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Biopharmaceutical Study of the Hepatobiliary Transport of Drugs. VI.[1] Inhibition of Active Biliary Excretion of Organic Cations by Retrograde Infusion

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The new method employed to organic anions was applied to investigate the active excretory process of organic cations from liver cells to bile canaliculi. Biliary excretion of acetyl procaineamide ethobromide (APAEB) and quinine in rats was studied after infusing retrogradely a solution of various reagents from the cannulated common bile duct into the biliary trees. Among the tested reagents, surfactants, sulfhydryl reagents and amino-reactive agents remarkably inhibited the active biliary excretion of organic cations. The concentrations in bile, the amounts excreted for 30 minutes and the bile/plasma ratios of APAEB were decreased to about 1/2—1/3 of their values at the sham operation by the treatment with these reagents. Because of the intensive decrease in the B/L ratios compared to the L/P ratios, the reagents are supposed to act locally on the bile canalicular site of the liver plasma membrane and to inhibit the active excretory process of organic cations. As compared with organic anions, the biliary excretion of organic cations was more sensitive to the treatment with sulfhydryl reagents and less sensitive to the solubilizing effect of surfactants and organic solvents.

Keywords——biliary excretion; active transport; organic cation; retrograde infusion; bile canalicular membrane; surfactant; sulfhydryl reagent; acetyl procaineamide ethobromide

A number of quaternary ammonium compounds and tertiary amines have been shown to be actively excreted from blood into bile. In the previous report,[1] the hepatic uptake of organic cations which were actively transported into bile was studied especially by *in vitro* method. The transport process of organic cations from the hepatocytes to bile canaliculi, however, has been absolutely obscure until recently. This laboratory already reported about a new method to specifically investigate the biliary excretory process of organic anions from liver cells into bile canaliculi.[3] By infusing surfactants or some other reagents retrogradely from the common bile duct into the biliary trees, the active excretion of organic anions, bromphenol blue and uranine, was found to be strikingly inhibited. The bile/plasma (B/P) concentration ratio was dramatically decreased mainly on account of the reduction of the bile/liver (B/L) concentration ratio. Furthermore in the bile collected after finishing infusion of surfactants, a number of proteins which did not appear in normal bile were discovered by the method of polyacrylamide gel electrophoresis.

In this report, the same method was applied to the biliary excretion of organic cations, acetyl procaineamide ethobromide (APAEB) and quinine. The new method has demonstrated that it is of great use to study the active excretion of not only organic anions but organic cations and that surfactants, sulfhydryl reagents and amino-reactive reagents are effective to inhibit the active excretion of organic cations.

1) H. Nakae, R. Sakata, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **24**, 886 (1976).
2) Location: *Yoshida shimoadachi-cho, Sakyo-ku, Kyoto.*
3) K. Takada, Y. Tokunaga, and S. Muranishi, *Chem. Pharm. Bull.* (Tokyo), **24**, 871 (1976).

Experimental

Materials——APAEB was synthesized in our laboratory according to the method of Hwang[4] from procaine amide which was kindly provided by Daiichi Seiyaku Co., Ltd. Triton X-100 was obtained from Wako Pure Chemical Co., Ltd and digestive enzymes from Sigma Chemical Co., Ltd. Other reagents were purchased from Nakarai Chemicals Co., Ltd.

Animal Experiments——Animal experiments were mostly performed according to the method of Takada.[3] Male Wistar rats weighing 220—250 g were anesthetized with urethane (0.9 g/kg). Renal pedicles were ligated and the common bile duct was cannulated with a polyethylene tube. Reagents were dissolved in Krebs-Ringer phosphate buffer solution unless otherwise noted. A 50 μl of reagent solution was gently infused retrogradely over 1 minute from the cannulated tube to the biliary trees and the handle of the syringe was held on for 15 minutes. After then the syringe was taken off from the tubing to let the bile flow freely. In the most cases the bile flow immediately recovered to a normal value. Then APAEB (8.8 μmol/300 g) or quinine (5 μmol/300 g) was injected intravenously and the bile sample was collected for two 15 minutes. A blood sample was taken via the aorta at 30 minutes after the injection and the liver was removed immediately. A concentration of APAEB or quinine in plasma, liver and bile was determined by the method described in the previous report.[1]

Results

Effect of Retrogradely Infused Surfactants on Hepatobiliary Transport of APAEB

APAEB is the cationic organic compound which is actively secreted into bile and is not metabolized in the body of a rat. Furthermore the concentration gradient of APAEB was especially high between liver and bile, while the concentration gradient was extremely low between plasma and liver. Accordingly at first APAEB was chosen to study the effect of intrabiliary retrograde infusion on the active secretion of organic cations. The concentrations of APAEB in plasma, liver and bile are shown in Table I. When the sham operation in which only Krebs-Ringer phosphate buffer solution was infused, was performed, the concentrations in plasma, liver and bile were slightly affected. When a potent anionic surfactant, sodium lauryl sulfate (SLS) solution (50 mM) was infused, however, the concentration in bile in 15—30 minutes was strikingly decreased from 1720 nmol/ml to 567 nmol/ml and the percentage excreted into bile for 30 minutes was also decreased from 7.6% to 2.3% as compared with the sham operation. Although concentrations in plasma and liver were also affected, the changes in those concentrations were very small. Therefore the B/L ratio was decreased from 8.2 to 3.2 in contrast to a slight decrease in the liver/plasma (L/P) ratio.

Table I. Effect of Surfactants on the Biliary Excretion of APAEB

Infused solution	Plasma level (nmol/ml)	Liver level (nmol/g)	Bile level (nmol/ml) 0—15 min	Bile level (nmol/ml) 15—30 min	L/P ratio	B/P ratio	B/L ratio	excreted for 30 min (%)
No treatment	75.1±10.9	203±41	1336±526	2006±491	2.4±1.1	20.3±8.2	10.0±2.6	9.3±1.9
Krebs-Ringer phosphate buffer	75.6± 7.0	213±20	855±190	1720±367	2.8±0.2	25.3±4.9	8.2±2.3	7.6±2.7
SLS 50 mM	92.4±19.9	170±15	328± 83	567±255	2.1±0.6	6.2±1.9	3.2±1.7	2.3±0.3
Triton X-100 4%	108 ± 8	191± 9	354± 39	369± 58	1.8±0.2	3.4±0.7	1.9±0.3	2.4±1.0
Sodium deoxycholate 100 mM	94.5± 7.8	162±12	372± 93	538± 35	1.7±0.1	5.7±0.5	3.3±0.2	2.3±0.9
SLS 50 mM after 1 hr[a]	101 ± 7	177±46	431±198	565±236	1.8±0.4	5.7±2.5	3.2±1.1	3.1±1.4

a) APAEB was injected 1 hr after the treatment with SLS.
Plasma and liver levels are values at 30 min.
Results are expressed as mean±S.D. of more than three experiments.

4) S.W. Hwang, R.H. Reuning, and L.S. Schanker, *XENOBIOTICA*, **1**, 265 (1971).

An unionized surfactant, Triton X-100 (4%), also gave the similar results. The concentration in bile in 15—30 minutes was decreased to 369 nmol/ml and the B/L ratio to 1.9. Triton X-100 had an inclination to stimulate the bile flow which was not the case in SLS infusion.

When sodium deoxycholate (100 mM), one of bile acids, was infused retrogradely, the bile concentration was decreased to 538 nmol/ml and the B/L ratio to 3.3.

These three surfactants were equally observed to be much more effective to decrease the B/L ratios than the L/P ratios. This indicates that surfactants retrogradely infused may depress especially the active excretory process of APAEB from liver to bile.

In order to investigate the duration of this inhibitory effect, an administration of APAEB was delayed for one hour after finishing the pretreatment with SLS. The result of this experiment was almost the same as the above experiment with SLS as shown in Table I, indicating no recovery from the inhibitory effect occurred.

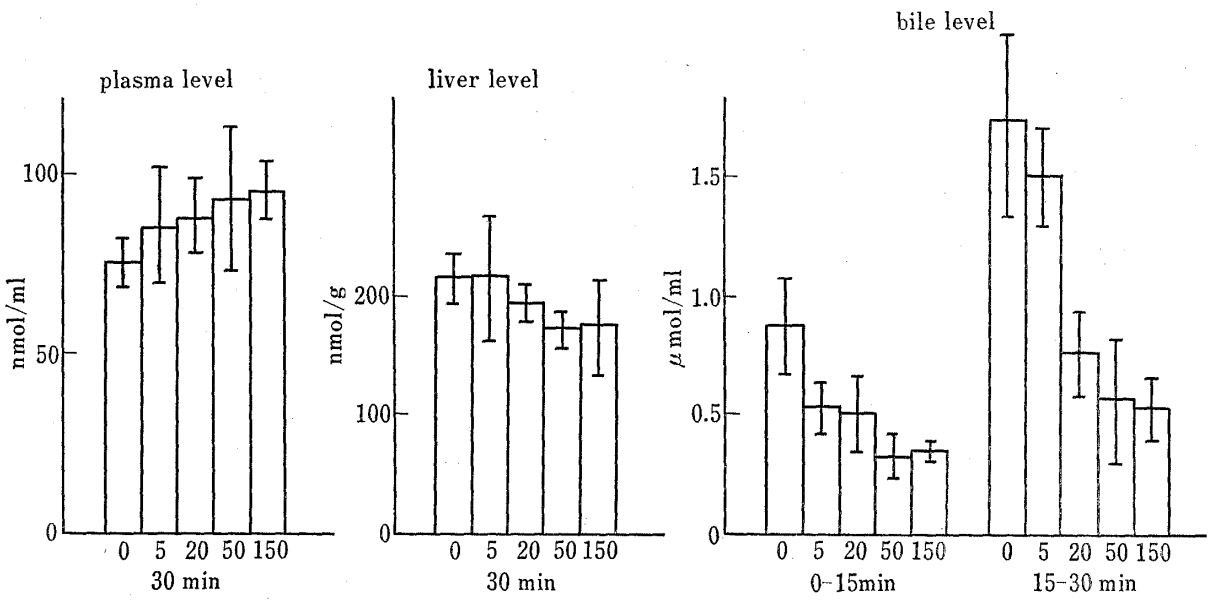

Fig. 1. Effect of the Concentration of SLS on the Concentrations of APAEB

The 0, 5, 20, 50, and 150 mM SLS solutions were infused retrogradely from the cannulated common bile duct before injection of APAEB (8.8 μmol/300 g of rat).

Effect of the Concentration of SLS

Effect of the concentration of SLS retrogradely infused is shown in Fig. 1. As the concentration of SLS was increased, the concentration of APAEB in bile was gradually decreased and the effect of SLS reached to the maximum around concentration of 50 mM of SLS, whereas concentrations in plasma and liver were not extensively changed. Therefore the B/L ratios decreased from 8.2 to 2.8 in accordance with the increase of the concentration of SLS as shown in Table II.

Effect of Various Reagents

The digestive enzymes, sulfhydryl reagents, amino-reactive reagents, metabolic inhibitors and the other reagents were utilized for modifying the excretory process in this study.

Digestive Enzymes——Proteolytic enzymes, trypsin (0.5%) and papain (1%) in Tris-HCl buffer solution (pH 7.4) gave no depressing effect on the concentration of APAEB in bile as shown in Table III. Phospholipase D (1%) in phosphate buffer solution (pH 5.6) did not show the significant inhibitory effect, either. As all these enzymes had an inclination to depress the bile flow, the percentages excreted for 30 minutes were decreased in all cases.

Sulfhydryl Reagents——When *p*-chloromercuribenzenesulfonate (PCMBS) (50 mM), N-ethylmaleimide (NEM) (25 mM) or fluorescein mercuric acetate (FMA) (10 mM) in 0.1 M sodium

Table II. Effect of the Concentration of SLS on the Concentration Ratios and the Excreted Percentage of APAEB

Cocn. of SLS	L/P ratio	B/P ratio	B/L ratio	% excreted for 30 min
0 mM	2.8±0.2	25.3±4.8	8.2±2.3	7.6±2.7
5 mM	2.3±0.3	17.9±3.8	7.3±1.7	6.0±1.6
20 mM	2.2±0.4	8.6±1.6	4.0±1.2	5.0±1.5
50 mM	2.1±0.6	6.2±1.9	3.2±1.7	2.3±0.3
150 mM	1.8±0.4	4.5±2.4	2.8±1.1	2.0±0.7

Results are expressed as mean±S.D. of more than three experiments.

Table III. Effect of Various Reagents on the Biliary Excretion of APAEB

Infused solution	Plasma level (nmol/ml)	Liver level (nmol/g)	Bile level (nmol/ml) 0—15 min	Bile level (nmol/ml) 15—30 min	L/P ratio	B/P ratio	B/L ratio	Excreted for 30 min (%)
Krebs-Ringer phosphate buffer	75.6± 7.0	213±20	855±190	1720±367	2.8±0.2	25.3±4.9	8.2±2.3	7.6±2.7
Trypsin 0.5%	80.4± 8.3	190±22	617±137	1731±335	2.1±0.5	21.1±6.8	9.6±1.9	4.2±0.8
Papain 1%	94.7± 3.6	237±22	720±151	1723±411	2.5±0.1	18.2±4.1	7.3±1.8	5.2±1.9
Phospholipase D 1%	84.9± 8.8	200±25	740±140	1468±129	2.3±1.5	16.6±2.4	7.4±0.3	5.4±1.6
PCMBS 50 mM	95.0± 4.1	155±14	470± 72	810±192	1.6±0.1	8.6±2.4	5.3±1.6	2.9±0.6
NEM 25 mM	103 ±11	196±13	405± 21	844±262	1.9±0.1	8.1±1.6	4.2±1.1	2.3±0.5
FMA 10 mM	93.7± 9.6	168±39	496± 20	941±174	1.8±0.6	9.2±3.2	5.6±2.8	2.9±0.1
PCMB 10 mM	88.3± 7.8	162±17	694±105	1525±186	1.9±0.3	17.3±2.3	9.4±0.7	5.2±0.9
IAA 100 mM	94.2±10.6	222±11	855±128	1976±231	2.4±0.4	21.2±0.4	8.9±1.5	5.6±1.3
PCMBS 50 mM+ SLS 50 mM	98.6± 6.2	184±12	427± 41	557±133	1.9±0.3	5.7±1.7	3.0±0.5	2.1±0.1
Acetic anhydride 100 mM	100 ± 6	139±14	369± 77	560± 62	1.4±0.2	5.6±0.7	4.0±0.2	3.1±0.3
Succinic anhydride 100 mM	86.4± 2.6	117±11	255± 12	575±115	1.4±0.1	6.7±1.5	5.0±1.3	2.4±0.4
DNP 1 mM	83.9± 4.9	183±15	730±247	1567±407	2.2±0.3	19.1±5.9	8.5±1.8	6.4±3.0
Ouabain 1 mM	83.9±16.6	209±22	802±207	1854±296	2.6±0.7	23.0±6.9	8.9±0.9	8.3±2.2
Ethanol–ether (3:1)	110 ± 6	150±22	423± 83	777±202	1.4±0.2	7.0±1.6	5.1±1.1	2.5±0.5
Potassium bicarbonate 50 mM	88.0± 9.3	121±15	389± 89	980±187	1.4±0.3	11.0±1.4	8.1±1.6	3.9±0.9

Results are expressed as mean±S.D. of more than three experiments.
Plasma and liver levels are values at 30 min.

pyrophosphate solution was infused, the concentration in bile and the percentage excreted for 30 minutes were decreased to 1/2—1/3 of those in the sham operation. The extent of the inhibitory effect of these reagents were equal to that of the surfactants. As the concentration more than 50 mM of NEM solution almost gave a stop of the bile flow, the experiment by this reagent was not proceeded. On the other hand, *p*-chloromercuribenzonate (PCMB) (10 mM) in 0.1 M sodium pyrophosphate solution and iodoacetamide (IAA) (100 mM) had no inhibitory effect. The effect of PCMB at the higher concentration could not be studied because of its poor solubility.

When the mixture of SLS (50 mM) and PCMBS (50 mM) was infused, the obvious inhibitory effect was observed as well as at the infusion of each reagent, but was no more than that of SLS alone. From this result these two reagents seem to modify the same site of the active excretory process of organic cations.

Amino-reactive Reagents——Acetic anhydride (100 mM) or succinic anhydride (100 mM)

showed the inhibitory effect. Both reagents distinctly decreased the bile concentrations and the percentages excreted for 30 minutes to less than 1/2 of the sham operation experiment as shown in Table III. In these cases the considerable decrease in the concentration in liver was observed. Consequently the L/P ratios gave the value of 1.4, which was distinctive from the effect of the other reagents. Acetic anhydride increased the bile flow about 20%.

Metabolic Inhibitors——Neither dinitrophenol (1 mM) nor ouabain (1 mM) had the effect to depress the biliary excretion of APAEB as shown in Table III.

Other Reagents——When ethanol–ether (3: 1) was infused, the bile concentration was decreased to 777 nmol/ml and the percentage excreted for 30 minutes to 2.5% (Table III). The bile flow was decreased about 20%. An infusion of potassium bicarbonate (0.05 M) in saline, which was reported to solubilize 70% of the liver cell plasma membrane *in vitro*,[5] was also observed to be effective. But in this case the effect was obtained on the L/P ratio because of the intensive decrease in the concentration in liver.

Effect of Surfactants and Various Reagents on Hepato-biliary Excretion of Quinine

Quinine is one of the organic cations which are excreted actively into bile[6] and was studied in our previous report.[1] Thus this compound was employed to investigate the inhibitory effect and unmetabolized quinine was determined in the series of experiments.

Table IV. Effect of Various Reagents on the Biliary Excretion of Quinine

Infused solution	Plasma level (nmol/ml)	Liver level (nmol/g)	Bile level (nmol/ml) 0—15 min	Bile level (nmol/ml) 15—30 min	L/P ratio	B/P ratio	B/L ratio	Excreted for 30 min (%)
No treatment	1.33±0.16	51.2±6.8	21.2±8.3	26.2±2.0	38.5±3.7	19.7±2.8	0.52±0.07	0.28±0.01
Krebs-Ringer phosphate buffer	2.06±0.13	43.6±8.9	21.3±6.6	25.5±5.3	21.1±3.9	12.3±2.0	0.61±0.19	0.19±0.03
SLS 50 mM	2.06±0.13	38.3±3.7	11.8±1.4	8.0±1.0	18.6±1.0	3.9±0.6	0.21±0.03	0.07±0.01
Triton X-100 4%	1.95±0.32	38.9±4.8	8.8±1.4	5.7±0.8	20.1±1.0	2.9±0.2	0.15±0.01	0.07±0.02
NEM 25 mM	2.27±0.10	52.5±5.1	18.2±1.9	14.6±2.7	23.1±1.8	6.4±1.0	0.28±0.03	0.15±0.01
Acetic anhydride 100 mM	2.10±0.37	50.1±3.0	20.7±2.9	18.3±5.8	24.4±4.7	9.0±3.4	0.37±0.14	0.21±0.03

Results are expressed as mean±S.D. of more than three experiments.

Table IV shows the concentrations of quinine in plasma, liver and bile when 5 μmol/300 g of quinine was injected after pretreatment with surfactants and various reagents which were proved to be effective in the case of APAEB. The results obtained were generally in accordance with those of APAEB. An infusion of SLS (50 mM) or Triton X-100 (4%) remarkably inhibited the biliary excretion of quinine. As the concentration in plasma and liver were affected only a little by the pretreatment with these surfactants, the B/P and the B/L ratios were decreased to about 1/3—1/4 of the sham operation. NEM (25 mM) and acetic anhydride (100 mM) were also effective to inhibit the biliary excretion of quinine.

Discussion

Surfactants (SLS, Triton X-100 and sodium deoxycholate), sulfhydryl reagents (PC-MBS, NEM and FMA), amino-reactive reagents (acetic anhydride and succinic anhydride), organic solvent (ethanol–ether 3: 1), alkaline solution (potassium carbonate) and phospholipase D were shown to inhibit the biliary excretion of APAEB when infused retrogradely from the common bile duct into the biliary trees. Some of them were also shown to inhibit the biliary

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6) P.K. Nayak and L.S. Schanker, *Am. J. Physiol.*, **217**, 1639 (1969).

excretion of quinine. The most remarkable point about this effect was that the concentration in bile was extremely decreased, ultimately to 1/4 of the sham operation as shown in Triton X-100, whereas the concentrations in plasma and liver were not affected so much. Consequently the B/P and the B/L ratios were extensively decreased to 1/3—1/4 of the sham operation, while the L/P ratios were not so much decreased. As the volume of the infused solution, 50 μl, was not more than the whole volume of biliary trees, the reagents did not seem to attain directly the inside of hepatocytes. Therefore the inhibitory effect of these reagents suggests that they might act mostly on the liver plasma membrane at the bile canalicular site and inhibited the active excretory process there.

On the other hand, the hepatic uptake process might have been also influenced by this treatment, for the L/P ratios were somewhat decreased. But it was little as compared with the effect on the excretory process.

The way to act on the plasma membrane seems to be different according to the reagents. Surfactants are considered to inhibit the biliary excretion bacause they solubilized the part of the plasma membrane. For the effect of SLS was gradually increased with its concentrations (Fig. 1) and a number of proteins which did not appear in normal bile were observed after the treatment with SLS or Triton X-100.[3] But the possibility that the potency of the active transport mechanism was suppressed by surfactants may not be excluded, as enzyme activities in liver plasma membrane are reported to have been suppressed by surfactants.[7]

Sodium deoxycholate, one of bile salts itself, also gave the marked inhibitory effect. This fact is interesting and corresponding to the report that states bile salt liberation is accompanied by a partial solubilization of the plasma membrane.[8] Inhibitory effect of organic solvent, ethanol–ether (3: 1), also seems to be due to the solubilizing effect, since the proteins observed after treatment with surfactants were found in this case, too.[3] Trypsin and papain, proteolytic enzymes, which have been often used to solubilize the biomembrane, failed in the inhibitory effect in this experiment. As those proteins were not found after treatment with these reagents,[3] the digestion of the active secretory system in the plasma membrane is though to be insufficient in this experimental condition. Sulfhydryl and amino-reactive reagents are considered to be able to react respectively with sulfhydryl groups and amino groups of proteins in the membrane. Therefore the inhibitory effect of reagents indicates that sulfhydryl and amino groups in the plasma membrane are involved in the active excretory mechanism of organic cations. It is interesting that PCMB did not inhibit the active excretion in this experiment, nevertheless it is reported that PCMB depressed tha accumulation of APAEB by liver slices in *in vitro* experiment.[9] Dinitrophenol has been reported to depress the accumulation of PAEB and chloroguanide-triazine by liver slices[10] and ouabain also to depress that of piribenzil.[11] It is notable, however, that both of these reagents had no effect at all.

From the results of the preceding and this report, the new method employed here was verified to be valuable to study the active excretory process of organic compounds. As compared with organic anions, the biliary excretion of organic cations was more sensitive to the treatment with sulfhydryl reagents and less sensitive to the solubilizing effect of surfactants and organic solvents. PCMBS (50 mM) decreased the concentration of APAEB in bile to about one half of the sham operation, whereas the concentration of BPB was decreased to 75% by the reagent.[3] The amount excreted for 30 minutes was decreased to 40% of the sham operation for APAEB and 73% for BPB. Accordingly sulfhydryl groups of proteins in the plasma membrane are considered to play the more important roles in the active excretion of organic

7) J.K. Dunnick, G.V. Marinetti, and P. Greenland, *Biochim. Biophys. Acta*, **266**, 684 (1972).
8) G. Holdsworth and R. Coleman, *Biochim. Biophys. Acta*, **389**, 47 (1975).
9) S.H. Hwang and L.S. Schanker, *Am. J. Physiol.*, **225**, 1437 (1973).
10) *a*) H.M. Solomon and L.S. Schanker, *Biochem. Pharmacol.*, **12**, 621 (1963); *b*) P.L. Gigon, P.K. Nayak, and L.S. Schanker, *Proc. Soc. Exp. Biol. Med.*, **132**, 1103 (1969).
11) A. Ryrfeldt, *Acta Pharmacol. Toxicol.*, **33**, 289 (1973).

cations than organic anions. On the other hand, by the treatment with SLS the concentration in bile and the amount excreted for 30 minutes of APAEB were decreased to 1/3 of the sham operation, while those of BPB were decreased indeed to 1/10 of the sham operation.[3] This difference is not explainable now, but the following fact might have some relation to it. Namely, although proteins solubilized with these surfactants were shown to have a high affinity to the organic anions,[3] they were not observed to bind to an organic cation, neutral red, by the method of the disc gel electrophoresis (unpublished data).

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