STUDIES ON THE METABOLISM OF CEPHALOSPORINS. II
BILIARY EXCRETION AND METABOLISM OF ^{14}C-CEPHACHLOMEZINE AND ITS PARENT COMPOUND

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The excretion and metabolic fate of two cephalosporin antibiotics, cephachlomezine (CEC), and its parent compound, *m*-chlorophenyl-acetamidocephalosporanic acid (*m*-Cl-PACA), was studied in rats, with emphasis being placed on biliary excretion and serum protein binding. When ^{14}C-*m*-Cl-PACA was given intramuscularly, about 40 percent of the administered radioactivity was found in the bile, which contained only 1.6 % of the administered antibiotic activity.

Lactone of *m*-Cl-PACA, one of the metabolites of *m*-Cl-PACA, has been isolated in a crystalline form as a result of hydrolysis by citrus acetylase. After a single intramuscular administration of ^{14}C-CEC, more than 60 % of the administered dose was excreted in urine in the unchanged form and 15 % was recovered either in the bile as shown by radioassay or in microbioassay. Orally administered ^{14}C-CEC underwent suspected degradation in the gut. Binding of CEC and *m*-Cl-PACA to serum protein was examined by means of an ultrafiltration technique chiefly in relation to the biliary excretion.

The metabolism of *m*-Cl-PACA in rats was reported in the preceding paper[1], in which the authors referred to the conversion of this antibiotic into some other less active metabolites including an O-deacetyl derivative. In the present paper we present additional information on the metabolism of this compound including that of the properties of lactone of *m*-Cl-PACA, one of the metabolites. We have also studied the metabolic fate of 4,4-dimethylpiperazinium-1-thiocarbonyl-thiomethyl derivative (CEC).

Materials and Methods

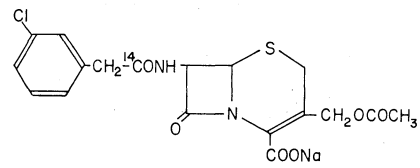
(1) Labeled antibiotics: The synthesis of ^{14}C-*m*-Cl-PACA [I] has already been described in the preceding paper[1]. The specific radioactivity of this compound is 1.34 μCi/mM. The labeled CEC was prepared as follows:

Sodium salt of 7-(*m*-chlorophenyl-acetamido)-3-(4-methylpiperazinium-1-thiocarbonyl-thiomethyl)-3-cephem-4-carboxylate, dissolved in formamide, was cooled to −20°C and then treated with ^{14}C-methyl iodide (6.6 mCi/mM; from Daiichi Pure Chemical Co., Ltd., Tokyo). As methylation proceeded, precipitation was noted of 7-(*m*-chlorophenyl-acetamido)-3-(^{14}C-4,4-dimethylpiperazinium-1-thiocarbonyl-thiomethyl)-3-cephem-4-carboxylate [II]. The precipitate was collected by filtration and washed with a mixture of tetrahydrofuran and methanol (2 : 1). The ^{14}C-CEC thus obtained had a specific activity of 3.30 mCi/mM, and showed a single radioactive peak on radioscanning the paper chromato-

grams. This was consistent with the results of paper electrophoresis. This substance also proved to be homogeneous when the chromatogram was bioautographed with *Streptococcus hemolyticus* S-23[1] as the test organism.

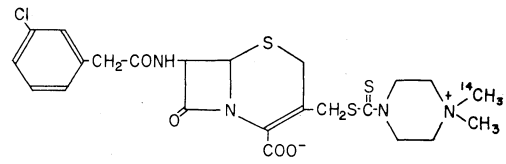
(2) Measurement of radioactivity: The radioactivity of materials was determined by a liquid scintillation counter (Nuclear Chicago Model 724) or a low background gas flow counter (Nuclear Chicago C-115 or Aloka LBC-22).

^{14}C-*m*-Cl-PACA; I



$C_{18}H_{16}O_6SClNa$ M.W. 446.9

^{14}C-CEC; II



$C_{23}H_{27}N_4O_4S_3Cl$ M.W. 555.1

Determination of radioactivity in urine samples were done as follows:

To a 200-μl aliquot of a urine sample, the followings were added in order: 1 ml of NCS solubilizer (Nuclear Chicago Corp.), 4.8 ml of ethanol, and finally, 14 ml of phosphor consisting of 3 g of 2,5-diphenyloxazole (PPO) and 180 mg of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene (dimethyl POPOP) per 420 ml of toluene. Quenching correction was made by the channel ratio method. The bile samples were dried on stainless steel planchets (TH type, Chiyoda Safety Co., Tokyo) for radioactivity determinations in the gas flow counter.

(3) Measurement of antibiotic activity: To each urine sample, an equal volume of ethanol was added to achieve bacterial sterilization, and the antibiotic activity of the urine was assayed by a turbidimetric method using *Staphylococcus aureus* FDA 209-P as the test organism. The bile samples were filtered through a Millipore filter (Millipore Filter Co.), and the activity was assayed by the disk method using *Bacillus subtilis* ATCC-6633 as the test organism.

(4) Paper chromatography and paper electrophoresis: Fifteen-μl portions of suitable urine- and bile-dilutions were spotted on strips of Toyo No. 51 paper (2×40 cm), and the chromatograms developed for 18 hours by ascending technique using a solvent system consisting of *n*-butanol, acetic acid, and water (4:1:5 in volume, upper layer). Paper electrophoresis of the urine was carried out in 0.05 M, pH 4.5 pyridine-acetate buffer (14 V/cm, 1.5 hour). Radioactivity on the paper strips was examined with Actigraph II radiochromato-scanner (Nuclear Chicago Corp.). Areas representing antibiotic activity were located by bioautographic techniques using *Streptococcus hemolyticus* S-23[1] as test organism.

(5) Biliary excretion: Male Wistar rats weighing between 150 and 200 g were used throughout this experiment. Non-fasted animals were restrained in the supine position on surgical boards. Their bile ducts were cannulated with polyethylene tubing (Clay-Adams Co., PE-10) under light ether anesthesia. After recovery they received intramuscularly an antibiotic dissolved in physiological saline. Over the test period of 48 hours, the bile and urine samples were separately collected at specified time intervals into conical glass tubes of 10-ml capacity graduated to 0.1 ml, and refrigerated until use. Tubes for collecting urine were affixed to the orifice of the urethra. Food was withheld but water was given at regular intervals throughout the test period.

(6) Urinary excretion: After an overnight fast, ^{14}C-CEC was given to male Wistar rats either intramuscularly into the hind thighs or orally *via* a stomach tube. For the former route, the ^{14}C-CEC was dissolved in a phosphate buffer solution containing 10% N,N-dimethylformamide to give a concentration of 2 mg/ml and, for the latter route, the substance was suspended in water to give 5 mg/ml. Five animals were placed in one

funnel type metabolic cage. They were supplied with water *ad libitum*, but food was withheld during the experiment.

(7) Serum protein binding: Non-fasted male rats received 20 mg/kg of an antibiotic intramuscularly. One hour later, blood was withdrawn from the femoral artery and femoral vein. The serum and ultrafiltrate from the blood sample were taken for microbioassay of the disk method employing *B. subtilis* ATCC-6633 as the test organism. Standard solutions were prepared in normal serum ultrafiltrate. Total antibiotic concentrations in serum were measured against standard solutions prepared in normal rat serum.

(8) Acetylase preparation and enzymatic hydrolysis of *m*-Cl-PACA: The *m*-Cl-PACA was mixed with citrus acetylase, which had been prepared from the peel of *Citrus hassaku* according to a modification of the method of JANSEN *et al.*[2] The conditions of this enzymatic hydrolysis were as described in the preceding paper[1]. The incubation mixture was then acidified to pH 2, neutralized, and extracted with an equal volume of ethyl acetate. The ethylactate layer was evaporated to dryness under reduced pressure at a temperature not exceeding 40°C. The residue was recrystallized from ethyl acetate. The crystals of colorless, fine platelets had m. p. 222°C (with decomp.) and these other characteristics: $\lambda_{max}^{95\%\ EtOH}$ 259 mμ, ε 7350. IR[Nujol]: 1770 cm^{-1} (β-lactam), 1750 cm^{-1} shoulder (lactone), NMR[D_6-DMSO]: two proton peak at τ=3.80 (singlet), for the lactone methylene at C-3.

Anal. Calcd. for $C_{16}H_{13}N_2O_4SCl$: C 52.67, H 3.59, N 7.68, S 8.79, Cl 9.72.
Found: C 52.52, H 3.36, N 7.58, S 8.52, Cl 9.99.

Results

1. Biliary excretion of *m*-Cl-PACA

Recovery of radioactivity from bile and urine after intramuscular injection of ^{14}C-*m*-Cl-PACA (100 mg/kg, 0.6 μCi/rat) into three cannulated rats is summarized in Table 1. Virtually all the radioactivity injected was recovered during the experimental period and about 40 % was in the bile and 62 % in the urine. Bile of nine cannulated rats receiving intramuscularly 20 mg/kg of non-radioactive *m*-Cl-PACA was examined for antibiotic activity by the disk method.

In contrast to the findings in excretion of radioactivity, only 1.62 % of the administered antibiotic activity was recovered in the bile. And it appeared likely that *m*-Cl-PACA underwent extensive metabolism in the liver.

2. Antibiotic activity of lactone of *m*-Cl-PACA

As was described previously[1], both O-deacetylated and lactone derivatives were recovered as metabolites in urine of rats receiving *m*-Cl-PACA intramuscularly.

Table 1. Excretion of *m*-Cl-PACA in the bile and urine following intramuscular injection in rats

(1) Radioassay 100 mg/kg (3 rats) % of dose (mean±S.E.)

	0~2 hrs.	2~4 hrs.	4~6 hrs.	6~8 hrs.	8~10 hrs.	10~24 hrs.	24~31 hrs.	31~48 hrs.	Total	Grand total
Bile	19.50 ±3.93	10.42 ±0.77	4.36 ±0.55	2.18 ±0.23	1.22 ±0.24	1.87 ±0.24	0.15 ±0.00	0.23 ±0.00	39.94 ±3.52	102.07 ±2.62
Urine	48.99±5.02				12.56±4.07		0.25 ±0.13	0.32 ±0.08	62.12 ±5.08	

(2) Bioassay* 20 mg/kg (3 rats) % of dose (mean±S.E.)

	0~2 hrs.	2~4 hrs.	4~8 hrs.	8~24 hrs.	Total
Bile	1.49±0.29	0.09±0.02	0.02±0.01	0.02±0.01	1.62±0.29

* Disk method using *B. subtilis* ATCC-6633 Urine: Not determined

Comparison of the autoradiogram with the bioautogram of urine samples indicated that the antibiotic activity of these two derivatives differ, and that the former has somewhat lower activity and the latter far lower than the parent compound. Extreme difficulties in isolating the O-deacetylated derivative in a pure form prevented investigation of the activity of metabolites other than the lactone. As Table 2 shows, this lactone was less active against gram-positive bacteria, and far less active against gram-negative bacteria, than the parent compound.

Table 2. Antimicrobial spectra of *m*-Cl-PACA and its lactone (Agar dilution method)

Organism	MIC (mcg/ml)	
	m-Cl-PACA	Lactone
Staphylococcus aureus FDA 209-P	0.06	5
〃 〃 Terashima	0.25	10
〃 〃 Newman	0.125	5
〃 〃 Smith	0.06	2.5
Streptococcus hemolyticus S-23	0.125	10
Diplococcus pneumoniae III	0.25	25
Bacillus subtilis ATCC-6633	0.06	2.5
Sarcina lutea PCI-1001	0.06	5
Salmonella typhosa T-287	5	>400
〃 〃 O-901	5	>400
〃 *enteritidis*	100	>400
Klebsiella pneumoniae ST-101	25	>400
Escherichia coli NIHJ	5	>400
Shigella flexneri 2a	50	>400
〃 *sonnei* I	50	>400
Proteus vulgaris IAM-1025	50	>400
Pseudomonas aeruginosa IAM-1095	>400	>400

3. Urinary excretion of CEC after intramuscular injection to rats

The presence of metabolites and unchanged CEC in urine was measured by two different methods: radioactivity studies for experiments I and II, and antibiotic determinations for experiment III. Data collected in this study is summarized in Table 3. The recovery rate in the 48-hour urine sample was 60 % of the radioactivity and 66 % of the antibiotic activity administered. These findings suggest that most of the administered dose remains in the body in an active form, and that the urinary excretion is relatively slow. Furthermore, CEC appears to be more stable in the body than *m*-Cl-PACA. About 40 % of the administered activity was not accounted for in the urine and we examined the intestinal contents for the presence of activity. Radioactivity in the intestinal contents shown by the oxygen combustion flask method, suggested the excretion of this antibiotic into the bile.

Table 3. Excretion of CEC in the urine following intramuscular injection (% of dose)

Interval (hrs.)	Radioassay		Bioassay*
	I	II	III
0~4	43.18	37.63	39.9
4~8	9.03	9.95	11.6
8~12	4.24	2.89	6.4
12~24	3.24	3.85	7.2
24~48	Not determined	4.99	1.3
Total	59.69	59.31	66.4

A uniform dose of 20 mg/kg to 5 rats in experiment.
* Turbidimetric method using *Staph. aureus* FDA 209-P.

4. Biliary excretion of CEC

In Table 4 is summarized data on the biliary and urinary excretion of radioactivity over a 48-hour period after a single intramuscular injection of ^{14}C-CEC into three cannulated rats. About 91 % of the administered dose was recovered; 16 % in bile and 75 % in urine. Most of the biliary excretion occurred in the early 4-hour observation period. Microbiological assay of samples for a group of seven rats showed that 15 % of the initial antibiotic activity was recovered in the 24-hour bile. This study showed

Table 4. Excretion of CEC in the bile and urine following intramuscular injection in rats

(1) Radioassay 20 mg/kg (3 rats) % of dose (mean±S.E.)

	0～2 hrs.	2～4 hrs.	4～6 hrs.	6～8 hrs.	8～10 hrs.	10～24 hrs.	24～31 hrs.	31～48 hrs.	Total	Grand total
Bile	8.38 ±1.32	5.77 ±1.21	1.35 ±0.05	0.32 ±0.05	0.10 ±0.01	0.10 ±0.02	0.01 ±0.00	0.02 ±0.00	16.05 ±2.50	91.47 ±3.13
Urine	61.67±7.26				13.20±4.85		0.27 ±0.06	0.25 ±0.04	75.42* ±4.65	

* Urine remaining in bladder at sacrifice : 0.03±0.03

(2) Bioassay** 20 mg/kg (7 rats) % of dose (mean±S.E.)

	0～2 hrs.	2～4 hrs.	4～6 hrs.	6～24 hrs.	Total
Bile	5.90±1.20	5.34±1.00	2.34±0.13	1.60±0.35	15.19±2.34

** Disk method using *B. subtilis* ATCC-6633 Urine : Not determined

that CEC was excreted into bile largely in its unchanged form.

5. Serum protein binding

Animals received CEC or *m*-Cl-PACA in a single intramuscular dose of 20 mg/kg. One hour later, the serum and ultrafiltrate were examined for their antibiotic activity. From the results, shown in Table 5, we conclude that CEC was highly bound to the serum protein, with only 1.4 % of its free form. In contrast, about 30 % *m*-Cl-PACA was found in the free form.

In order to examine the nature of this protein binding, the following experiment was undertaken : A buffer solution of CEC (3.2 mg/ml) was incubated with equal volume of rabbit serum at 37°C for 2 hours. An aliquot of the product was chromatographed on a Sephadex G-10 column at room temperature, and then eluted with distilled water. The radioactivity and optical density measured at 260 nm and 280 nm revealed the presence of two peaks, one from the protein-bound fraction, and the other the free form. Another aliquot of the incubation product was added to twice the volume of acetone and, after adequate dilution, assayed for antibiotic activity. The initial antibiotic activity was quantitatively recovered from the incubation product. Paper chromatography (solvent system : *n*-butanol - acetic acid - water, 4 : 1 : 5) followed by bioautography showed an Rf value consistent with that of the authentic CEC, indicating that no enzymatic degradation occurred under these conditions.

Table 5. Serum protein binding of *m*-Cl-PACA and CEC *in vivo* (Mean±standard errors of 3 rats)

Antibiotic	Antibiotic activity (mcg/ml)		Protein binding %*
	Serum	Ultrafiltrate	
m-Cl-PACA	4.2, 3.2, 2.8 → 3.4 ±0.42	1.1, 0.9, 1.0 → 1.0 ±0.06	73.8, 71.9, 64.3 → 70.0 ±2.90
CEC	23, 22, 22 → 22.3 ±0.33	0.7, 0.05, 0.2 → 0.3 ±0.02	97.0, 99.8, 99.1 → 98.6 ±0.84

Rats were sacrificed at one hour after 20 mg/kg intramuscular injection

* Protein binding % $= \frac{\text{serum} - \text{ultrafiltrate}}{\text{serum}} \times 100$

Table 6. Excretion of CEC in the urine following oral administration in rats* (% of dose)

Interval (hrs.)	Experiment I		Experiment II	
	Radio-assay	Bio-assay**	Radio-assay	Bio-assay**
0～4	1.07	0.24	0.84	0.55
4～8	3.59	0.72	4.37	0.26
8～12	2.89	0.36	2.77	0.14
12～24	5.55	0.43	5.76	0.31
Total	13.10	1.75	13.74	1.32

* 100 mg/kg to 5 rats in each group

** Turbidimetric method using *Staph. aureus* FDA 209-P

6. Absorption and urinary excretion of CEC after oral administration to rats

Data on the recovery of radioactivity and antibiotic activity in urine after a single oral dose of ^{14}C-CEC (100 mg/kg) is summarized in Table 6. Each figure is the average value obtained from samples for five rats. The excretion of radioactivity into the urine was quite slow, as only 13 % was noted in urine collected in the first 24-hour period. On the other hand, the antibiotic activity of the same urine sample accounted for only about 1.5 % of administered dose. Examination of animals sacrificed at the end of experiment I showed that the urine remaining in the bladder still retained appreciable levels of radioactivity. In order to pursue the fate of radioactivity in areas outside urine, the gastrointestinal tract and its contents were homogenized with saline, lyophilized, and analyzed by the oxygen combustion flask method. About 85 % of the administered radioactivity was recovered. We conclude that since the rate of recovery of administered dose as measured by microbioassay to that measured by radioassay decreases gradually with time, formation of microbiologically inactive metabolites increased in the present experiment.

7. Metabolic pattern of CEC in urine or in bile

Urine specimens collected from rats receiving ^{14}C-CEC (intramuscularly or orally) were examined by paper chromatography and paper electrophoresis. The paper chromatograms produced a single radioactive peak of Rf 0.76, corresponding to ^{14}C-CEC, which, under electrophoretic condition used, migrated by 2.0 cm toward the anode (Fig. 1 (a), Fig. 2 (d)). The patterns of behavior of samples of urine and bile in paper electrophoresis are presented in Figs. 1~3 (from (a) to (i)). As these charts show, the major radioactive peak (peak I), corresponding to the original antibiotic, was found in all the specimens. Chart (b) showed that the radioactivity in the first 8-hour urine was almost unchanged. The pattern in 8~24-hour urine was closely similar to that in (b). The peak in (c) is derived from unaltered CEC. These charts unanimously demonstrate that intramuscularly given CEC does not undergo any extensive metabolism in the body. Studies using bioautography revealed that the unchanged CEC was the only antimicrobial component recovered in urine after intramuscular injection.

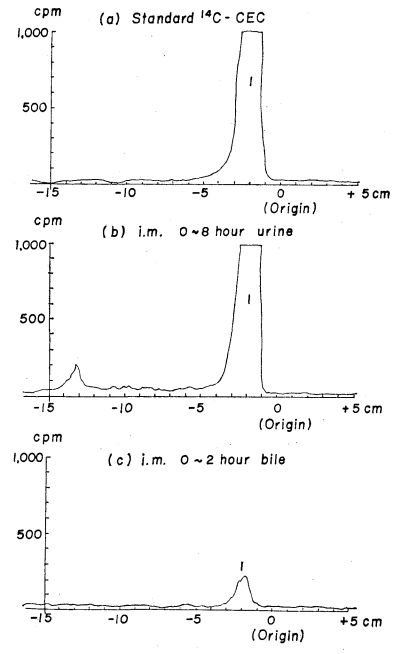


Fig. 1. Metabolic fate of ^{14}C-CEC in the urine and bile after intramuscular administration (paper electrophoresis).

Urinary radioactivity recovered during the early period (0~8 hours) after ^{14}C-CEC oral administration was derived mainly from the ^{14}C-CEC itself, and partly from metabolites which were evidenced by two peaks in paper electrophoresis, at 9.8 cm and 12.9 cm toward the anode (Fig. 2 (e)). The relative amount of the latter two peaks increased with time (Fig. 2 (f), Fig. 3 (i)). The corresponding bioautography revealed that these two metabolites were

microbiologically inactive.

Fig. 2. Metabolic fate of ^{14}C-CEC in the urine after oral administration (paper electrophoresis).

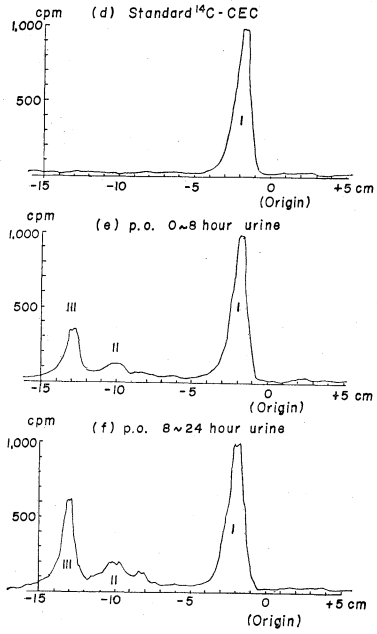
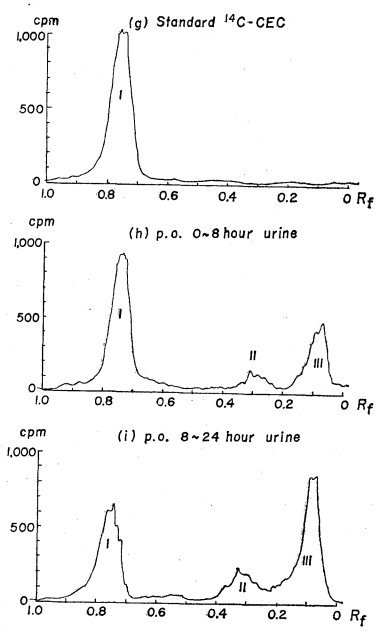


Fig. 3. Metabolic fate of ^{14}C-CEC in the urine after oral administration (papar chromatography).



Discussion

O'CALLAGHAN *et al.*[3] concluded that *in vivo* antibiotic activity of cephalosporin analogues having an acetoxymethyl group at C-3 position of 7-ACA is dependent on the activity of the corresponding deacetyl metabolite and on the rate of this metabolite formation. As indicated in our previous paper[1], *m*-Cl-PACA is deacetylated very easily *in vivo* and undergoes further decomposition to a microbiologically inactive form. It may be concluded that substitution at the C-3 position of cephem nucleus with dimethylpiperazinium dithiocarbamate would affect significantly not only its physicochemical or antibiotic property[4] but also its pharmacological behavior including oral absorption, distribution, excretion, and metabolism. When given orally to rats, one remarkable change effected by this substitution was its relatively better absorption than in its parent compound. The total recovery of radioactivity in the 24-hour urine amounted to about 13 % of the orally administered dose. Chromatographic analysis of the urine revealed the presence of two radioactive metabolites having no antibiotic activity.

On the other hand, when given intramuscularly, this antibiotic underwent little metabolism. It appears likely that this antibiotic is extensively degraded in the gut and then absorbed into the blood stream. Even though the definite structure of these metabolites is not established, one could assume that the parent compound might be converted into somewhat more polar forms retaining a positively charged structure as judged from chromatographic and electrophoretic findings.

The conversion of *m*-Cl-PACA into CEC resulted in a derivative which possesses certain advantages over the parent compound. CEC was found to have a relatively long retention-time in the rat body, because urine remaining in the bladder at the time of sacrifice (48 hours after intramuscular administration) showed that considerable radioactivity was retained therein. Furthermore, the presence of radioactivity apparently showed that the excretion of the metabolite was slower than that of the parent compound. It is of interest that CEC is excreted into bile in the biologically active form. Enterohepatic circulation also might be concerned in the delayed excretion of the radioactivity derived from CEC. BRAUER[5] advocated a theory on relationship between biliary excretion and chemical structure. He presumed that class B compounds, which he intended to include carboxylic acids with the molecular weight exceeding 300 and a property of high serum-protein binding, are actively transported from the blood into the bile. CEC is an amphoteric compound, has a molecular weight of 555 and high protein-binding property. Consequently CEC applies satisfactorily to BRAUER's class B compound.

FLYNN[6] reported that the MIC values of dithiocarbamate derivatives of cephalosporin were substantially altered by the presence of serum. The MIC of CEC increased from 0.1 mcg/ml to 0.2 mcg/ml by the presence of serum. CEC showed a high degree of protein binding, and only less than 2 % was available as the free form in rat serum. The nature of protein binding seems to be essentially reversible when judged from the results of Sephadex gel-filtration and dissociation by acetone.

Acknowledgement

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