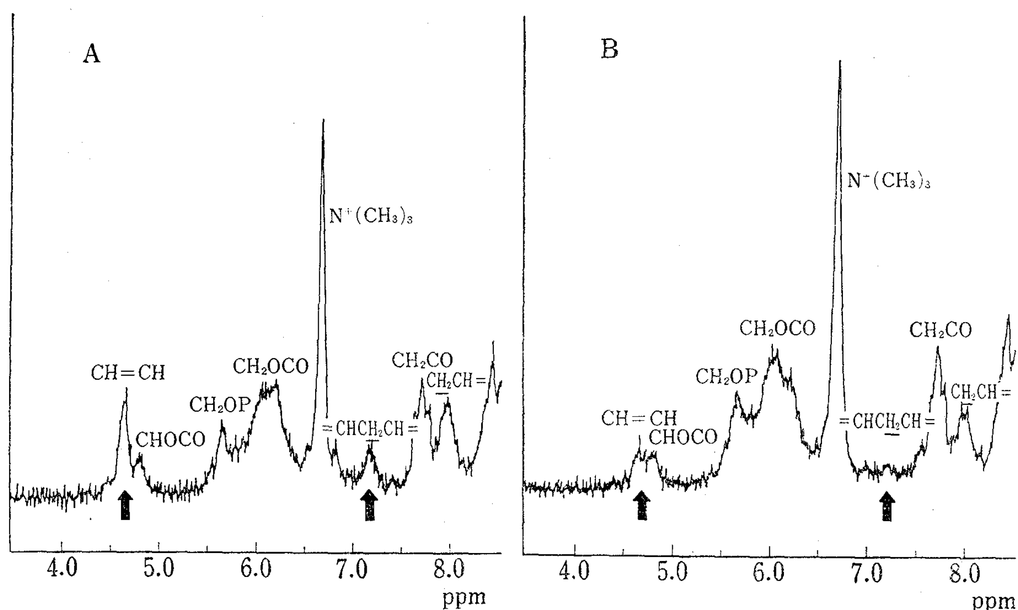

Fig. 2. NMR Spectra of Egg Phosphatidylcholine after Exposing Its Dispersion in: A. pH 6.5 Phosphate Buffer; B. 0.1 mM FMN Solution in pH 6.5 Phosphate Buffer

concentration of egg phosphatidylcholine: 0.005% time of exposure: 8 hours source of light: 4 tungsten electric lamps 60 W each

Thereafter, experiments using liposomes as a model of biological membranes were performed. Two different kinds of lecithins were used, Merck egg lecithin and dimyristoyllecithin (DML) representing the unsaturated and saturated forms respectively. Time course experiments were done. As it is clear in Fig. 3A, the release of lactose-INH entrapped inside DML liposomes was not affected by the presence of RF and light. On the contrary, when the unsaturated form of lecithin was used, a remarkable enhancement in the rate of release of lactose-INH on irradiationwas occurred (Fig. 3B) and it is demonstrated by the noticeable upward inflection of the curve. Control experiments in the absence of RF and presence of light or the reverse showed no detectable change in the rate of release of lactose-INH. As it has been well known that, the presence of double bond weakens the carbon-hydrogen bond of the carbon atom adjacent to a carbon with an unsaturated bond, therefore, changes in the permeability of the liposomes was attributed to an attack caused by the photolysates of RF on the α methylene carbons and the double bonds of the olefinic carbons of the unsaturated fatty acid side chain. The proposed alteration in the chain length which can be expected to lead to its shortening was considered to be responsible for the changes in the permeability of the liposomes. This result provided an additional evidence consolidating our previous concept and is in consistant with the previous work of Gier and others[13] who demonstrated that, the introduction of double bonds and shortening of the chain length of the fatty acid side chain of phospholipids increase the fluidity of lipid barrier and consequently enhance the permeability. Also, a

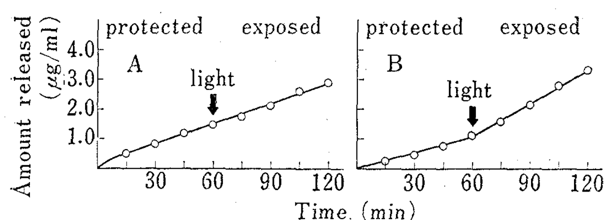

Fig. 3. Effect of Riboflavine Photolysates on the Release of Lactose-INH Entrapped into Liposomes of: A. Dimyristoyllecithin B. Commercial Grade Egg Lecithin

Liposomes were composed of lecithin A or B and dicetyl phosphate in molar ratio of 1 : 0.1.

13) J. DeGier, J.G. Mandersloot, and L.L.M. Van Deenen, *Biochim. Biophys. Acta*, **150**, 666 (1968).

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recent publication of Teige and others[14] indicated that, the attack of ozone on PC liposomes produced molecules in which the unsaturated fatty acid in position 2 was shortened at the double bond with the formation of aldehyde or acid as the terminal group. A similarity between the mechanism of action of ozone and that of RF photolysates on the lecithins of the liposomes could be expected.

From the previous works[14–16] it was clear that, shortening of the fatty acid chain of phospholipid molecules is mostly a consequence of lipid peroxide formation. Therefore it was of importance to detect whether they were formed. TLC separation revealed formation of lipid peroxides when egg PC was treated with RF and light. This was also confirmed by the thiobarbituric acid test of Wilbur and others[17] for detection of malonaldehyde formation where it gave a positive material. No lipid peroxides could be detected in the absence of RF and presence of light or the reverse, and also in the presence of both RF and light when DML was used. These findings are in agreement with several previous publications[14–16,18–20] stated a relation between lipid peroxidation and changes in the permeability of either the erythrocytes or liposomes which was found to be increased. Surface tension measurements were done and showed a decrease in the surface tension of egg PC dispersion in the presence of RF and light which could be ascribed to the shortening of the fatty acid side chain. This might also contributed to a certain extent in enhancing the absorption where a decrease in the interficial tension between the intestinal membrane and the adjacent solution of drug can be expected.

The effect of RF photolysates on the other constituents of the intestinal membrane and involvement of other factors can not be excluded and this requires further investigations.

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