9. Zaffaroni, A., Barton, R. B., Keutmann, E. H., J. Biol. Chem., 1949, v177, 109.
10. Watson, D. J., Romanoff, E. B., Kato, J., Bartosik, D. B., Anal. Biochem., in press.
11. Kabara, J. J., McLaughlin, J. T., J. Lipid Res., 1961, v2, 283.
12. Mares, S. E., Zimbelman, R. G., Casida, L. E., J. Animal Sci., 1962, v21, 266.
13. Levin, L., Jailer, J. W., Endocrinology, 1948, v43, 153.
14. Claesson, L., Hillarp, N.-A., Acta Physiol. Scand., 1947, v13, 115.

Received May 8, 1967. P.S.E.B.M., 1967, v126.

Probenecid Binding by Renal Cortical Slices And Homogenates.* (32382)

W. O. BERNDT† (Introduced by R. E. Gosselin)
Department of Pharmacology and Toxicology, Dartmouth Medical School, Hanover, N.H.

Previous studies from this laboratory have implicated tissue binding as a mechanism for probenecid accumulation by isolated rabbit renal tissue(1,2). This information is relevant to studies of organic acid transport since probenecid is often used as a specific inhibitor of those transport processes. In fact, the ability of probenecid to reduce the secretion of a substance has been taken as experimental evidence that the substance in question was handled by the renal organic acid transport system(3).

Although under *in vivo* conditions there are clearance data to support renal secretion of probenecid(4), unequivocal evidence for carrier mediated transport of this acid by isolated tissues is not available. For example, although bromcresol green does reduce probenecid accumulation by renal cortex slices, p-aminohippurate (PAH) does not(1). In addition various alterations in experimental conditions which influence the transport of PAH do not affect probenecid uptake or run-out(2). For example, several substances that produce biphasic affects on PAH efflux have no effect on probenecid efflux.

Because of these discrepancies the following experiments were undertaken. It was hoped that the use of a standard equilibrium analysis as well as the homogenate studies would give direct evidence concerning the importance of tissue binding in the probenecid transport process.

Methods. Probenecid uptake was determined by procedures reported previously(1). Free-hand cortical slices (less than 0.5 mm thick) were prepared from freshly isolated kidneys and were stored in cold Krebs-Ringer phosphate solution until used. Slices (100-300 mg) were incubated in 3.0 ml of a modified Krebs-Ringer phosphate solution for 60 minutes. This time period has been shown in earlier studies(1) to be adequate for attainment of the steady state. The incubation medium contained 40 mM potassium and 10 mM sodium acetate in addition to the usual constituents. Probenecid was added to this medium to give the various final concentrations employed in this study. All incubations were carried out in a Dubnoff incubator at a shaker speed of 95 cycles/minute, a temperature of 25°, and with 100% oxygen as the gas phase.

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. After centrifugation an aliquot of the supernatant solution was acidified and extracted for probenecid according to a modification of the method of Dayton *et al*(1,5). This involved shaking of the acidified sample with ethylene dichloride, washing of the organic phase 3 times with a citrate-phosphate buffer and finally shaking the ethylene dichloride with sodium hydroxide. The probenecid determin-

* This work was supported by Am. Heart Assn. Grant 64 G 76.

† The work was done during the tenure of an Established Investigatorship of Am. Heart Assn.

ation was then conducted on the sodium hydroxide by spectrophotometric analysis at 244 mμ in a Beckman DU-2 spectrophotometer. A similar extraction was performed on the media supernatants after protein precipitation. All tissue probenecid values were corrected for appropriate tissue blanks. Recoveries of probenecid ranged between 85 and 97%.

When probenecid concentrations of 28.5 μg/ml or lower were used, uptake was determined using probenecid-C^{14} which was supplied by Dr. C. F. Rosenblum of Merck, Sharpe & Dohme Co. For these studies probenecid extractions were not performed. After homogenizing the tissue, precipitating the proteins, and centrifugation the clear supernatant, solutions were plated and counted by conventional techniques using a Nuclear-Chicago gas-flow detector and automatic sample changer. Probenecid-C^{14} was demonstrated to be a valid tracer for the chemical species by using both chemical and radiochemical analyses in a few experiments. Regardless of the analytical procedure employed the same results were obtained.

For the binding experiments, the tissues were sliced, weighed and then homogenized in Krebs-Ringer phosphate solution. After homogenizing the tissue samples were diluted to a total volume of 4.0 ml and probenecid was added to a final concentration of 10^{-4} M. Each tissue homogenate was obtained from 200 to 400 mg of tissue slices. After 60 minutes of incubation 3.0 ml aliquot of the homogenate was pipetted into a dialysis bag and tissue binding of probenecid was determined by use of an ultrafiltration apparatus according to the procedure of Toribara *et al* (6).

Where appropriate, statistical analyses were performed using Student's t test.

Results and discussion. The interactions of proteins with small molecules has been extensively studied from a quantitative viewpoint(7,8), and Scatchard has presented the mass-action relationships of these interactions. Many workers(8,9,10,11) have applied these fundamental relationships to more complicated biological systems than those originally studied, and a similar application was used here for the equilibrium analysis.

The simplest equilibrium between probenecid and the kidney cortex slice can be given as:

$$P + T \leftrightarrows PT$$

where P is the free probenecid concentration, T the concentration of free tissue binding sites and PT the concentration of bound drug. The mass action relationship is:

$$K = \frac{(P)\,(T)}{(PT)} \qquad (1)$$

where K is the dissociation contant and the other items are as defined above.

Where stable complexes are formed the binding curves show a sharp inflection point and present a well defined asymptote as saturation of the binding sites is approached(10). In the case of probenecid uptake by kidney slices this is not the case. The binding curve for this drug over a 100-fold concentration range is presented in Fig. 1. The data here only start to show an approach to an asymptote over the concentration range studied; in fact the data are essentially linear over this range. It is not possible, therefore, to determine the binding capacity from these data as presented.

A more useful modification of the mass action relationhip is a linear transformation of the following type:

$$\frac{(PT)}{(P)} = \frac{(T)}{K} - \frac{(PT)}{K} \qquad (2)$$

from which T, the tissue binding site concentration can be determined by extrapolation. This relationship is similar to that presented originally by Scatchard(7) to describe the protein binding of small molecules. Rothstein and Hayes(10) used equation 2 in their demonstration of the nature of manganese binding by yeast cells.

In Fig. 2 the data of Fig. 1 are presented as a "Scatchard plot", *i.e.;* according to equation 2 above. The two straight lines suggest the existence of at least 2 distinct populations of binding sites for probenecid. The line with the steeper slope represents that population with the greater affinity for the drug, but

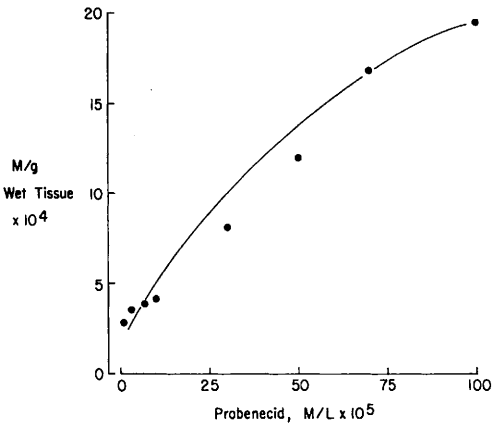

FIG. 1. Uptake curve of rabbit kidney cortex slices for probenecid. The tissue values were corrected for extracellular contamination. Each point is the mean of from 6 to 10 experiments.

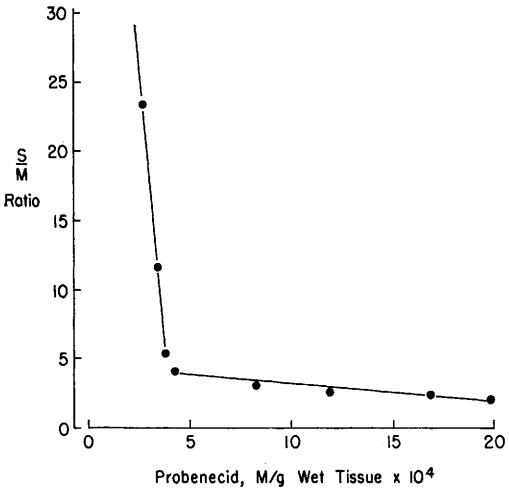

FIG. 2. Effect of bathing solution concentration of probenecid on the S/M ratio, *i.e.*, the tissue probenecid concentration divided by the bathing solution probenecid concentration. This plot conforms to a modified "Scatchard plot" as given in Equation 2.

with the lesser capacity. The dissociation constant was calculated to be 7×10^{-6} M for this population of sites, a quantity about one-hundreth that for the second population.

Because the usual interpretation of curves of this type is that a binding phenomenon has occurred, a direct examination of this possibility was attempted by studying probenecid accumulation by tissue homogenates. In Table I are presented the results of several homogenate binding experiments. The extent of binding by kidney cortex homogenates was near 30% whereas liver and kidney medulla failed to bind probenecid to a significant degree. The addition of 10^{-4} M bromcresol green to the homogenate before the addition of probenecid appeared to reverse the binding.

It was indicated previously(1) that constant S/M ratios were observed at low probenecid levels (*i.e.*, below 10^{-4} M in the bathing solution), a situation which was not noted here. This discrepancy was probably due to the different analytical procedures employed. The chemical determinations used in the earlier study were not sensitive enough for valid analyses at probenecid concentrations below 10^{-4} M; the radiochemical analyses are. In the present study lower probenecid concentrations could not be studied because of specific activity limitations of the labeled material.

The data in Table I demonstrate a significant degree of binding by renal cortex homogenates. The nearly 30% binding noted with this tissue was not approached by either liver or renal medullary homogenates. This observation is at least in qualitative agreement with previous findings wherein it was reported that slices of medulla and liver accumulated less probenecid than did cortex slices. From a quantitative point of view this degree of binding by cortex homogenates cannot account for total uptake as seen with tissue slices. This lack of quantitative agreement might be taken as evidence for the existence of at least two mechanisms of accumulation, only one of which is protein binding. On the other hand, destruction of tissue binding sites by the homogenization process is a possibility wherein a lesser degree of uptake would be noted here than with slices. No direct evi-

TABLE I. Binding of Probenecid to Tissue Homogenates. Probenecid conc. in incubation media $= 10^{-4}$M.

	% Bound ± S.E.	N	P*
Kidney cortex	28.4 ± 3.19	5	
" medulla	9.65 ± 3.81	4	<.01
Liver	6.60 ± 2.63	3	<.01
Kidney cortex + 10^{-4}M BCG	2.50 ± 1.16	3	<.01

* P values were determined relative to the value for kidney cortex.

dence is available to substantiate one or the other of these viewpoints.

Summary. The uptake of probenecid by kidney cortex slices was examined over a 100-fold concentration range. This study offers direct evidence implicating tissue binding in the slice uptake process. For example, the steady-state accumulation conformed to a modified "Scatchard plot". The data were interpreted to mean that two populations of binding sites exist. In addition it was shown that probenecid was bound by kidney cortex homogenates, but not by liver or renal medulla homogenates, an observation in keeping with slice uptake data reported previously.

The technical assistance of Mrs. Dace Grote is gratefully acknowledged.

1. Berndt, W. O., Biochem. Pharm., 1966, v15, 1947.

2. ———, Nephron, 1967, in press.

3. Rennick, B., Yoss, N., J. Pharmacol., 1962, v138, 347.

4. Weiner, I. M., Washington, J. A. II, Mudge, G. H., Bull. Johns Hopkins Hosp., 1960, v106, 333.

5. Dayton, P. G., Yü, T. F., Chen, W., Berger, L., West, L. A., Gutman, A. B., J. Pharmacol., 1963, v140, 278.

6. Toribara, T. X., Terepka, A. R., Dewey, P. P., J. Clin. Invest., 1957, v36, 738.

7. Scatchard, G., Ann. N. Y. Acad. Sci., 1949, v51, 660.

8. Scatchard, G., Hughes, W. L. Jr., Gurd, F. R. N., Wilcox, P. E., In: Chemical Specificity in Biological Interactions, F. R. N. Gurd, ed., Academic Press, Inc., New York, 1954, p193.

9. Rothstein, A., Larrabee, C., J. Cell. Comp. Physiol., 1948, v32, 247.

10. Rothstein, A., Hayes, A. D., Arch. Biochem. & Biophys., 1956, v63, 87.

11. Weed, R. Eber, J., Rothstein, A., J. Gen. Physiol., 1962, v45, 395.

Received May 10, 1967. P.S.E.B.M., 1967, v126.

Ionic Interaction with Bone Mineral. III. Reversible Calcium Exchange with Bone Powder. (32383)

CHARLES Y. C. PAK AND FREDERIC C. BARTTER
Clinical Endocrinology Branch, National Heart Institute, Bethesda, Md.

In *in vivo* kinetic studies of intravenously administered radiocalcium in man(1-3), a change of slope in the specific activity (SA) curve of blood is sometimes encountered at about the 7th day after isotope is given, with a subsequent slower rate of decline. Some workers have attributed this phenomenon to the transfer of isotope from bone back into blood as a result of bone resorption(1,4).

An alternative explanation has been offered by Nordin and associates on the basis of *in vitro* studies with bone slices(5). They believed that the "bend" in the SA curve can be accounted for by "back diffusion" of radiocalcium from bone into solution rather than by physiologic bone resorption. The curve before the "bend" was considered to reflect only "diffusion" into bone.

The present experiments were designed to test the validity of the hypothesis of Nordin and associates(5). From the wash-out studies (6,7) of defatted bone powder and of synthetic hydroxyapatite labelled with ^{45}Ca, the release of radiocalcium from the sample was measured at various times after labelling. Contrary to the findings of Nordin and associates, our results suggest that "back-diffusion" is not limited to the period after the inflection in the SA curve, but occurs before the inflection as well.

Materials and methods. Fresh rat femur and tibia were cleaned of adhering tissue and freeze-dried. The specimen was then ground to powder in a mortar, defatted with ethanol-ether and dried. Bone powder obtained between 105 and 250 μ sieves (Central Scientific Co.) was employed in the study. The calcium-to-phosphate molar ratio of the sample was 1.70. Synthetic hydroxyapatite (B-R Apatite) was the same as that used previously(6,7).

The method for measuring ^{45}Ca exchange was reported previously(6,7) and will be described here only briefly. 500 mg of bone powder (or B-R Apatite) were preincubated