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PROBENECID UPTAKE BY SLICES OF RABBIT KIDNEY CORTEX*

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Abstract—The present studies were undertaken to provide a detailed analysis of probenecid transport. It was anticipated that a study of the interaction of probenecid with renal tissue would help clarify certain aspects of organic acid transport. Probenecid was analyzed chemically by a modification of the method of Dayton *et al.* Incubation of freehand slices was performed in a phosphate buffer system containing in most experiments 10^{-4} M probenecid. Slices of rabbit kidney cortex showed steady-state probenecid levels four to five times as high as the bathing solution, i.e. slice/medium ratios of 4:5. Rabbit kidney medulla and liver failed to show this degree of uptake. Unlike the accumulation of *p*-aminohippurate (PAH), probenecid uptake was uninfluenced by cyanide, succinate, or iodoacetamide. Furthermore, the removal of potassium or acetate from the bathing solution did not alter uptake of the drug. Although PAH did not reduce probenecid accumulation, the organic dye bromcresol green did significantly reduce uptake by about 40 per cent. Ouabain also produced a 40 per cent reduction in probenecid accumulation.

FOR MANY years probenecid has been cited as an inhibitor of *p*-aminohippurate transport and as the prototype inhibitor of the classical organic acid transport system in the kidney. Indeed, the generally accepted explanation of some excretory mechanisms is based at least partly on whether or not probenecid interacts in the process.[1]

That probenecid lacks specificity in its actions was stated in 1960 by Weiner *et al.*[2] and was discussed in detail by Despopoulos in 1965.[3] For example, probenecid was reported to enhance uric acid excretion in man[4] and the dog[5] but to reduce urate excretion in the rabbit.[6] In an appropriate concentration, probenecid inhibits the excretion of DL-epinephrine by the chicken without affecting *p*-aminohippurate (PAH) excretion.[7] Furthermore, there is a variety of substances with nothing in common except that their excretion is inhibited by probenecid: urea (in the frog),[8] nitrofurantoin,[9] creatinine,[10, 11] and thiosulfate (in sheep and goats).[12] In addition to interfering with various transport processes, probenecid has been reported also to inhibit certain specific chemical reactions, e.g. conjugation[13] and acetylation.[14]

In addition to the effects of probenecid on renal function, a few studies of probenecid transport also have been conducted. Weiner *et al.*[2] concluded from clearance

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experiments in the dog that probenecid was excreted by the same transport mechanism as PAH. Indeed, probenecid/inulin clearance ratios greater than one indicated that tubular secretion was involved. The evidence indicated that a competitive interaction of probenecid with PAH could exist.

If dog kidney can accumulate probenecid, this was not shown by Dayton *et al.*[15] with experiments *in vivo*. After systemic administration of probenecid, simultaneous tissue and plasma analyses indicated no net drug uptake. Braun and Schniewind[16] on the other hand found in the guinea pig that kidney levels of probenecid exceeded those in plasma and skeletal muscle. Furthermore, these workers showed that probenecid accumulation did occur *in vitro* and could be partially blocked by bromcresol green. Further studies by Braun[17] indicated that the inhibitory effect of probenecid on PAH uptake was not competitive in nature. A kinetic analysis of the inhibition demonstrated it to be of a noncompetitive type in surviving renal cortex slices.

Because of the nonspecificity of the probenecid effects as well as the inconclusive evidence on the nature of its transport, the present studies *in vitro* were undertaken. By a detailed analysis of probenecid transport it was anticipated that its interaction with renal tissue and perhaps with the transport of other organic acids could be clarified.

METHODS

Colored rabbits of either sex were sacrificed by a blow to the base of the skull. The kidneys (and livers in a few instances) were removed promptly and placed in cold Krebs–Ringer phosphate solution. Freehand tissue slices of renal cortex were prepared and were kept cold until the incubation. When renal medulla and liver were used, the preparation and handling of the slices were identical with that for the renal cortex.

Tissue slices weighing from 100 to 300 mg were incubated in 3·0 ml of a modified Krebs–Ringer phosphate. This solution, similar to that used in earlier studies,[18] contained 40 mM potassium and 10 mM sodium acetate. The pH was maintained at 7·3 with a sodium phosphate buffer. Except where noted otherwise, incubations were performed in a Dubnoff shaker at 25° and gassed with 100% oxygen.

After incubation, the tissues were blotted, weighed, and homogenized in distilled water with a Potter–Elvehjem homogenizer. Protein precipitation was accomplished with zinc sulfate and sodium hydroxide, and after centrifugation 3·0 ml of the clear supernatant was pipetted off and acidified with 0·2 ml of 6 N HCl and the probenecid extracted as described below.

The incubation media were transferred to centrifuge tubes and the $ZnSO_4$–NaOH protein precipitation was performed. A volume of 2·0 ml of the clear supernatant was diluted with 1 ml water, acidified and assayed for probenecid.

Probenecid was determined by a modification of the method of Dayton *et al.*[15] The volume of ethylene dichloride (Fisher, reagent grade) was reduced from 25 ml to 10 ml, and the extraction was performed in a 15-ml glass-stoppered centrifuge tube. After addition of the acidified protein-free samples to the ethylene dichloride, the tubes were shaken for 20 min, after which the samples were centrifuged briefly to facilitate separation of the two solutions. The ethylene dichloride was then washed three times; each wash involved 5–10 min of shaking with 3·0 ml of a sodium citrate (0·1 M)–disodium phosphate (0·2 M) buffer at pH 4·8. After the final wash, 3·0 ml of 2·5 N NaOH was added to the ethylene dichloride and the tubes were shaken for

15 min. Two milliliters or less of the NaOH phase was read at 242 mμ in a Beckman DU-2 spectrophotometer.

This modified extraction procedure yielded a linear relationship between optical density and the probenecid concentration of standard solutions. When working with biological material, tissue blanks were routinely analyzed in addition to the usual reagent blanks. All tissue probenecid values were corrected for the tissue blanks, which on the average gave optical density values of about 0·02 when compared to reagent blanks. Blanks obtained by extracting incubation solutions did not differ significantly from reagent blanks. Recoveries of probenecid from the experimental incubations ranged from 82 to 105 per cent.

All tissue respiration measurements were conducted by means of standard Warburg techniques. In each case the incubation conditions were the same as for the probenecid accumulation experiments, although no measurements of drug uptake were made.

The probenecid used throughout this study was generously supplied by Merck, Sharpe and Dohme Co.

In general the data were expressed as the ratio of the probenecid concentration in tissue to that in the external bathing solution, i.e. the slice/medium or S/M ratio. Statistical analyses were performed according to Student's *t*-test.

RESULTS

In Fig. 1 the time course of probenecid accumulation at three different temperatures is presented, with the data expressed as S/M ratios. The bathing solution contained 10^{-4} M probenecid. It can be seen that the S/M ratios reached a plateau by at least 1 hr. Unless otherwise indicated, all subsequent measurements were made at this time when the tissue accumulation was in the steady state. Although the usual effect of temperature was noted, i.e. an enhanced uptake with increased temperature,

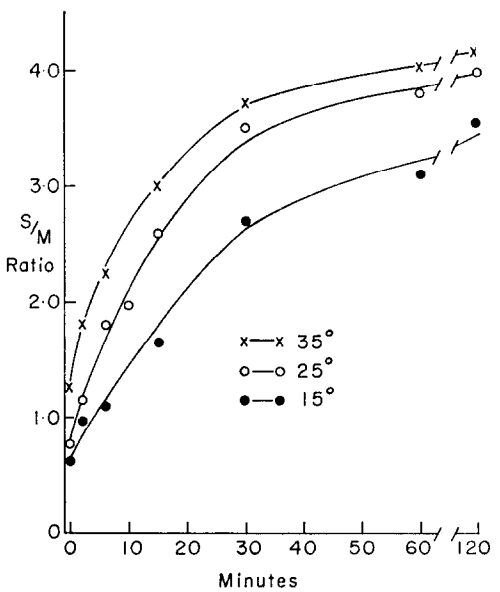

FIG. 1. Accumulation of probenecid as a function of time at three temperatures. Each point is the mean of four to ten observations.

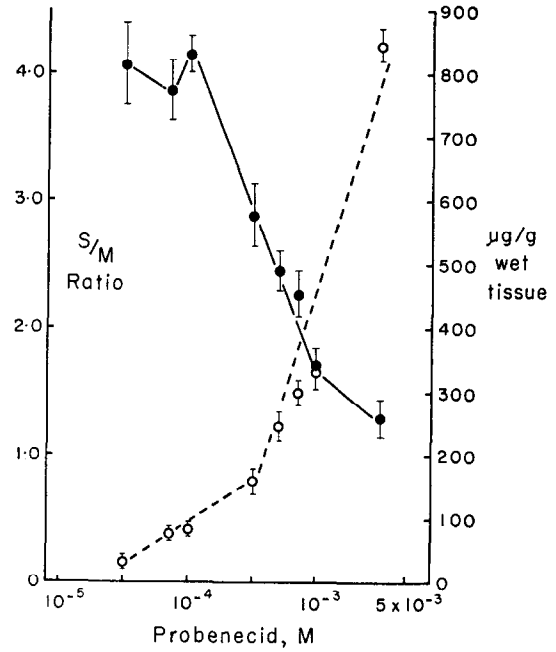

FIG. 2. Uptake of probenecid as a function of the probenecid concentration in the bathing solution. Values are means ± S.E. Incubations were for 60 min at 25°. Solid points are S/M ratios, and circles are the quantity per unit weight of tissue. Each point is the mean of six to eighteen determinations.

the magnitude of this effect was not great. Calculated Q_{10} values from the estimated initial uptake rates varied from 1·5 between 25° and 35° to 1·7 between 15° and 25°.

The influence of probenecid concentration in the bathing solution on its uptake was studied; the data are presented in Fig. 2. The final S/M ratio (solid line in Fig. 2) after 1 hr remained constant from the lowest concentration tested up to media concentrations of 10^{-4}. Above this concentration the ratios were significantly depressed. The broken line in Fig. 2 represents the accumulation of probenecid as expressed on a weight basis. For all subsequent experiments a probenecid concentration of 10^{-4} M was used in the bathing solution.

Various inhibitors of the renal organic acid transport system have been investigated for their effects on the net accumulation oi probenecid. In Fig. 3 the mean S/M ratios obtained after 60-min incubation in the presence of N_2 and O_2 are presented. Incubation with nitrogen for 1 hr significantly ($P < 0{\cdot}01$) reduced the S/M ratio from that found in the presence of oxygen. In Table 1 the accumulations in the presence of nitrogen and oxygen at three time periods are compared. It can be seen that in each case the tissue has reached a steady state at least by 60 min, although the steady-states values in the two experimental conditions were different.

The effects of various other inhibitors on probenecid accumulation are presented in Table 2. In addition, the results of parallel experiments measuring slice respiration are presented. The metabolic poisons cyanide and iodoacetamide failed to alter probenecid uptake. The concentration of cyanide was an effective one, however, as indicated by the marked reduction of tissue oxygen consumption to less than 25 per

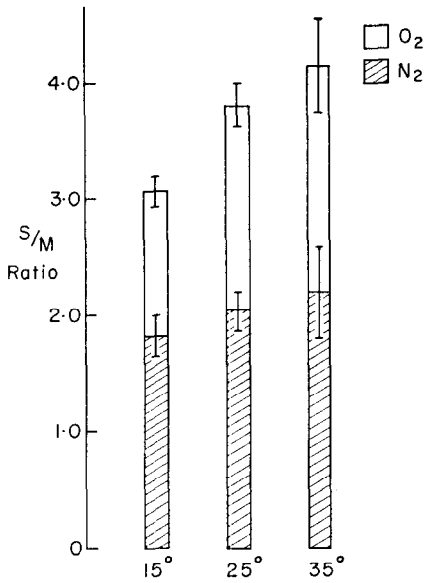

FIG. 3. Effect of incubation in nitrogen on probenecid accumulation. Values are means ± S.E. Incubations were for 60 min as indicated.

cent of the control value. The addition of succinate or the removal of acetate from the bathing solution, two procedures which retard *p*-aminohippurate and uric acid accumulation,[19, 20] did not influence probenecid uptake. The lack of effect of succinate was not due to failure of tissue penetration, since an increase tissue respiration was noted. The importance of media potassium concentration in the accumulation of other organic acids (e.g. PAH, urate) has been well documented.[18, 21] From Table 2 it can be seen that the probenecid uptake after 60 min in the absence of extracellular potassium did not differ significantly from the control accumulation where a potassium concentration of 40 m-moles/l. was employed.

TABLE 1. COMPARISON OF S/M RATIOS AT THREE TIME INTERVALS IN THE PRESENCE OF OXYGEN AND NITROGEN

Time (min)	S/M Ratio ± S.E.	
	O_2	N_2
30	3·50 ± 0·19	1·74 ± 0·16
60	3·81 ± 0·21	2·05 ± 0·18
120	4·06 ± 0·39	1·86 ± 0·16

The oxygen values are the same as those in Fig. 1 for 25°. Each nitrogen value is the mean of five experiments.

The effects of pH on probenecid uptake are depicted in Fig. 4. The hydrogen ion effect noted here was unlike that seen with other organic acids.[18, 22] The S/M ratios found at pH 4·5 were the highest encountered in this study and were significantly greater than the values at physiological pH's. It must also be noted that these maximal

TABLE 2. EFFECT OF VARIOUS FACTORS ON PROBENECID ACCUMULATION AND TISSUE RESPIRATION*

Inhibitor	M	N	S/M ± S.E.	*P*	Q_{O_2} ± S.E.	N	*P*
None		6	3·73 ± 0·54		0·86	10	
Cyanide	10^{-3}	8	3·23 ± 0·33	>0·4	0·20	6	<0·01
Succinate	10^{-2}	5	3·55 ± 0·55	>0·8	1·91	4	<0·01
Iodoacetamide	5×10^{-4}	4	3·76 ± 0·57	>0·8			
K^+-free Ringer's		10	3·21 ± 0·21	>0·3	0·84	3	<0·8
Acetate-free Ringer's		4	4·19 ± 0·86	>0·4			

Incubations were performed for 60 min at 25°.

* S/M = Probenecid concentration in tissue slices ÷ concentration in incubation medium. All *P* values were determined relative to the situation where no inhibitor was employed. Q_{O_2} = μl O_2 consumed/mg wet tissue per hr.

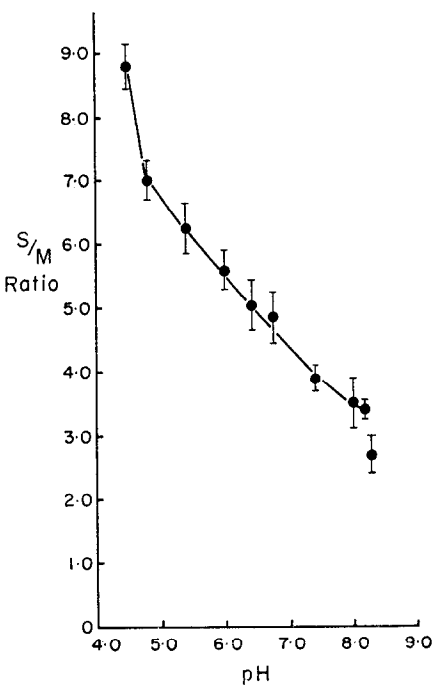

FIG. 4. Influence of pH on probenecid uptake. Values are means ± S.E. plotted against final pH of incubation solution. Incubations were for 60 min at 25°. Each point is the mean of six to eighteen determinations.

S/M values were found when the tissue respiration was significantly depressed from that at pH 7·3 (Table 3).

The accumulation of probenecid by tissues other than kidney cortex was also investigated. In Table 4 are presented the mean S/M ratios for slices of kidney cortex, kidney medulla, and liver after 60-min incubation. Only the cortical tissue accumulated large amounts of probenecid, with mean values significantly greater than those for medulla ($P < 0·01$) or liver ($P < 0·01$).

TABLE 3. EFFECT OF pH ON RESPIRATION OF RENAL CORTEX SLICES

Final pH	(μl O_2/mg wet tissue/hr)	N	P*
5·0	0·23 + 0·062	5	<0·01
6·2	0·93 + 0·055	5	
7·3	0·86 + 0·033	5	
7·6	0·90 + 0·047	5	
8·1	0·90 + 0·050	5	
8·5	0·70 + 0·032	5	<0·05

Values given as mean Q_{O_2} ± S.E.

* P Values were determined relative to the mean Q_{O_2} at pH 7·3.

TABLE 4. ACCUMULATION OF PROBENECID BY THREE DIFFERENT TISSUES

	N	S/M ± S.E.	P*
Kidney cortex	4	5·24 ± 0·089	
Kidney medulla	4	1·75 ± 0·68	<0·01
Liver	4	1·31 ± 0·24	<0·01

Data are given as the S/M ratio obtained after 60-min incubation at 25°.

* P Values were determined relative to the mean S/M ratio for kidney cortex.

The effect of PAH on probenecid uptake was also studied, and the data are summarized in Table 5. PAH failed to reduce the accumulation of probenecid under these experimental conditions. Bromcresol green is an organic dye which, although sluggishly accumulated, is avidly bound by certain tissues.[23] This substance was studied by Braun and Schniewind[16] for its effects on probenecid-S^{35} uptake by slices from guinea pig kidney cortex. Contrary to the response seen with PAH, bromcresol green significantly depressed the S/M ratio at a concentration of 10^{-4} M (Table 5).

TABLE 5. EFFECTS OF THREE INHIBITORS ON S/M RATIO FOR PROBENECID IN KIDNEY CORTEX

	M	S/M Ratio ± S.E.	(% cont.)	N
PAH	0	4·02 ± 0·33		6
	3×10^{-4}	3·61 ± 0·58	90·0	4
	7×10^{-4}	3·74 ± 0·38	93·0	6
	10^{-3}	4·32 ± 0·31	107·5	6
Bromcresol green	0	4·41 ± 0·49		5
	10^{-5}	4·09 ± 0·50	99·6	4
	10^{-4}	2·73 ± 0·40	66·4*	4
	3×10^{-4}	2·80 ± 0·24	68·1*	3
Ouabain	0	3·90 ± 0·39		4
	3×10^{-6}	3·80 ± 0·33	97·5	4
	10^{-5}	3·15 ± 0·49	80·8	4
	3×10^{-5}	2·32 ± 0·36	59·5*	4
	10^{-4}	2·54 ± 0·34	65·2*	4

* These values are significantly different from controls at the 0·05 level.

Finally the effect of the cardiotonic glycoside ouabain was tested (Table 5). The mechanism of action of this substance in inhibiting organic acid transport by surviving kidney tissues is not well understood, but it is known to reduce both PAH and urate uptake.[18, 21] Ouabain in the higher concentrations tested (3×10^{-5} and 10^{-4} M) significantly reduced probenecid uptake.

DISCUSSION

The experimental data presented in Fig. 1 demonstrate net accumulation of probenecid by slices of rabbit kidney cortex. This observation is in essential agreement with that of Braun and Schniewind[16] even though the two sets of experiments were performed on different animal species and employed different analytical techniques.

The effect of the probenecid concentration in the bathing solution on probenecid uptake is shown in Fig. 2. As the external concentration was increased, the absolute quantity taken up by the tissue increased from about 30 μg/g wet tissue at 3×10^{-5} M to 840 μg/g at 3×10^{-3} M. The S/M ratio, however, remained relatively constant up to a medium concentration of 10^{-4} M, above which the ratio was markedly depressed to 1·3 at 3×10^{-3} M. Thus, the pattern noted here is unlike that for uric acid. Platts and Mudge[19] showed that the maximal urate S/M ratio was found at a bathing solution urate concentration of 7×10^{-4} M. With urate levels both above and below this the S/M ratio was depressed. It was not possible in the present study to employ probenecid concentrations lower than those presented in Fig. 2, because of limitations of the chemical analysis. It is possible, therefore, that the use of lower concentrations of probenecid would reveal S/M ratios below 4·0, although this is unlikely. The use of radioactive probenecid would facilitate resolution of this problem.

The estimated Q_{10} values obtained from the initial uptake rates (Fig. 1) are consistent with values found in other transport systems,[24, 25] although higher values than those found here have been reported. Temperature had no statistically significant effect on the final steady-state values (i.e. 60 and 120 min). The final quantity of material accumulated was essentially the same at each of the temperatures. At earlier time periods, however, statistically significant differences were found at least between the two temperature extremes. All the values obtained earlier than 30 min at 15° were significantly different from those at 35°.

The pH studies (Fig. 4) produced an interesting relationship. It is difficult to invoke a physiological explanation for this phenomenon, particularly in view of the reduced tissue respiration at the low pH values. Furthermore, throughout the pH range studied probenecid was at least 90 per cent ionized ($pK = 3{\cdot}4$)[26] and, therefore, it is unlikely that alterations in the ionized state of probenecid could explain the results if this is a binding or trapping process. What is likely, however, is that as the pH, was reduced, changes in the ionization of tissue components were responsible for the enhanced probenecid uptake. On the other hand, these data are also consistent with an active transport process if probenecid is transported in the un-ionized state. In this situation a reduction in the medium pH would result in an increased availability of probenecid for the uptake process. There are at least two difficulties with this interpretation, however. The pH optimum for probenecid uptake is considerably different from that for other organic acids such as PAH[22] and uric acid.[18] In addition, this phenomenon proceeds in the presence of a significantly reduced oxygen consumption.

The data obtained with the use of nitrogen, various presumed inhibitors, and the cardiotonic glycoside ouabain are complicated and difficult to interpret. In addition alterations in electrolyte composition of the bathing solution and variations in the metabolic substrates employed failed to produce the results that have been noted with other organic acids.

The metabolic inhibitors sodium cyanide and iodoacetamide failed to inhibit significantly the probenecid accumulation. In separate experiments it was determined that this concentration of cyanide did significantly reduce oxygen consumption as well as the transport of other organic substances. Both these substances are capable of interfering with organic acid transport.[20] On the other hand, when the bathing solutions were equilibrated with nitrogen rather than oxygen, significant reductions in the probenecid S/M ratios were observed at each of the temperatures studied (Fig. 3). This effect is consistent with the findings for other organic acids.[19, 20]

Removal of potassium ion from the bathing solution in the present experiments did not alter probenecid uptake. Previously it was suggested that the potassium effect on urate transport might be explained by an influence on the urate efflux.[27] Although no effect of potassium on probenecid efflux would be anticipated, probenecid efflux experiments are at present being conducted in an attempt to evaluate this and other problems.

The mechanism of action of ouabain in inhibiting the transport of certain organic acids[18, 21] is poorly understood. It has been suggested, however, that such an action is a reflection of the well-known effect of the cardiotonic glycosides on tissue electrolytes,[28, 29] presumably through inhibition of a specific sodium–potassium-activated ATPase. If this interpretation is accurate it might be concluded that probenecid uptake, like that of urate and PAH, is inhibited by ouabain through an effect on the Na–K-activated ATPase.

Two substances, both of which are transported by the renal organic acid system, were tested for their effects on probenecid accumulation. PAH was investigated at several different concentrations, but even in a tenfold molar excess (PAH = 10^{-3} M) this substance failed to reduce the probenecid uptake.

On the other hand, the organic dye bromcresol green in an equimolar concentration produced a 35 per cent inhibition of probenecid uptake. This appeared to be a maximal effect, since an additional threefold increase in the dye concentration did not enhance the inhibition. These data are in essential agreement with those of Braun and Schniewind.[16] That bromcresol green retarded probenecid uptake while PAH was incapable of doing so is not surprising. Bromcresol green is known to bind very tightly to kidney tissue,[23] while no such phenomenon is noted for PAH although this substance is effectively transported. It is conceivable, therefore, that bromcresol green could displace probenecid whereas PAH could not, even when present in a tenfold molar excess.

From the data presented here and information in the literature, certain differences are noted between PAH and probenecid transport. These data might be taken as evidence that the probenecid accumulation is due only to nonspecific binding and is therefore unrelated to the classical organic acid transport process. This explanation is hard to reconcile with other data, however. Exclusion of oxygen from the system significantly reduced probenecid accumulation, an effect in direct contrast to the results seen with the metabolic inhibitors, although this might reflect only tissue

destruction with the consequent loss of tissue binding sites. The effect of ouabain is difficult to relate to a nonspecific binding if its mechanism of action is associated with ATPase inhibition.

It appears likely, therefore, that probenecid accumulation by isolated renal cortex slices may involve both nonspecific binding and active transport presumably by the organic acid transport system. It must be pointed out, however, that if these explanations are valid, the overall accumulation process must be peculiar. In Table 4 are presented the uptake data by various tissues. It is noteworthy that, although probenecid is known to interact with the liver organic acid transport system,[30] there was only modest probenecid accumulation by isolated liver slices compared to renal cortex slices. Furthermore, renal medullary slices also did not accumulate probenecid to as large an extent as cortical tissue. These relationships indicate that the binding process responsible for probenecid uptake is more or less specific for renal cortex and that essentially no interaction with the liver acid transport system was noted.

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