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UPTAKE OF PROBENECID BY RAT LIVER SLICES

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Abstract—The uptake of ^{14}C-probenecid by rat liver has been studied by standard tissue-slice methods. No metabolites of this transport inhibitor accumulated in the slices, but the parent drug and a single metabolite appeared in the incubation media. The metabolite was identified by GLC-MS procedures as the glucuronide of side-chain hydroxylated probenecid. Maximum slice/medium (S/M) ratios of 2 to 3 were obtained at 20–60 min. Metabolic inhibitors (*N*-ethylmaleimide, iodoacetate, and DNP, but not NaCN) decreased the S/M ratios after 1hr incubation. Of 7 anionic compounds tested as potential inhibitors of probenecid uptake, only iopanoic acid (telepaque) and sulfobromophthalein (BSP) produced significant depression of S/M ratios in 20 min incubated slices. Neither of the three cationic compounds tested depressed the S/M ratios of any time period studied (20, 60, or 120 min). All of the potential inhibitors were also tested for their effects on probenecid binding to rat liver homogenates. Iopanoic acid and DNP dramatically decreased probenecid binding, while enhanced binding to homogenates was observed in the presence of tyropanoate, procainamide ethobromide, or chloroquanide triazine. On the basis that various inhibitors could decrease binding and/or transport, the conclusion was reached that the liver slice uptake of probenecid occurs both by nonspecific binding and by organic acid transport mechanisms.

ALTHOUGH probenecid is often employed as a prototype inhibitor of organic acid transport systems in many organs, in comparison with studies on the kidney, there is a paucity of data on the effects of this inhibitor on the liver. Detailed studies such as those of Berndt[1, 2] on renal uptake and binding of probenecid have not been published for the liver. For this reason and because of the reported biliary transport of probenecid[3] and other classic inhibitors such as ouabain,[4] it was thought to study, in greater detail, probenecid uptake and binding in the rat liver.

METHODS

Preparation of slices and incubation procedures. Male Sprague–Dawley rats (NIH colony) weighing 250–350 g, were allowed free access to Purina laboratory chow and tap water at all times. Animals were sacrificed by decapitation and the excised liver was rinsed in ice-cold Krebs–Ringer phosphate solution. Slices, about 0·4 mm thick, were prepared with a Stadie–Riggs microtome. Three slices (400–600 mg total weight)

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were suspended in 10 ml of oxygen-saturated Krebs–Ringer phosphate solution (pH 7·4) containing 1 g glucose/l. and various concentrations of probenecid, where the ratio between ^{14}C-probenecid and unlabeled probenecid was kept constant.

Incubations were carried out in 50 ml beakers placed in a Dubnoff metabolic shaker (90 oscillations/min) at 37° in an atmosphere of pure oxygen. For some experiments, one of the following compounds was added to the incubation mixture: Iodoacetic acid, 2,4-dinitrophenol (DNP), *N*-ethylmaleimide, sodium cyanide (NaCN), iopanoic acid (telepaque), tyropanoate (bilipaque sodium), phloridzin, *p*-acetylaminohippuric acid (PAAH), chlorothiazide (CTZ), phenol red, sulfobromophthalein Na (BSP), chloroguanide triazine HCl (CGT), procainamide ethobromide HBr (PAEB) and procainamide HCl.

Both nonradioactive and ring-labeled ^{14}C-probenecid (3·1 μc/mg) were kindly supplied by Merck Institute for Therapeutic Research, Rahway, N. J. Iopanoic acid and tyropanoate were gifts from Sterling Winthrop Research Institute, Rensselaer, N.Y.

In incubations done under nitrogen, the incubation medium was previously saturated with nitrogen.

Radioactivity determinations. At the end of the incubation period, slices were removed from the beakers, blotted on slightly moistened filter paper and weighed. For the measurements of radioactivity, the slices were homogenized in 2 ml of distilled water by means of a Thomas tissue homogenizer fitted with a Teflon pestle. A 0·5 ml aliquot of the homogenate was then transferred to a counting vial and 2 ml NCS reagent (Nuclear Chicago tissue solubilizer) was added and allowed to stand overnight at room temperature before adding 15 ml of scintillation fluid.[5] Similarly, a 0·5 ml aliquot of the incubation medium which had been centrifuged (10 min at 4000 rpm) was mixed with 1 ml NCS reagent and treated as above. The radioactivity was measured in a Packard Tri-Carb liquid scintillation spectrometer, model 3375.

For all measurements, the total number of counts was at least 40 times the background value; quenching corrections were made by the external standardization method. When expressed as a slice per medium (S/M) concentration ratio, the amount of drug taken up by the slices was calculated on a wet weight basis.

Identification of probenecid and its metabolite. In order to determine the nature of the radioactivity being counted under these conditions, liver slices and media from control incubations conducted as above were subjected to extraction and identification procedures as described previously.[3,6] The initial concentration of probenecid in the incubation medium was 10^{-4}M. Homogenization in 3N HCl and extraction into ethylene dichloride removed 93–100 per cent of added ^{14}C-probenecid from slices. Gas–liquid chromatographic and mass spectrographic (GLC-MS) procedures conducted as described previously for rat bile samples[6] showed that only probenecid was present in rat liver slices incubated for either 20 or 60 min. Ethyl acetate extracts of the incubation medium contained 43 per cent probenecid, while 57 per cent of the radioactivity occurred as the glucuronide of side-chain hydroxylated probenecid. This metabolite A, *p*-(*N*-propyl, *N*-2 hydroxypropyl-sulfamoyl)-benzoic acid, also is found in rat bile,[6] but none of the other biliary metabolites occurred in detectable quantities in slices or media. Since about half of the radioactivity in the media is not probenecid, the S/M ratios in this report may be biased toward lowered values by a factor of one-half.

GLC-MS procedures could not be applied to all of the individual experiments and thus the possible effects of various inhibitors on liver slice metabolism of the drug could not be assessed. The low-biased values for S/M ratios, therefore, can be considered as minimum values expected for the conditions indicated. Thus, ratios corrected for the presence of metabolite range from 4 to 5·6 in control slices.

Studies of the binding of probenecid in rat liver homogenates. Rat livers were homogenized in Krebs–Ringer phosphate solution (pH 7·4, 1 g glucose/l. resulting in 10, 20 and 40 per cent suspensions of liver tissue. Various concentrations of probenecid (radioactive + unlabeled) and other compounds used in this study were added and the homogenates were centrifuged for 20 hr at 30,000 rpm (rotor #40; average rotational centrifugal force: 59,000 *g*) in a Spinco model L centrifuge at 0°. The amount of probenecid in three resulting layers was assayed for radioactivity as described above for the tissue slices. The three layers are designated here in the following manner:

1. Particulate—the layer at the bottom of the centrifuge tube.
2. Supernatant—the uppermost 0·5 ml of the centrifuged solution.
3. Infranatant—all of the fluid between the particulate and supernatant layers.

Statistics. Where there were more than two values, means were compared by the Students *t*-test and were regarded as significant if $P < 0{\cdot}05$.

RESULTS

Incubation of slices. When rat liver slices were incubated for periods up to 20 min in Krebs–Ringer phosphate solution containing probenecid in concentrations ranging from 5×10^{-5}M to 10^{-2}M, the drug readily entered the tissue in amounts directly proportional to the concentration of the drug from the 5×10^{-5}M to the 5×10^{-3}M solution. At this higher concentration, the uptake process of the drug by the slices reached a saturation level. At a concentration of 10 μmoles/ml incubation medium (10^{-2}M), the amount of drug present in the tissue is essentially the same as that at the next lower concentration (5 μmoles) (Fig. 1). It is also noted that the rate of uptake reached a plateau with increasing time. Since the amount of drug taken up is not linear with time and since the S/M values for more dilute concentrations of probenecid are relatively higher (Fig. 1 insert), it seemed to be of great importance to report the values after correction for the amount of probenecid taken up at zero time (nonspecific binding). In order to determine the time zero values, the slices were dipped into the incubation medium, then quickly removed and treated as described above. All values in Fig. 1 are corrected for these zero time values.

The insert in Fig. 1 shows a rectilinear plot of S/M values at various times vs. concentration of probenecid in the media. For the early incubation times, 5, 10 and 20 min, the shape of the curves clearly indicate that accumulation continues throughout a 100-fold range of drug concentrations. The nonlinearity of these curves suggests saturation of the uptake process. To complete the range of incubation times used in this study, the asymptotic (120 min) point for control slices also appears in this insert (see Fig. 2). The range of S/M ratios (1–3) seen in this insert can be compared with the values obtained by Berndt[1] in rabbit liver slices (1·3), but interestingly contrasted with those obtained by this same worker in rabbit renal cortex slices (up to 5·2).

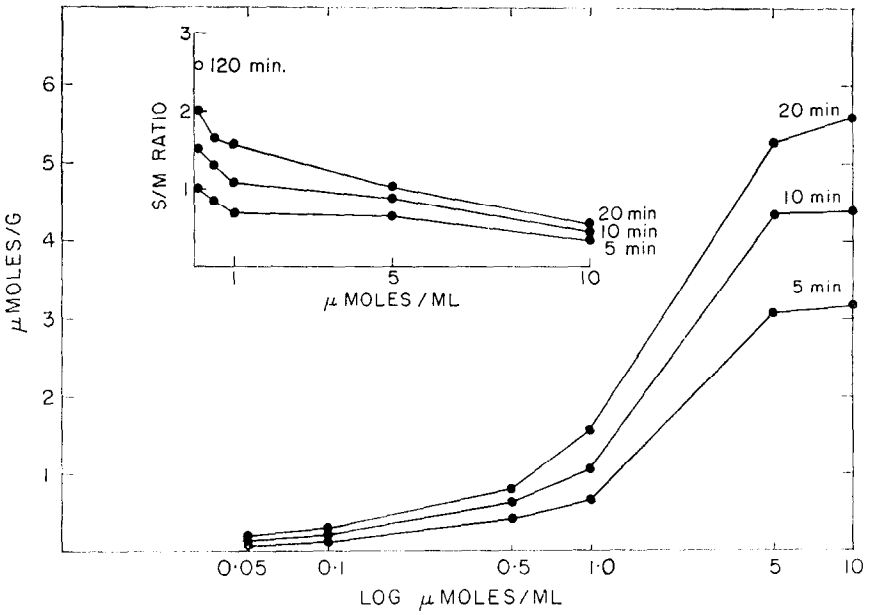

FIG. 1. Influence of concentration of probenecid in incubation medium (μmoles/ml) on uptake of liver slices (μmoles/g). Insert: Influence of concentration of probenecid on S/M ratio. Each point is the mean of at least four determinations for 20, 10, or 5-min incubation times. O is the asymptotic (120-min value) at the indicated concentration.

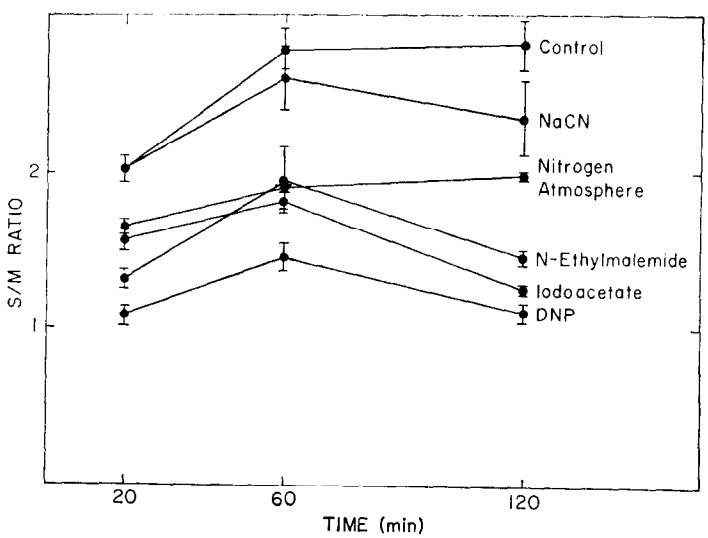

FIG. 2. Effect of metabolic inhibitors or nitrogen on the uptake of probenecid by liver slices. Each point is the mean of four determinations; brackets indicate the S.E.

From values obtained in the 5-min studies in the Fig. 1 insert an estimated K_m of $1{\cdot}9 \times 10^{-3}$M and V_{max} of 116 μg/g/min were obtained by means of a computerized least squares method in which the data were fitted to the Michaelis–Menten model as reported previously.[7]

For the following assays where the incubation period was extended to 2 hr, the concentration of probenecid in the incubation medium was fixed at 10^{-4}M. Based on the values represented in Fig. 1, this concentration was not limiting. This concentration of probenecid was also employed so that even inhibitory compounds of low aqueous solubility could be added to the incubation medium in a 10:1 molar ratio of inhibitor to drug. Fig. 2 shows the uptake of probenecid, reported as an S/M ratio, in the presence of a variety of metabolic inhibitors and under an atmosphere of nitrogen. It can be seen that the uptake of probenecid in controls reaches saturation after 1 hr; the S/M ratio at that time is about 2·8 or 5·6, if corrected for presence of the metabolite.

At a 10-fold higher concentration than that of probenecid, NaCN does not change the rate of uptake within the first hour; however, after this time the presence of NaCN appears to cause a release of the drug into the incubation medium, resulting in an S/M ratio about 15 per cent lower than that of the control. These 2-hr values also could have been depressed because some of the cyanide volatilized from the beakers. *N*-ethylmaleimide, iodoacetate and DNP significantly inhibit the uptake of probenecid within the first hour of incubation. In the presence of these inhibitors, the S/M ratios after 1 hr are 50–70 per cent that of control. In addition to slowing down the process of uptake, all three compounds appear to cause a release of probenecid back into the incubation medium since after 2 hr the S/M ratios are 40–50 per cent that of control levels. Substitution of nitrogen for oxygen as gas phase also inhibits the uptake of the drug since the S/M ratio after 1 hr is 69 per cent that of the control. In contrast to the above-mentioned compounds, however, under a nitrogen atmosphere the S/M ratio remains essentially unchanged through the 2-hr time period.

Further incubations were carried out in the presence of a variety of anionic compounds, all at a concentration of 10^{-3}M. The values in Table 1 show that only iopanoic acid gave a pattern of uptake similar to the above-mentioned inhibitors. After 20 min, 1, or 2 hr the S/M ratio in the presence of iopanoic acid was 61–70 per cent of the control at each time period.

While phloridzin, PAAH, and chlorothiazide did not significantly change the S/M ratios from controls at any of the measured time periods, tyropanoate and BSP had some noteworthy effects on the rate of uptake of probenecid at 1 and 2 hr. In the presence of tyropanoate, there was no significant change from the control S/M ratio at 20 min. The rate of uptake within the next 100 min increased significantly to a 2-hr S/M ratio, which was nearly 2-fold higher than the control. BSP had an effect similar to that of iopanoic acid since the 20-min S/M ration was significantly less than the control, after 1 hr the S/M ratio reached a value practically identical to that of the control, but the 120-min levels were significantly higher than the 20-min values. Phenol red tended to have effects similar to that of iopanoic acid and BSP, except that the 20-min levels were not significantly less than those of controls.

The presence of cationic compounds (10^{-3}M) such as CGT, PAEB, and procainamide in the incubation medium did not remarkably change the 20-min S/M ratios

compared with controls. The presence of CGT or PAEB was associated with significantly elevated S/M ratios at 60 and 120 min.

Binding of probenecid to liver homogenates. Table 2 presents values obtained in experiments where both the concentration of tissue in the homogenates and the overall probenecid concentrations were varied. The values shown are the ratio of probenecid present in 1 g wet particulate material divided by the amount of probenecid remaining

TABLE 1. EFFECTS OF VARIOUS INHIBITORS ON S/M RATIO FOR PROBENECID IN LIVER SLICES*

Inhibitors	Time (min) 20	60	120
Control	2·12 ± 0·17	2·46 ± 0·28†	2·56 ± 0·26†
Anionic drugs			
Iopanoic acid	1·30 ± 0·12‡	1·73 ± 0·11†,‡	1·66 ± 0·04†,‡
Tyropanoate	1·99 ± 0·09	3·01 ± 0·24†	4·04 ± 0·27†,‡
Phloridzin	1·79 ± 0·63	2·58 ± 0·70	2·68 ± 1·08
PAAH	1·87 ± 0·46	2·99 ± 0·97†	2·81 ± 1·00
Chlorothiazide	1·92 ± 0·54	2·79 ± 0·75	2·88 ± 0·90
Phenol red	1·84 ± 0·42	2·99 ± 0·64†	3·39 ± 0·49†,‡
BSP	1·59 ± 0·21‡	2·53 ± 0·24†	3·32 ± 0·44†,‡
Cationic drugs			
CGT	2·10 ± 0·08	3·63 ± 0·27†,‡	3·49 ± 0·25†,‡
PAEB	2·55 ± 0·33	3·52 ± 0·10†,‡	3·67 ± 0·21†,‡
Procainamide	2·41 ± 0·35	3·26 ± 0·35†,‡	3·34 ± 0·21†,‡

*The initial concentration of probenecid in the incubation medium was 10^{-4}M. The concentration of each inhibitor was 10^{-3}M. Each S/M value is the mean ± S. D. of nine experiments for the control and four to six experiments in the presence of the inhibitors.
†Significantly different ($P < 0·05$) from corresponding 20-min value.
‡Significantly different ($P < 0·05$) from corresponding control group.

TABLE 2. BINDING RATIO OF PROBENECID TO RAT LIVER HOMOGENATES*

Concentration of probenecid	Homogenate % 40	20	10
10^{-2}M	2·42	2·19	2·25
10^{-3}M	3·05	2·86	2·67
10^{-4}M	4·77 ± 0·71	4·90 ± 0·68	4·63 ± 0·60
10^{-5}M	7·77	8·17	7·60

*Calculated as $\frac{\mu\text{moles probenecid/g particulate}}{\mu\text{moles probenecid/ml supernatant}}$.

Each value is the mean of duplicate determinations except at 10^{-4}M where five determinations(± S. D.) were done.

in 1 ml of supernatant after centrifugation. The value obtained for probenecid in the supernatant was found by analyzing only 0·5 ml of the uppermost supernatant phase. This supernatant was collected by means of careful aspiration out of the centrifugation tube by means of a micro pipette. It can be seen that for a given concentration of drug, the binding ratio was virtually independent of the tissue concentration. It became necessary to show the validity of this method for determining tissue binding and the method for constructing these ratios. Before these experiments

were conducted, the similarity between the use of the supernatant values after centrifugation and those obtained by the use of a standard ultrafiltration technique was established. The decreasing ratios in the presence of increasing concentrations of the drug suggested saturation of the binding sites in the homogenates. Protein concentrations measured by the method of Lowry *et al.*[8] in all fractions of the homogenates are presented in Table 3. Homogenates of all three tissue concentrations and constant probenecid concentrations of 10^{-4}M were subjected to ultrafiltration at 0°.

TABLE 3. COMPARISON OF PROTEIN VALUES (mg/ml OR g) OBTAINED BY ULTRACENTRIFUGATION AND ULTRAFILTRATION TECHNIQUES*

Fraction	Homogenate % 40	20	10
Homogenate	90·3 ± 4·3	45·1 ± 0·3	23·4 ± 0·6
Ultracentrifugation			
Particulate	246·6 ± 2·8	238·4 ± 7·9	239·0 ± 9·9
Infranatant	22·8 ± 3·5	9·5 ± 0·8	4·9 ± 0·2
Supernatant	5·4 ± 0·6	2·9 ± 0·3	1·6 ± 0·2
Ultrafiltration			
Dialysate	< 0·03†	< 0·03†	< 0·03†

*Techniques as described in text. Each value is mean ± S. D. for four experiments.
†Below the sensitivity of the Lowry *et al.*[8] procedure.

Visking tubing containing 10 ml of homogenate was suspended in plastic tubes and centrifuged at 4000 rpm for 5 hr. Probenecid controls containing no tissue were subjected to the same procedure. These data in Table 3 show that the protein concentration of the supernatant and infranatant phases are negligible compared with the particulate protein concentration, although this former value increased with increasing homogenate concentration. Hence, in the centrifugation procedure employed here, it appears that most of the liver protein becomes concentrated in the particulate fraction. The ratios obtained by utilizing the concentration of probenecid (10^{-4} M) in the ultrafiltrate (dialysis) of 40, 20 and 10% liver homogenates were 4·25 ± 0·20, 4·36 ± 0·25 and 4·43 ± 0·25, respectively (N = 3 in each group). The good correlation of the ratios obtained with the values obtained by use of the supernatant (Table 2) on one hand and the ultrafiltrate on the other hand seemed to justify the substitution of the supernatant technique for that of the ultrafiltrate in the following experiments. The former technique was more convenient since centrifuge tubes were prepared in large quantities much faster than one could prepare samples in Visking tubing.

At a probenecid concentration of 10^{-4} M, similar assays were carried out where the homogenates were centrifuged after addition of various compounds (10^{-3} M). Table 4 shows the ratios obtained with tissue concentration of 40 per cent in the homogenates. Because of the 14·9 per cent variability of the controls, and since some samples sizes did not allow for the usual statistical analysis, only changes of more than 15 per cent were considered noteworthy. Among the metabolic inhibitors used, DNP was the only one which strikingly reduced the binding of probenecid to the

TABLE 4. EFFECT OF METABOLIC INHIBITORS, ANIONIC AND CATIONIC DRUGS ON THE BINDING OF PROBENECID TO RAT LIVER HOMOGENATES*

Inhibitors	$\frac{\mu\text{moles probenecid/g wet particulate}}{\mu\text{moles probenecid/ml supernatant}}$	Difference %
Control	4·77 ± 0·71	+14·9
Metabolic inhibitors		
N-ethylmaleimide	5·15	+ 8·0
Iodoacetate	5·36	+11·2
DNP	3·90	−18·2
NaCN	4·86	+ 1·9
Anionic drugs		
Iopanoic acid	3·92	−17·8
Tyropanoate	5·56	+16·6
Phloridzin	4·52	− 5·2
PAAH	4·77	0·0
Chlorothiazide	4·55	− 4·6
Phenol red	4·36	− 8·6
BSP	4·50	− 5·7
Cationic drugs		
CGT	5·79	+21·4
PAEB	6·01	+26·0
Procainamide	5·20	+ 9·0

*The tissue concentration was 40 per cent; the initial concentration of probenecid was 10^{-4}M and that of the added compounds was 10^{-3}M. Each value is the mean (± S. D.) of five experiments for the control and of duplicate experiments in the presence of each of the drugs. Per cent difference represents maximum variation of individual values from the control mean.

particulate. Among the anionic drugs, iopanoic acid reduced the ratio to 18 per cent of the control value. On the other hand, the closely related compound, tyropanoate, appeared to exert the unusual effect of enhanced binding of probenecid to the particulate. The other compounds did not produce any noteworthy effects. The results expected from the presence of cationic drugs[9] in the homogenates did not occur for all compounds tested; procainamide failed to alter the binding characteristics of probenecid compared with control, whereas, both CGT and PAEB increased the ratio substantially.

DISCUSSION

The results shown in Fig. 1 suggest that the uptake of probenecid by rat liver slices is dependent upon both the concentration of drugs in the incubation medium and the incubation time. Saturation characteristics are very obvious when uptake is reported in terms of S/M ratios as in Fig. 1 insert and Fig. 2. The saturation level is reached within a relatively short time (20 min) at high substrate concentration (10^{-3} M), or after 1 hr at the low concentration (10^{-4} M). The fact that for short time periods (up to 20 min) the uptake is linear with time suggests that the uptake is in part because of some type of concentrative process such as carrier-mediated transport, since tissue binding after passive diffusion into the liver slice reaches its maximum within a relative shorter period of time.

The decreased rate of uptake within 1 hr in the presence of some compounds and when nitrogen is substituted for oxygen as the gas phase further suggest (Table 1)

that the uptake consists of two processes, either of which can be modified. Metabolic inhibitors like *N*-ethylmaleimide and iodoacetate decrease the maximal S/M ratio (1 hr) but do not have any substantial effect on the binding characteristics of probenecid in liver homogenates (Table 4). This fact suggests that these two compounds inhibit only that portion of uptake due to the transport mechanism. DNP, however, reduces the binding rate of probenecid in homogenates. Since the S/M ratios obtained with DNP are the lowest values found in this study, it therefore seems likely that this inhibitor interferes with both the tissue binding and the active transport mechanism of organic acids. These data are consistent with the reports of Mudge and Taggart[10] and Berndt and Grote.[11] Both groups have shown active uptake of DNP by renal tissues and the latter workers showed decreased DNP uptake of kidney slices in the presence of probenecid. Under nitrogen only the transport step is expected to be inhibited; hence, the maximum S/M ration reached after 1 hr remained constant for the following hour. Therefore, the binding and storage mechanisms appeared to be unaffected. In contrast to this, all four metabolic inhibitors, regardless of their respective sites of action, appear to affect the storage capacity of the slices for probenecid since more of the drug appears in the medium after 2 hr.

Among the anionic drugs added to the incubation medium, iopanoic acid might inhibit both transport and binding, since it showed a reduction of the binding ratio of the control experiments, and the S/M values are much lower than those found in the presence of nitrogen. The related compound, tyropanoate, had no effect on the transport portion at early time periods, as is seen in the 20-min S/M ratio. On the other hand, tyropanoate appeared to enhance the binding of probenecid to homogenates (Table 4). Since the rate of uptake in the presence of tyropanoate between 20 min and 2 hr is proportional with time, this may indicate a facilitation of the diffusion and binding processes. BSP had no effect on homogenate binding, but the 20-min S/M ratio is significantly lower than control values. This drug may compete for a carrier-mediated transport mechanism of probenecid at early times and at later times there may be facilitation of the diffusion and binding mechanism, as with tyropanoate. The significant elevation of S/M ratios when slices are incubated with CGT or PAEB (Table 1) might be related to the higher ratio of binding (Table 4) in the presence of these compounds. The enhanced binding noted here and other aspects of the above discussion may be viewed in light of the recent work of Levi *et al.*[12] These workers have separated by gel filtration 2 hepatic cytoplasmic protein fractions which they have designated Y and Z. These soluble proteins are suggested to have a role in the transfer of bilirubin and other organic anions from plasma into the liver. It is interesting to note that when they studied bilirubin binding in the presence of bunamiodyl or iodipamide (two compounds resembling tyropanoate and iopanate used in the present investigation), both compounds significantly enhanced binding in the Y fraction while binding was diminished in the Z fraction. Hence, many of the unexpected results obtained after addition of various anionic or cationic drugs to the media may be the result of a combination of interactions of these ions, probenecid and the Y and Z proteins. The end result of these interactions apparently can give a spectrum of results which may suggest anything from inhibited to enhanced binding.

The technique used here to determine the binding ratios of probenecid to liver homogenates may provide an alternative to the ultrafiltration technique when it is desired to determine drug binding in large numbers of samples.

In conclusion, it has been attempted to bring some light into the mechanisms *in vitro* by which probenecid uptake occurs in the liver; the process appears to involve two mechanisms, carrier-mediated transport of a concentrative type and diffusion followed by binding, where either one or the other mechanisms may be interfered with by the presence of suitable inhibitors.

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