Implication of Phospholipids in Rat Proximal Tubule Reabsorption.* (31320)

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Active transport has been defined as a movement of solute against an electrochemical gradient, such movement requiring metabolic energy. If the proposed models of the mechanism of transport are examined, one feature usually appears, *i.e.*, that there occurs an interaction between the solute species which is transported and some molecular species of the cell, often considered to be a membrane component. It would seem then that the identification of the molecular species with which solute may interact constitutes a major problem to be solved before our understanding of the detailed molecular events of active transport can advance beyond the stage of speculation. It is to the identification of such substances that the present work is directed.

The approach utilized in these experiments was that used by Tobias(1) in studying which cellular constituents may be responsible for maintenance of resting and action potentials of nerve. Tobias was able to systematically degrade the nerve membrane by local application of enzymes, and thereby obtained evidence that phospholipid may be critical for maintaining axonal function. Results of similar experiments carried out on the proximal tubule of the rat kidney will be the subject of this report. In addition, results of experiments are reported wherein kidney homogenates have been treated with enzymes and the subsequent ability of extracted phospholipids to bind sodium has been studied (2).

Materials and methods. I. *In vivo* experiments. Male rats weighing between 400 and 520 g were anaesthetized with sodium pentobarbital. The animals were placed on a steel plate through which they were warmed to maintain a rectal temperature of 38°C. The kidneys were exposed, decapsulated and mounted in a lucite holder according to procedures previously described(3). Warm light mineral oil bathed the kidneys to prevent drying.

To measure the rate of tubular reabsorption, the change in length of an intratubular droplet of solution trapped between oil columns was measured as a function of time, as originally described by Gertz(4). Sudan black saturated oil was introduced into single proximal convoluted tubules through one channel of a double-barreled capillary pipette of 12 to 15 μ outer diameter. The oil column was then split by test solution, and the pipette tip and puncture site was then sealed by a further injection of oil. In the original procedure, Gertz used timed photography to monitor column length; in these experiments, however, length measurements were made with a filar ocular micrometer. Since the rate of reabsorption of saline and other test solutions has been found to be a logarithmic function of time, the time of 50% reabsorption ($T_{1/2}$) becomes an index of reabsorptive rate.

All enzymes used in this study were obtained from Nutritional Biochemicals Corp. Since the primary purpose of this investigation was limited to attempting to define the gross nature of the molecular species which may be involved in the transport process, no attempt was made to purify the enzymes beyond the levels which were commercially available. All test solutions were prepared to contain 0.5 mg of enzyme powder per ml. It was not possible to dissolve all of the commercially available material, however. Consequently, all the test solutions were centrifuged and the supernatants were used in the experiments. Enzymes were selected to attack broad classes of structural components which might possibly play a role in the transport process either as constituents with which electrolytes associate or by acting as structural barriers to the backdiffusion of transported

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TABLE I. Effect of Enzymes on Rate of Reabsorption of Fluid Droplets from Proximal Tubules.

Test solution	N	$T_{½}$*	Range	Osm	P†
Saline	17	11.3 ± .5	7–14	280	
1% $CaCl_2$ in saline	16	12.8 ± .69	6–16	506	>.1
Hyaluronidase	15	13.4 ± .86	7–18	285	<.02
Collagenase	18	13.8 ± .70	9–21	295	<.01
Chymotrypsin	17	14.0 ± .98	7–21	287	<.02
Lipase	17		17–>200	516	
Phospholipase C	12		96–>200	285	
" D	20	10.0 ± .76	6–17	289	>.1
Ribonuclease	19	41.4 ± 1.9 ‡	30–59		<.001

* Mean ± standard error in sec.
† P values calculated using student's t test.
‡ $T_{½}$ calculated from slope of early part of reabsorptive curve. See Fig. 1 and text.

substance. The enzymes used are listed in Table I.

Since pancreatic lipase activity is optimal in the presence of Ca^{++}, this enzyme was dissolved in saline containing 1% $CaCl_2$. Appropriate control experiments were carried out with this solution. One would expect when hypertonic solutions were used as test solutions that there would first occur an increase in droplet volume as the fluid became diluted to the final osmolality equal to that of plasma. Reabsorption of fluid would then take place. This sequence was in fact observed, and the $T_{½}$s for $CaCl_2$ containing saline summarized in Table I were obtained from the downslope of the reabsorptive curves.

II. *In vitro* experiments. Krebs-Ringer phosphate buffer was used throughout. Rat kidney was prepared as a 1:1 homogenate (kidney weight:volume of buffer) in a Potter-Elvehjem homogenizer (50% homogenate). Enzyme solutions in buffer in a concentration of 2 mg/ml were prepared fresh every other day. Three milliliters of buffer and one milliliter of the 50% homogenate were incubated in the control flasks. The experimental flasks contained 3 ml of the indicated enzyme solution and 1 ml of homogenate. All incubations were at 37°C under 100% oxygen for 30 minutes. At the end of this time, the action of the enzymes was stopped by addition of 20-25 cc of absolute ethanol. The mixture was then heated on the steam bath for 15 minutes, and the extraction continued according to the method previously described (5). Sodium and potassium were estimated by flame photometry.

Results. I. *In vivo* experiments. Mean $T_{½}$ along with statistical evaluation of enzyme effects are shown in Table I. Although the $T_{½}$ of saline reabsorption is somewhat longer than the 9.8 seconds found by Gertz(4), the range of values of single experiments overlaps and it is highly unlikely that the difference is meaningful. The probability that the reabsorptive rate for a given enzyme solution is the same as that of saline is given in the last column of Table I. It is clear that solutions containing $CaCl_2$ and phospholipase D were without effect on transport. Hyaluronidase, collagenase and chymotrypsin seemed to have a small but significant effect. Since the range of individual half-times overlaps the values obtained with saline, the meaningfulness of this difference is subject to question. Further doubt is suggested by the fact that the osmolality of these solutions is slightly higher than that of saline which could of itself cause a slight increase in the measured $T_{½}$.

It may be argued that the injection of a small droplet at a given tubular site does not result in any measurable increase in reabsorptive rate because the amount of enzyme is too small or that the enzyme does not act for a long enough time to produce appreciable effects. This possibility has been tested in two ways. In one series of experiments, the tubule was flushed with the enzyme-containing solution by continuous infusion for a minimal time of one minute and as long as 3

minutes. The $T_{1/2}$ was then determined and found to be the same as without previous flushing. This control has a deficiency, however. The enzyme may be able to act only if it is absorbed and reaches some intracellular site, and no evidence of reabsorption of enzyme can be obtained from this experiment. To meet this objection, a sequence of repeat determinations was carried out at single tubular loci. If the argument concerning inadequate amount of enzyme and inadequate time of action was valid, one would then predict that $T_{1/2}$ would increase as a function of the number of determinations. When phospholipase D, hyaluronidase, collagenase or chymotrypsin were the test enzymes, as many as 6 repeat reabsorptions did not alter reabsorptive rate.

When lipase was used as the degradative agent, different results were obtained as compared to the enzymes considered above. The $T_{1/2}$ of the first determination was found to be slightly longer, *i.e.*, reabsorption was reduced, or in the same range as that found with the control $CaCl_2$-containing solution. Upon repeat determinations at the same tubular spot, however, reabsorptive rate decreased to give the range of final values presented in Table I.

The most potent inhibitor of transport was found to be phospholipase C. A single infusion of a droplet generally resulted in almost complete inhibition of reabsorption. The lower range of $T_{1/2}$ at 96 seconds is a single experiment where $T_{1/2}$ was less than 200 seconds.

When the proximal tubule was treated with ribonuclease, a distinct alteration in kinetics of reabsorption was evident. As shown in Fig. 1, during the early part of a reabsorptive curve, the semi-logarithmic relationship of reabsorption as a function of time was approximated. The $T_{1/2}$ was, however, increased to 41 seconds (range 30 to 59 seconds). As reabsorption progressed, the rate decreased further so that the volume remaining in the tubule remained constant. The volume remaining averaged 20% of the injected volume with a range of 0 to 37%. Complete reabsorption was observed in only one experiment.

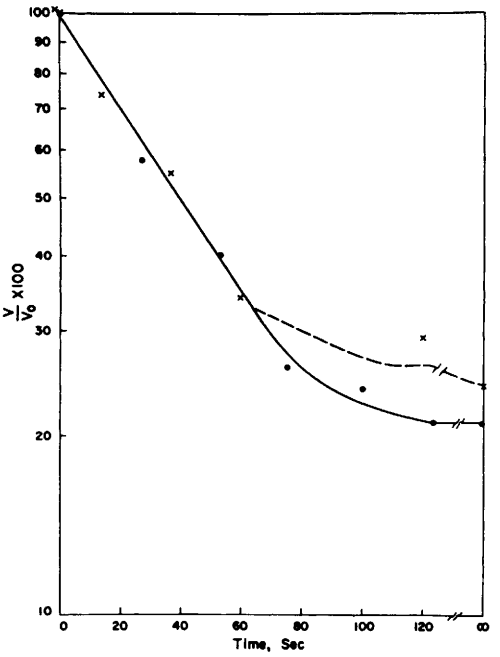

FIG. 1. Reabsorptive curves of saline droplets containing ribonuclease. Dashed line shows reabsorption of a second droplet infused into the same tubular region from which the solid curve was obtained.

It may be argued that the time delay in the attainment of a steady state volume represents the time of action required for ribonuclease to attack nucleic acids somehow involved in the transport process. One might then expect that a repeat determination at the same tubular region would result in an increased half-time of reabsorption and an increase of the final steady state volume. As can be seen in Fig. 1, however, when such experiments are done there exists essentially no difference between 2 consecutive curves. No readily apparent explanation of the action of ribonuclease is available.

II. *In vitro* experiments. The effect of the enzymes on the amount of sodium and potassium bound to the extracted phospholipids is shown in Table II. The experiments were done in 3 sets, and the control group is shown at the top of each set. Highly significant reductions in the bound Na were found as a result of incubation with phospholipase C, chymotrypsin, and lipase. There was also an associated decrease in the amount of bound

TABLE II. Effect of Enzymes on Na and K Binding by Extracts.

Treatment	Bound Na (μeq/g)	p	Bound K (μeq/g)	p
Control	17.7 ± 1.8*		5.8 ± .6	
Phospholipase C	6.6 ± .5	$<5.7 \times 10^{-7}$	1.9 ± .3	$<2.0 \times 10^{-9}$
" D	20.3 ± .8	< .05†	6.6 ± .8	> .20†
Chymotrypsin	8.3 ± 1.8	< .01	1.8 ± .5	$<5.7 \times 10^{-7}$
Control	15.8 ± .8		4.1 ± .2	
Ribonuclease	17.6 ± 1.3	> .20	5.3 ± .3	< .01
Lipase	.6 ± .1	$<2.6 \times 10^{-12}$	.1 ± .1	$<2.6 \times 10^{-12}$
Control	16.3 ± 1.4		14.9 ±1.9	
Collagenase	16.6 ± 1.9	> .90	14.9 ±2.6	> .90
Hyaluronidase	15.1 ± 1.2	> .50	11.4 ± .8	> .10

* All values averages of 10 experiments ± standard error.
† These 2 p values calculated on data calculated for daily variation of control samples; all others calculated on uncorrected samples.

potassium in each of these instances. There was no significant decrease in the bound sodium or potassium produced by any of the other enzymes. There was a significant increase in the sodium bound when the homogenates were incubated with phospholipase D, and a similar increase in potassium when incubation was carried out with ribonuclease. The significance of these latter two observations is not apparent.

Discussion. I. *In vivo* experiments. Since the terminology characterizing phospholipases is somewhat confusing, depending on whether one reads early or late literature, a word is in order about the designations used in this paper. The terminology used by Tobias(1) is used here, *i.e.,* phospholipase C is that enzyme which is involved in attacking the linkage between phosphate and glycerol, and the D enzyme is that which is involved in splitting nitrogenous base from phosphate.

On the basis of the experiments reported above, it is tempting to speculate that phospholipid phosphate constitutes a carrier site involved in the movement of electrolyte across the tubular epithelium. Such need not be the case, however. Attainment of constant volume can be achieved by either decreasing the rate of reabsorption by the tubule or by increasing the rate of backflux of salt and water. An alternative explanation of the phospholipase C effect might be that the removal of the hydrophilic portion of the lipid results in the removal of a barrier to backflux. Independent of mechanism, whether as carrier or as a critical structure maintaining the appropriate cell geometry, it seems reasonable to conclude that phospholipids are somehow involved in the transport process. This conclusion is consistent with previous observations made on a variety of transporting systems(6,7,8, for example).

Because of the design of the experiments, it is not possible to conclude that those molecular entities which would be attacked by the enzymes found to be ineffective are not involved in maintaining transport. Although it is not known if the protein (enzymes) used in these experiments is absorbed by the tubular cells, it has been shown the rat kidney is able to absorb protein(9). If the enzyme is absorbed and in its transit across the tubule was compartmentalized, no effect would be seen. For example, it could be held in pinocytotic vesicles. Another alternative is that the substance to be degraded by the enzyme may itself be sandwiched between non-degradable materials.

The question must be raised as to whether those enzyme solutions which did not effect transport actually had activity. The ideal control for demonstrating enzymatic effects would be an experiment wherein end products of enzyme action were detected in reaspirated droplets. Because of the small volumes involved, such an experiment is not feasible. As a result, the compromise control was done to determine if the enzyme preparations which showed no effects had activity in macro-scale experiments. It was found that the solutions did in fact show enzyme action. Unless the microinjection process it-

self alters the enzyme, it is reasonable to suggest that they would be active in the tubular droplet.

One final point for consideration is the control experiment with high concentration of $CaCl_2$. Experiments have been reported the results of which are consistent with the view that Ca^{++} and Na^{+} may be absorbed by a common path(10,11). One would expect then that increasing the concentration of Ca^{++} in the infusate would lead to a decreased reabsorptive rate, since Ca^{++} and Na^{+} would compete for the reabsorptive sites. If Ca^{++} and Na^{+} are indeed handled by a common mechanism, the failure of high Ca^{++} levels to affect the rate of fluid reabsorption will require explanation.

II. *In vitro* experiments. Christenson and Hastings(12) reported on the binding of sodium by phospholipids. It is a well-known phenomenon that phospholipid extracts carry many substances with them and these have been viewed as contaminants absorbed upon the phospholipid molecules. More recently, from the work of Elworthy(2) it would appear that these substances might be trapped within micelles formed by the phospholipids.

The fact that the binding of sodium by the phospholipid extracted from rat kidney is affected by phospholipase C, which splits the phospholipid at the phosphorus-glyceryl junction, and is not affected by phospholipase D, which splits the nitrogen-containing moiety from the phosphate group, would not in itself differentiate between the sodium being specifically bound, as opposed to being nonspecifically bound or absorbed to the phospholipid. The fact that such a difference in behavior is predicted from the work on the perfused proximal convoluted tubule in the same species is consistent with a specific binding site which plays a functional role in the reabsorption of sodium in the intact kidney. The reduction in binding capacity by lipase and the failure to obtain any decrease in binding by hyaluronidase, ribonuclease and collagenase as predicted from the perfusion experiments similarly support this view.

The one finding with regard to the actions of enzymes which is not predicted by the *in vivo* experiments is the effect of chymotrypsin on sodium binding. The difference in the effect of chymotrypsin in the 2 experiments can be explained by the difference in the design of the experiments. It is probable that the phospholipid exists in the intact tissue as a portion of a lipoprotein complex. The lipid portion of this complex might be so oriented in the membrane that it presents itself for action by an enzyme in the lumen of the proximal tubule, but the protein portion of the complex is not acted upon by chymotrypsin. In the homogenate, both portions of the complex are available for enzyme activity.

There is experimental evidence for such an interpretation of an action of a proteolytic enzyme. Rojas and Luxoro(13) studied the effect of trypsin on the resting and action potentials of the squid axon. Tobias(1) has shown that trypsin does not affect these potentials of the squid axon when the enzyme is applied externally. Rojas and Luxoro injected a trypsin solution under the membrane of the axon and after 5 minutes the local response to a stimulation disappeared although the resting potential was not modified. This would indicate that the protein portion of the molecule was important to some aspect of the ion transport but was not attacked by trypsin from the external surface of the membrane.

The fact that the value of bound sodium in the phospholipase D sample is higher than the controls suggests that this enzyme may have a stabilizing effect upon the compound binding sodium. The effects of the enzymes on the binding of potassium are noted to parallel the effect of sodium except for the stabilizing effect of phospholipase D on bound sodium and the higher value of bound potassium in the ribonuclease experiments. Unpublished work by Vanatta indicates that there are binding sites on compounds obtained from dog kidney which will not accept potassium ions, thus indicating that these binding sites have some specificity. The independent effects of phospholipase D and of ribonuclease would be consistent with this point of view also.

The control and experimental values for bound potassium in the third series of ex-

periments are quite different from the first two series of experiments. A check on possible factors producing this has not revealed any explanation for this variation.

If the *in vivo* results are actually produced by enzymatic degradation of the tubular cells, the high degree of correlation between the *in vivo* and *in vitro* experiments supports the hypothesis that the bound sodium of the phospholipids is not an artifact of the extraction process but indeed represents some function of the molecule in the intact kidney. This function could be a role in sodium transport.

Summary. Phospholipase C and pancreatic lipase reduce the reabsorption of fluid droplets infused into rat proximal tubules. Phospholipase D, chymotrypsin, hyaluronidase, collagenase and $CaCl_2$ appeared to have no effect on reabsorption rate. Ribonuclease also reduced fluid reabsorption. When kidney homogenates are treated with the same enzymes, phospholipase C and chymotrypsin reduce the ability of extractable phospholipids to bind sodium and potassium. The data suggest that phospholipase D and ribonuclease may enhance cation binding. It is suggested that phospholipids play a critical role in salt transport by the proximal tubule.

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Acid Phosphatases of Rat Polymorphonuclear Leucocytes.* (31321)

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Since lysosomes were first proven to be a liver cell organelle rich in a variety of hydrolytic enzymes(1), numerous studies have been directed toward detection and separation of lysosomes or similar particles from other mammalian tissues. Cohn and Hirsch showed that granules isolated from rabbit polymorphonuclear (PMN) leucocytes possess properties and functions very similar to those of hepatic lysosomes(2). Other investigators have reported similar results(3). At present, granules in PMN leucocytes are considered to be "primary lysosomes," or storage sacs of hydrolytic enzymes(4).

Among the hydrolytic enzymes present in lysosomes, acid phosphatase has frequently been used as a marker enzyme to detect lysosomes by histochemical methods. As much as 70% to 80% of acid phosphatase was found in the lysosomal fraction upon fractionation of rat liver homogenate(5), and approximately 70% was recovered from the granule fraction of rabbit PMN leucocytes(2). Although the heterogeneous nature of acid phosphatase associated with lysosomal and soluble fractions of rat liver was indicated by Shibko and Tappel(6), the properties of this enzyme in subcellular fractions of PMN leucocytes have not been clarified.

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