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ETHACRYNIC ACID ACCUMULATION BY RENAL TISSUE

J. S. CHARNOCK and A. F. ALMEIDA
Department of Pharmacology, University of Alberta, Edmonton, Canada

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Abstract—During incubation in isotonic glucose–salt medium, thin slices of rabbit kidney tissue accumulate [2-^{14}C]ethacrynic acid against a concentration gradient. Saturation does not occur in 60 min of incubation, by which time the accumulation is about 10-fold when expressed as a slice : medium ratio. Electron microscopic examination of tissue integrity revealed that there was some swelling and other associated changes after 60 min of incubation also, and the experiments were thus not carried out for longer periods. [2-^{14}C]ethacrynic acid uptake was markedly lowered by reduction of incubation temperature, omission of glucose from the medium or anoxia. Addition of the inhibitors of cellular metabolism, 2,4-dinitrophenol or sodium azide, or addition of either probenecid or ouabain, which are known to block transport processes, also resulted in a striking decrease in drug accumulation. These results strongly suggest that a metabolically dependent active transport process is involved in the accumulation *in vitro* of ethacrynic acid by thin slices of rabbit kidney tissue. When slices of rabbit liver tissue were substituted in these experiments, the degree of [2-^{14}C]ethacrynic acid uptake was greatly reduced and may represent only nonspecific binding to tissue slices. It is suggested that the capacity of renal tissue to accumulate ethacrynic acid *in vitro* may reflect a process which *in vivo* enables significant concentration of this drug at pharmacological receptor sites within renal cells. Thus, observed plasma levels of this drug in whole animals may not represent effective pharmacological concentrations.

ALTHOUGH the diuretic and natriuretic action of ethacrynic acid is well documented, the biochemical mechanism by which these effects are mediated is not fully understood.[1–3] Several recent studies have shown that the distribution of water and the movement of electrolytes in renal tissue are effected by ethacrynic acid,[4,5] but whether these effects follow an inhibition of cellular energy production[4,6] or a more direct action upon membrane transport systems is not clear.[3,7]

In addition, several recent reports[8,9] have confirmed the inhibitory action of ethacrynic acid upon isolated microsomal preparations of the cardiac glycoside sensitive sodium-plus-potassium activated adenosine triphosphatase, ($Na^+ + K^+$)-ATPase, which is thought to be involved in the so-called "sodium pump".[10] However, this effect can only be demonstrated *in vitro* at a drug concentration which greatly exceeds that reported in the plasma of experimental animals during diuretic episodes.[11]

A number of investigators have suggested that this discrepancy might be explained by some form of drug accumulation by renal tissue,[11,12] although the data available do not provide direct evidence for this hypothesis.[3,9]

We have sought further information regarding possible drug accumulation *in vivo* by examining the uptake of [2-^{14}C]ethacrynic acid by thin slices of rabbit kidney tissue under a variety of experimental conditions, which include the effects of inhibitors of cellular metabolism and transport. The greatly increased level of cellular organization

present in these experiments, compared to those with isolated enzyme preparations, has enabled us to show that ethacrynic acid is actively accumulated against a concentration gradient, that this process is dependent upon temperature and substrate, and can be markedly reduced by anoxia, dinitrophenol, azide, ouabain and probenecid.

MATERIALS AND METHODS

Slices of fresh rabbit kidney or liver were cut by hand microtome[13] to a uniform thickness so that when trimmed to 1 cm^2 the slices had a mean initial weight of 50 $\pm$ 5 mg. The first slice was discarded so that all slices used had two cut surfaces. The trimmed weighed slices were kept ice-cold in isotonic glucose–salt medium of the following composition: 143 mM NaCl, 5 mM KCl, 2 mM $MgSO_4$, 10 mM CH_3-$COONa$, 5 mM Na_2HPO_4, 1 mM $CaCl_2$ and 10 mM glucose, adjusted to pH 7·4 by the addition of 0·1 N HCl. The osmolarity of this solution was determined by a Standard laboratory osmometer freezing point depression apparatus (Advanced Instruments Inc.) and was 314 mOsmoles/l.

For the incubation of tissue, [2-^{14}C]phenoxy-acetic ethacrynic acid (obtained as a gift from Merck, Sharp & Dohme Research Laboratories, Rahway, N.J., U.S.A.) was adjusted to pH 7·4 by the addition of solid Tris base (Schwarz/Mann ultrapure) and added to the incubation medium to give a final concentration of ethacrynic acid of $7{\cdot}4 \times 10^{-7}$ M. This concentration corresponds to that plasma level which might reasonably be expected to follow administration of a low clinical dose (10–50 mg) of this drug to man.

Either pure oxygen or nitrogen (for the anoxic experiments) was vigorously bubbled through this solution for at least 1 hr prior to use. When used, inhibitors were added to the medium prior to gassing. The concentrations employed are given in the text. Each tissue slice was incubated in a final volume of 5 ml of solution. The flasks were placed into a Dubnoff metabolic shaking apparatus at the required temperature and continuously flushed with gas throughout the incubation period. After incubation for the required times, the tissue slices were removed from their flasks with flat-nosed forceps and twice dipped into 10 ml of fresh medium without ethacrynic acid; the slices were then drained on absorbant tissue and transferred to vials for radioactivity counting. The tissue was dissolved overnight in 0·5 ml of commercially available NCS tissue solubilizer (Amersham/Searle) and 10 ml of toluene fluor was added (containing 5% BBS2, Beckman Biosolv and 0·5% 2,5-diphenyloxazole). Two drops of 4% $SnCl_2$ in 0·1 N HCl were then added and the tissues dark adapted overnight prior to counting in a Beckman LS-100 liquid scintillator. The samples were counted to a preset error of 3 per cent. The incubation medium was also counted to enable expression of results as slice : medium ratio at all time points during the experiment.[14]

Chromatography. After incubation in [2-^{14}C]ethacrynic acid, some slices were resuspended in 2 ml of ice-cold distilled water for at least 48 hr. The tissue was then separated by centrifuging at 0°, and the protein-free aqueous extracts so obtained were concentrated by evaporation under reduced pressure and spotted on strips of Whatman No. 1 paper. Ascending chromatography in butanol–acetic acid–water solvent (120:3:50) was carried out for 3 hr, according to the procedure of Bourke *et al.*,[15] using authentic ethacrynic acid as a marker. Spots were visualized under ultraviolet light, cut from the paper, and counted for radioactivity as above.

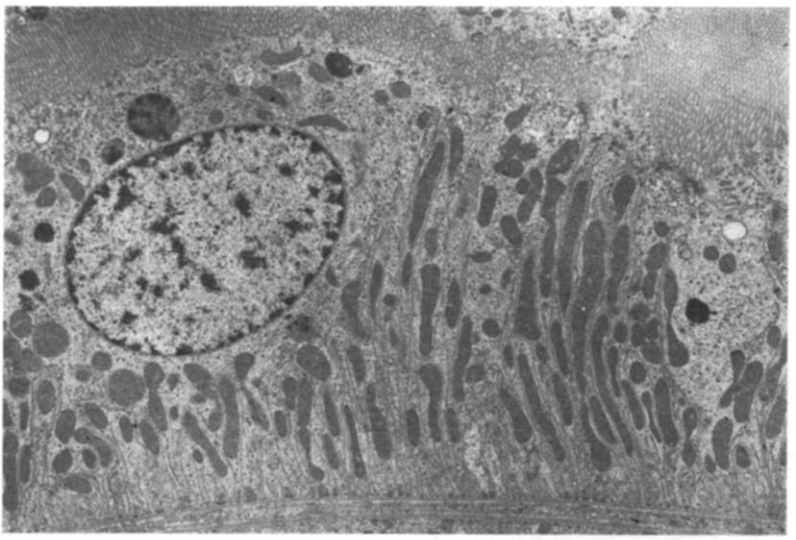

FIG. 1. Electron micrograph of untreated rabbit kidney tissue slice before incubation (magnification 5×10^3).

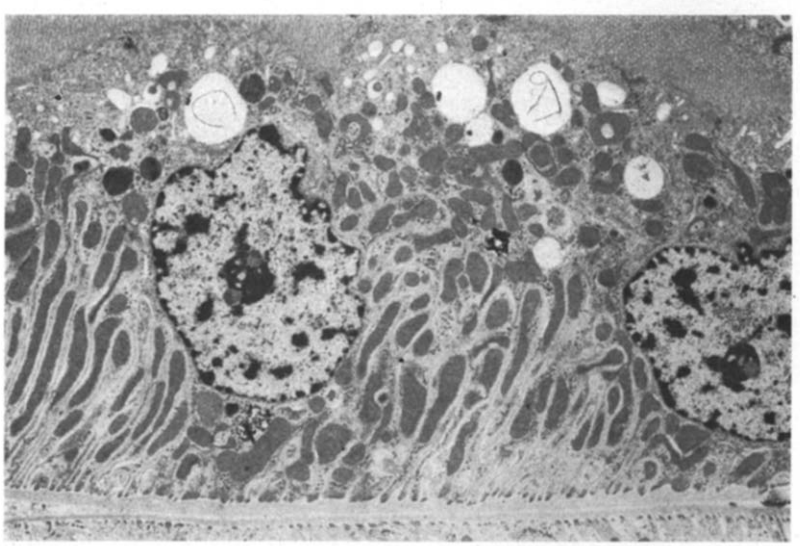

FIG. 2. Electron micrograph of untreated rabbit kidney tissue slice after 10 min of incubation at 37° in an oxygenated isotonic medium containing the salts and glucose as described in the text (magnification 5×10^3).

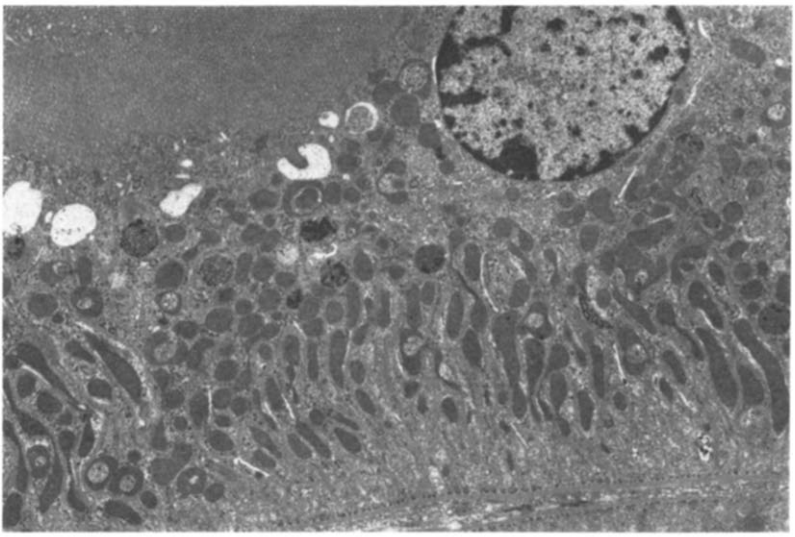

FIG. 3. Electron micrograph of rabbit kidney tissue slice after 10 min of incubation at 37° in an oxygenated isotonic medium containing the salts and glucose as described in the text, and with the addition of $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid. The medium was buffered to pH 7·4 by the addition of Tris base (magnification 5×10^3).

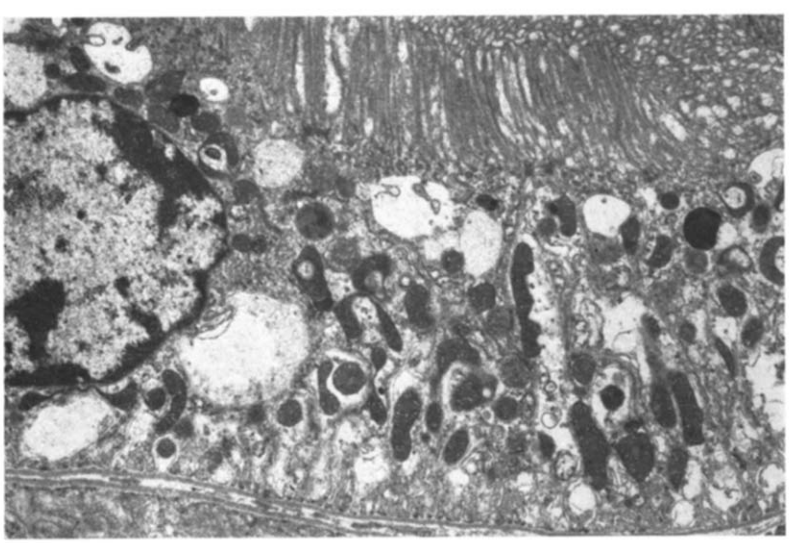

FIG. 4. Electron micrograph of rabbit kidney tissue slice after 60 min of incubation in the medium and under the conditions described in Fig. 3 (magnification 7×10^3).

Extracellular space. A measure of extracellular space was obtained from the distribution of [1-^{3}H]mannitol as described by Rosenberg *et al.*,[14] who made use of the observation that mannitol was not actively transported into cells.[16] The distribution ratio (disintegrations per minute per gram wet weight of tissue: disintegrations per minute per milliliter of incubation medium) was obtained after various incubation times.

Electron microscopy. Tissue slices were fixed in 5% glutaraldehyde in phosphate buffer (990 mOsmoles/l.), post-fixed in OsO_4, and dehydrated by serial treatment with graded ethanol before embedding in Epon. Sections were stained with uranyl acetate and lead citrate before examination in the electron microscope.

RESULTS

Tissue integrity. Electron microscopic examination was carried out on slices of rabbit kidney used in these experiments. Both freshly cut tissue and incubated slices were examined. Figure 1 shows control tissue before incubation. The basal membranes were intact with the nucleus, nuclear envelope, microvilli and mitochondria apparently normal. No signs of swelling or cellular disruption, were evident. After short-term incubation, there were some changes in mitochondrial morphology with a few organelles appearing vacuolated (Fig. 2). No marked differences were seen when ethacrynic acid was present in the medium, other than a slight increase in mitochondrial vacuolation (Fig. 3).

After 60 min of incubation, however, there was evidence of tissue swelling. The basement membrane, plasmalemma and nuclear membrane were intact, but cell organelles and microvilli were swollen. There were many more vacuolated mitochondria, although others appeared unchanged from the control before incubation. Occasional myelin figures were now present. Although no clear differences were seen, the addition of ethacrynic acid to the incubation medium may have enhanced these changes (Fig. 4). Marked cellular disruption or membrane damage was not observed under any experimental conditions, but sufficient changes and swelling had occurred to suggest that incubations should not be prolonged beyond 1 hr.

Some investigators have attributed the experimental effects of ethacrynic acid to direct interaction with anaerobic[6] or oxidative[17] metabolism of the cell. In both studies cited, the concentration of ethacrynic acid was at least 1000-fold greater than that used in the experiments reported here. The pharmacological relevance of experimental effects observed with such high drug concentrations must remain conjecture until a drug-accumulating mechanism is demonstrated. In our experiments, morphological evidence did not suggest significant cell damage by ethacrynic acid at a concentration of $7{\cdot}4 \times 10^{-7}$ M. Furthermore, the extracellular space, as measured by the distribution of trace amounts (8×10^{-8} M) of [1-^{3}H]mannitol, was also determined in the presence and absence of ethacrynic acid. The data given in Fig. 5 show that there was a marked uptake of [1-^{3}H]mannitol over the first 45 min of incubation, but that no significant further increase occurred by 60 min. The uptake of [1-^{3}H]mannitol was identical in the presence and absence of $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid and the mean distribution ratio (given as a percentage) at equilibrium was 48 per cent. Thus, there were no abrupt changes in membrane permeability or water distribution during the period of our experiments. Further confirmation of this conclusion came from

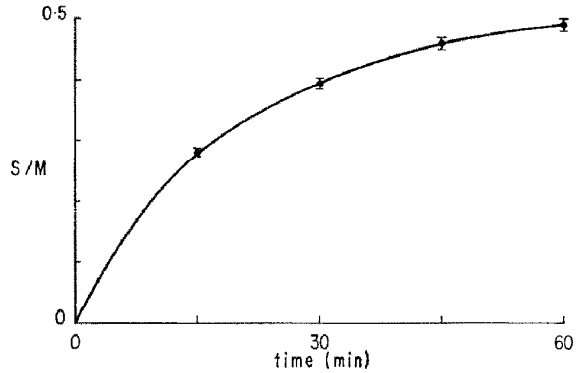

FIG. 5. Distribution (slice:medium) of [1-^{3}H]mannitol by thin slices of rabbit renal tissue. Observations are the mean ± S. E. where N = 8. The data were identical ± 7·4 × 10^{-7} M ethacrynic acid.

routine determination of total tissue water before and after incubation, which also indicated no significant change during incubation and was not influenced by the low concentration of ethacrynic acid employed in this investigation.

Uptake of [2-^{14}C]*ethacrynic acid.* The uptake of radioactively labeled ethacrynic acid by thin slices of rabbit kidney cortex was followed in an oxygenated isotonic medium containing salts and glucose. Incubations were for the times and at the temperatures described in the text and figures; the assays were performed in at least duplicate. Tissue was obtained from different rabbits and incubations were carried out on different days. The collected data from 29 experiments with 7·4 × 10^{-7} M ethacrynic acid are shown in Fig. 6 which, although by no means the total number of such experiments performed, serves for reference as our bulked control.

Although a concentration of 7·4 × 10^{-7} M drug represents that which might reasonably occur after administration of a low dose of this diuretic to man, we also

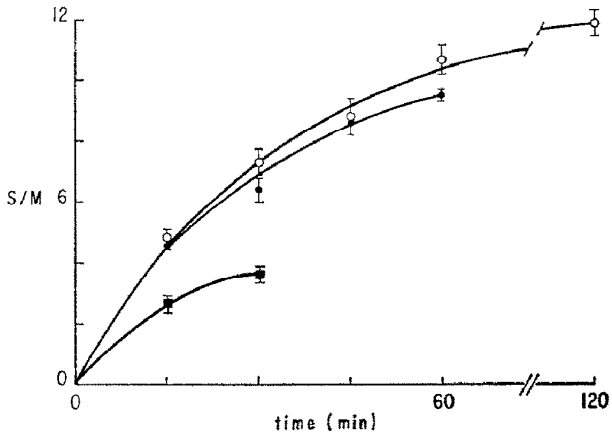

FIG. 6. Time course of uptake of [2-^{14}C]ethacrynic acid by thin slices of rabbit renal tissue under control conditions described in text, with a 100-fold increase in drug concentration, and with glucose omitted from the medium. The points shown are mean ± S. E.: ○, 7·4 × 10^{-7} M ethacrynic acid (N = 29); ●, 7·4 × 10^{-5} M ethacrynic acid (N = 8); ■, 7·4 × 10^{-7} M ethacrynic acid but in the absence of glucose (N = 7).

examined the effect of a 100-fold increase in drug concentration *in vitro* to explore the possibility of saturation. No such effect was observed, although there was a reduction of about 10 per cent in relative concentrating effect after 60 min of incubation at the higher drug concentration ($7{\cdot}4 \times 10^{-5}$ M). These data are also shown in Fig. 6, where the results are presented as slice : medium ratio plotted against time. Chromatographic recovery and identification of radioactively labeled material[15] after incubation of the tissues showed that all the counts were essentially in the form of ethacrynic acid rather than in possible degradation products, since no other ^{14}C- labeled spots were detected and recovery of total counts was greater than 98 per cent. This finding is in agreement with that of Beyer *et al.*,[11] who also reported little $^{14}CO_2$ production after administration of "acetate"-labeled ethacrynic acid to rats, and with a more than 90 per cent recovery of drug in unchanged form.

The slice : medium ratios obtained with $7{\cdot}4 \times 10^{-7}$ M ethacrynic acid were greatly in excess of unity, with a mean value of $10{\cdot}7 \pm 0{\cdot}5$ after 60 min of incubation. When considered with the extracellular space measurement of approximately 0·5 as determined previously (Fig. 5), these values show that this degree of uptake cannot be accounted for by diffusion alone. Some form of accumulation, either binding or transport or both, must have occurred.

In a smaller series (N = 7) of experiments, which are also shown in Fig. 6, the effect of omitting glucose from the medium was observed. After 30 min of incubation, the uptake of [2-^{14}C]ethacrynic acid was markedly reduced, being only 55 per cent of that of untreated controls, thus demonstrating the need for glucose as substrate for this reaction.

Unless otherwise stated, all further experiments were conducted for 1 hr with glucose present in the medium. They were always carried out as paired studies, including untreated controls, but for ready comparison between experiments are usually discussed as percentages of the uptake of the bulked controls.

Effect of temperature. Some earlier experiments had shown a marked temperature dependence of ethacrynic acid uptake by guinea-pig kidney slices, an effect which was not apparent with slices of liver obtained from the same animal (Table 1). The effect of temperature upon [2-^{14}C]ethacrynic acid uptake by rabbit tissue was therefore examined in more detail. The results are shown in Fig. 7, where after only 15 min of incubation a marked effect of temperature was apparent which persisted throughout the entire period of observation. After 60 min of incubation at 0°, the drug uptake

TABLE 1. EFFECT OF INCUBATION TEMPERATURE ON THE UPTAKE OF [2-^{14}C]ETHACRYNIC ACID BY SLICES OF GUINEA PIG KIDNEY CORTEX AND LIVER*

Tissue	Temp.	No. of experiments	Per cent mean dry wt.	Ethacrynic acid ($\mu\mu$moles/mg dry wt.)†	Per cent control
Kidney	37°	11	$19{\cdot}7 \pm 0{\cdot}8$	$2{\cdot}32 \pm 0{\cdot}62$	100
Kidney	0°	6	$21{\cdot}5 \pm 1{\cdot}6$	$0{\cdot}78 \pm 0{\cdot}18$	34
Liver	37°	7	$22{\cdot}0 \pm 1{\cdot}0$	$1{\cdot}01 \pm 0{\cdot}19$	44
Liver	0°	4	$22{\cdot}5 \pm 0{\cdot}3$	$0{\cdot}67 \pm 0{\cdot}11$	29

* Previously unpublished results of J. S. Charnock, H. A. Potter and D. McKee (1968).
† Means ± S. E.

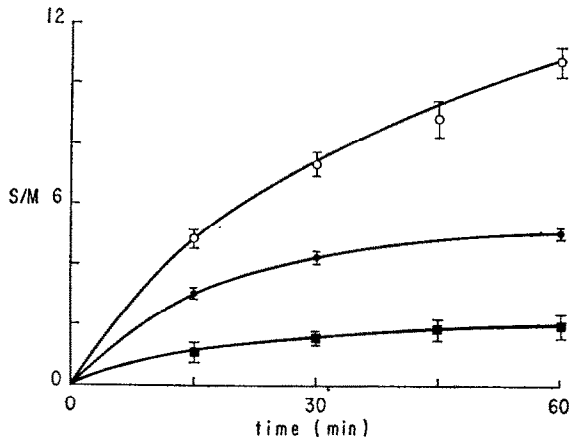

FIG. 7. Effect of temperature on [2-^{14}C]ethacrynic acid uptake. ○, Uptake by rabbit kidney at 37° where N = 29; ■, uptake by rabbit kidney at 0° where N = 6; ●, uptake by rabbit liver at 37° where N = 5.

represented less than 20 per cent of that seen at 37°. It is likely that this fraction represents the nonspecific drug binding previously reported by Beyer *et al.*[11] When slices of rabbit liver were substituted for kidney, the uptake of ethacrynic acid at 37° was also greatly reduced, reaching only 47 per cent of that found with kidney, thus confirming our earlier observations with guinea-pig tissue (cf. Table 1).

Effect of anoxia and metabolic inhibitors. The experiments in the absence of glucose and the marked effect of temperature had suggested a dependence upon cellular metabolism for ethacrynic acid uptake. This possibility was further examined by substitution of nitrogen for oxygen in the gas phase, both before and during incubation. The results of incubation under nitrogen are given in Table 2. There was some uptake of labeled drug during the first 15 min of incubation, which, however, did not increase appreciably during the remainder of the incubation period. At 60 min, the level under nitrogen was 50 per cent of that found under control conditions with oxygen. The observation that this level of uptake is greater than that found at low temperature presumably indicates only a partial block of metabolism by the substitution of nitrogen

TABLE 2. EFFECT OF INCUBATION CONDITIONS AND INHIBITORS ON THE UPTAKE OF [2-^{14}C]ETHACRYNIC ACID BY SLICES OF RABBIT KIDNEY CORTEX

Incubation conditions	No. of experiments	*Slice/medium ratio*			
		15 min	30 min	45 min	60 min
Oxygen	29	4·85 ± 0·03	7·34 ± 0·04	8·81 ± 0·07	10·69 ± 0·49
Nitrogen	4	3·64 ± 0·22	3·95 ± 0·04	4·92 ± 0·21	5·31 ± 0·17
2,4 Dinitrophenol, 0·1 mM	4	3·64 ± 0·19	3·85 ± 0·28	5·07 ± 0·64	5·03 ± 0·29
Sodium azide, 5 mM	4	2·21 ± 0·17	3·87 ± 0·34	4·36 ± 0·23	4·77 ± 0·19
Ouabain, 1 mM	7	2·95 ± 0·21	3·45 ± 0·13	4·45 ± 0·31	5·11 ± 0·33
Probenecid, 0·1 mM	4	1·80 ± 0·11	2·54 ± 0·13	3·25 ± 0·19	3·74 ± 0·26

* Values given are means ± S. E.

for oxygen. Certainly no attempt was made to remove possible traces of oxygen from the nitrogen supply.

The effects of the metabolic inhibitors, dinitrophenol (0·1 mM) and sodium azide (5 mM), are also shown in Table 2. Both agents markedly reduced ethacrynic acid uptake with the effect of azide being somewhat more pronounced (45 per cent of control at 60 min). Interestingly, at the concentration of inhibitors employed, both agents reduced the uptake to the level seen with oxygen deprivation but not to that found at low temperature (cf. Fig. 7). When taken together, these experiments strongly suggest a major dependence upon oxidative tissue metabolism for the accumulation of ethacrynic acid by slices of rabbit kidney cortex.

Effects of probenecid and ouabain. Finally, the action of two other pharmacological agents was studied, both of which have been known for many years to block cellular transport processes.[18–21] The results are also shown in Table 2, and again clearly indicate the metabolic dependence of ethacrynic acid uptake in this tissue. The action of probenecid (0·1 mM) seemed to occur more rapidly than that of ouabain (1 mM) and to be somewhat more pronounced, in that by 60 min it had reduced the mean level to 35 per cent of the control, whereas with ouabain the mean level was 49 per cent of control.

The action of these inhibitors of active transport and that of the inhibitors of oxidative metabolism reinforce the observations of dependence upon substrate, oxygen and temperature and strongly suggest that in large part the accumulation of [2-^{14}C]-ethacrynic acid observed in these experiments is due to the presence of an active transport system in rabbit renal cortex.

DISCUSSION

These studies *in vitro* were undertaken to determine whether renal tissue is capable of significantly accumulating the diuretic agent ethacrynic acid from an incubation medium, a process which may reflect the capacity of the organ *in situ* to accumulate the drug from the plasma and to raise its effective concentration at some pharmacological receptor site within kidney cells.

Earlier experiments with kidney slices of guinea-pig, a species not thought to be particularly sensitive to ethacrynic acid,[11] had suggested that some accumulation of this drug had occurred against a concentration gradient, and these observations were confirmed and extended using tissue from the more drug-sensitive rabbit species.

The data show that a marked drug accumulation is possible, with concentration factors of 10-fold being commonplace. Even when a concentration of drug was used which exceeded by 50–100 times that which might be expected in man, no evidence was obtained to indicate that the uptake process was saturated. Thus, very large increments may exist between the concentration of drug in the plasma of animals and that at some pharmacological target site or receptor within the renal cells.

In this regard Nechay *et al.*,[3] who seriously questioned the hypothesis that renal ($Na^+ + K^+$)-ATPase was the pharmacological site for ethacrynic acid action, showed that the plasma level of this drug in dogs during maximal diuretic response was only about 1 μg/ml, whereas that in urine collected at the same time was approximately 20-fold greater. This observation seems to support our contention that considerable drug accumulation may be possible within renal tissue. The actual concen-

tration achieved at a particular receptor site could conceivably be greater than that observed here, as our data do not indicate anything about the possible distribution of drug within the tissue slice, merely reflecting the overall concentrating effect achieved on the assumption of homogeneous distribution throughout the whole tissue. It seems not unreasonable to consider that some form of compartmentalization could exist within the kidney which would effectively raise the drug concentration further.

Because Beyer *et al.*[11] had reported that substantial amounts of radioactivity were found in the liver as well as in the kidneys of dogs after intravenous administration of 10 μc of "acetate"-labeled ethacrynic acid, we also examined the liver of both rabbits and guinea-pigs for drug accumulation in our system. The degree of concentration observed did not reach half of that found with the kidney of either of these species, perhaps suggesting a measure of nonspecific drug binding to tissue, but more importantly, some organ specificity for the ethacrynic acid accumulation process in the kidney.

That drug uptake must result in accumulation against a concentration gradient is clearly shown by the magnitude of the slice:medium ratios encountered and by concurrent measurement of "extracellular space". Even if mannitol substantially underestimates this space, and it is generally agreed that some overestimation is more likely, these concentrations of drug cannot be accounted for by diffusion alone. Quite clearly from our results a portion of the uptake observed was not abolished by removal of substrate from the system, substitution of nitrogen for oxygen, reduction of incubation temperature to zero or introduction of metabolic inhibitors. Presumably this fraction reflects the so-called nonspecific uptake reported by Beyer *et al.*[11] and may be related to the binding of drug to the cut surfaces of the tissue slices.

Although subsequent experiments were not corrected for this fraction, the action of the metabolic inhibitors and transport inhibitors is unequivocal in that both types of agents reduce the uptake of the drug profoundly, and argues strongly in favor of a metabolically dependent active uptake process.

Whether this process is specific for ethacrynic acid or reflects a more general renal system for the uptake and perhaps excretion of organic acids of this type cannot be stated at present. Certainly probenecid is widely regarded as the prototype inhibitor of classical organic acid transport systems in the kidney, but its action is also known to lack specificity.[21] Similarly, the action of ouabain is of interest, as this may reflect a more general cardiac glycoside sensitivity of an organic acid transport system, perhaps by dependence of the system upon the maintenance of adequate sodium ions gradients as is the case for sugars and many other substances.[22] On the other hand, a direct involvement of the ouabain-sensitive renal ($Na^+ + K^+$)-ATPase in ethacrynic acid accumulation cannot be excluded,[8,9] and this enzyme system must remain a potential target site for at least part of the pharmacological action of ethacrynic acid as well as the cardiac glycosides.

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