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Multiple pumps for sodium reabsorption by the perfused kidney

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Multiple pumps for sodium reabsorption by the perfused kidney. Several distinct transport mechanisms responsible for sodium reabsorption by the rat kidney can be identified by studying the function of isolated perfused kidneys. Approximately one-half of the fractional sodium reabsorption by the isolated perfused rat kidney appears to depend on Na-K-adenosine triphosphatase (ATPase) and is inhibited by ouabain. About 15 to 20% is associated with the reabsorption of bicarbonate and is blocked by acetazolamide. This fraction of transported sodium is unaffected by ouabain and therefore does not involve Na-K-ATPase. Neither furosemide nor ethacrynic acid produce further inhibition of sodium reabsorption in a kidney already exposed to ouabain and acetazolamide. Most of the residual transport of sodium is inhibited by cooling the perfused kidney, suggesting that it is powered by metabolic rather than physical sources of energy.

Multiplicité des pompes qui assurent la réabsorption du sodium par le rein perfusé. Plusieurs mécanismes de transport distincts responsables de la réabsorption de sodium par le rein de rat peuvent être identifiés par l'étude du fonctionnement de reins isolés perfusés. La moitié, approximativement, de la réabsorption fractionnelle du sodium par les reins isolés perfusés semble dépendre de la Na-K-ATPase et est inhibée par l'ouabaïne. Environ 15 à 20% sont associés à la réabsorption du bicarbonate et bloqués par l'acetazolamide. Cette fraction du sodium transporté n'est pas affectée par l'ouabaïne et donc n'implique pas la Na-K-ATPase. Ni le furosémide ni l'acide éthacrynique ne produisent d'inhibition supplémentaire de la réabsorption de sodium par un rein déjà exposé à l'ouabaïne et à l'acetazolamide. La plus grande partie du transport résiduel du sodium est inhibée par le refroidissement du rein perfusé, ce qui suggère une source d'énergie métabolique plutôt que physique.

The reabsorption of salt and water from the glomerular filtrate is thought to be accomplished by one or more ion pumps located within renal tubular cells. The transport mechanism that has been best characterized is that dependent upon sodium-potassium-activated adenosine triphosphatase (Na-K-ATPase). Studies of renal function in intact anesthetized dogs [1], in rats subjected to micropuncture [2] and in the isolated perfused rat kidney [3] suggest that approximately one-half of all the sodium reabsorbed by the kidney in these species is transported by a process involving Na-K-ATPase, while the remainder depends on some other mechanism.

The present experiments with the isolated perfused rat kidney were undertaken to examine further the nature of this residual transport process that continues when Na-K-ATPase is inhibited. The results indicate that about 40% of it can be related to the reabsorption of bicarbonate from glomerular filtrate. Little or none of the remainder is susceptible to inhibition by furosemide or ethacrynic acid. Most of the remaining reabsorption is blocked by cooling the kidney, suggesting an active process requiring metabolic energy.

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Methods

Male Sprague-Dawley rats weighing 290 to 480 g, fed standard rat chow (Purina) and provided with free access to water were used for all the experiments. Perfusion of the right kidney was performed according to the technique of Nishiitsutsuji-Uwo, Ross and Krebs [4] as modified by Ross, Epstein and Leaf [5]. The animals were anesthetized i.p. with pentobarbital (60 mg/kg). Mannitol (50 mg/100 g) and heparin (1000 U) were injected into the femoral vein. The peritoneal cavity was opened, a PE10 catheter was placed in the right ureter, and the inferior vena cava and the right renal artery were cannulated. The arterial cannula was inserted into the superior mesenteric artery and threaded through the aorta into the right renal artery. Perfusion medium was recirculated continuously with pulsatile flow at a pressure of 100/80 mm Hg distal to the tip of the arterial cannula. Temperature was maintained at 37°C ± 1° unless otherwise stated. The standard medium consisted of Krebs-Ringer-bicarbonate solution containing sodium, 143; phosphate, 1.2; bicarbonate, 25; and chloride, 123 mmoles/liter at a pH of 7.40 when gassed with 5% CO_2/95% O_2. The medium without bicarbonate was prepared in similar fashion, substituting chloride for bicarbonate, with 1.2 mM phosphate as

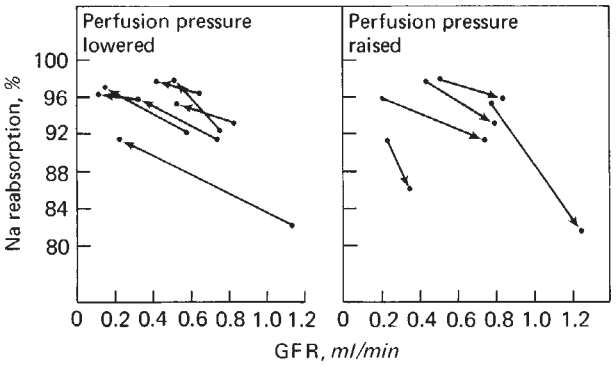

Fig. 1. *Effect of changing GFR on fraction reabsorption of sodium in the isolated perfused rat kidney.* Reducing GFR by lowering perfusion pressure was always associated with a rise in fractional sodium reabsorption (left-hand side of figure) while increasing perfusion pressure and GFR caused a fall in the percentage of filtered sodium that was reabsorbed.

buffer at pH 7.4, using 100% O_2 as the gas phase. The media were initially prepared as a 10% solution of albumin (bovine albumin, fraction V, Pentex Ltd.) and diluted to a final concentration of 6.7%. Glucose, 5 mM, was used as the sole exogenous substrate in all experiments. Ouabain (K & K Laboratories) was dissolved in 0.5 to 2 ml of boiling water just prior to adding it to the medium. Ethacrynic acid (Merck, Sharp and Dohme), either pure or in combination with cysteine in 1:1.1 M ratio, was added to a final concentration of 2 mM. Furosemide (Lasix, Hoechst) was used at a final concentration of 2 mM. Acetazolamide (Diamox) as the sodium salt was prepared in stock solutions of 10^{-2} M appropriately diluted and the pH adjusted to 7.4, and was added to the recirculating medium to a final concentration of 1 mM.

Glomerular filtration rate was determined using carboxyl-labelled ^{14}C-inulin (New England Nuclear). Sodium and potassium were measured in a flame photometer (IL-113). Osmolality of urine and perfusion medium was measured in an osmometer (Advanced Instruments). pH of the samples was read on a pH meter (Beckman Expandomatic).

The protocol for any given experiment consisted of an initial control period of 40 min of perfusion with three to four clearance periods of 5 to 10 min each, followed by the addition of inhibitors. Four to five subsequent periods were collected prior to administration of a second inhibitor. Data for analysis were obtained from those clearance periods in which the vasoactive changes induced by the inhibitors had abated (return of perfusion pressure to within 20 mm Hg of control with restoration of the normal rate of perfusion). Results are the average of two consecutive clearance periods in a steady state. All figures represent mean ± SEM. Student's *t* test was used for statistical analysis.

Results

Effect of changing glomerular filtration rate (GFR) on fractional sodium reabsorption in the isolated perfused kidney (Fig. 1). Because the maneuvers used to alter transport and metabolism of the kidney in these experiments also tended to change GFR, it was important to establish what influence a change in GFR per se might have on fractional sodium reabsorption in this preparation. Accordingly, in 11 experiments, filtration rate was raised or lowered by varying the arterial perfusion pressure; the results are shown in Fig. 1. A fall in GFR invariably produced an increase in fractional sodium reabsorption, while an increase in GFR was always associated with an increase in urinary output and sodium excretion and a fall in the fractional reabsorption of sodium.

Effect of ouabain and acetazolamide (Table 1 and Figs. 2 and 3). In earlier experiments ouabain was shown to inhibit Na-K-ATPase in rat kidney microsomes completely at a concentration of 5×10^{-3} M. When this concentration of ouabain was added to the medium perfusing the isolated rat kidney, sodium reabsorption was inhibited by about 50%, and when more ouabain was added in several experiments, no further inhibition of fractional reabsorption was obtained [3]. In experiments on the whole kidney, it is difficult to be certain that Na-K-ATPase is completely inhibited, since ouabain may not have access to the enzyme in the same way as in broken-cell homogenates. Furthermore, the concentration of ouabain required for enzyme inhibition *in vitro* is higher in the rat than in other species, and close to the limits of solubility for ouabain in water, presumably

Table 1. Effect of ouabain and acetazolamide on sodium reabsorption in the isolated perfused rat kidney[a]

	Control (N = 15)	P	Ouabain, 25 mM (N = 15)	P	Ouabain, 25 mM + acetazolamide, 1 mM (N = 12)
Fractional sodium reabsorption × 100	95.2 ± 1.3	<0.01	57.9 ± 2.5	<0.01	37.2 ± 3.5
GFR, *ml/min*	0.64 ± 0.05	<0.05	0.33 ± 0.02	<0.01	0.22 ± 0.02
V, *ml/min*	0.032 ± 0.005	<0.01	0.147 ± 0.011	NS	0.153 ± 0.010

[a]Values are mean ± SEM. P values represent calculated probability figures between adjacent columns.

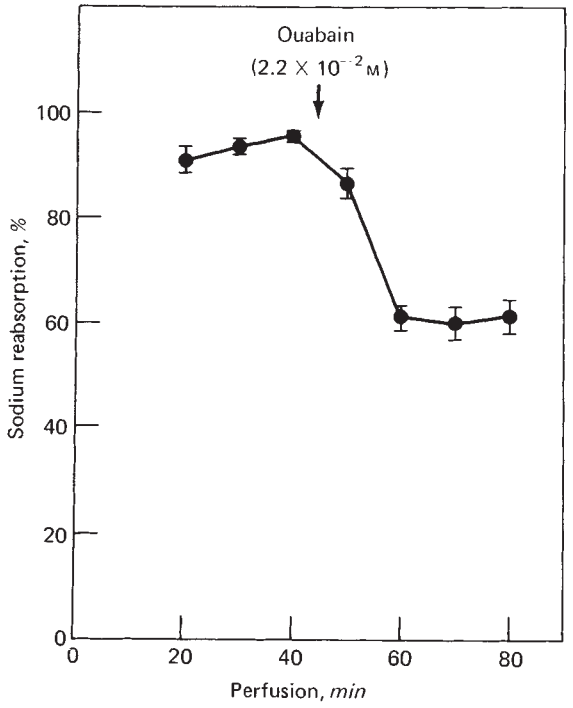

Fig. 2. *Effect of ouabain on fractional sodium reabsorption in the perfused rat kidney.* Values are mean ± SEM of ten experiments.

because the enzyme-inhibitor complex is readily dissociated. For these reasons the inhibitory effect of ouabain on sodium reabsorption by the isolated kidney was tested systematically at high concentrations of the cardiac glycoside. At 4×10^{-3} M, fractional sodium reabsorption averaged 57.7 ± 3.6% (mean ± SEM; $N = 23$), while at 2.2×10^{-2} M it was not significantly different (55.1 ± 3.5%; $N = 12$). Thus, inhibition of sodium transport was unchanged by a fivefold increase in the concentration of ouabain, suggesting that at these concentrations transport related to Na-K-ATPase was maximally inhibited in the perfused kidney.

In subsequent experiments ouabain was added to the perfusion medium at a concentration of 2.5×10^{-2} M. The addition of ouabain was followed by a precipitous and sustained fall in sodium reabsorption

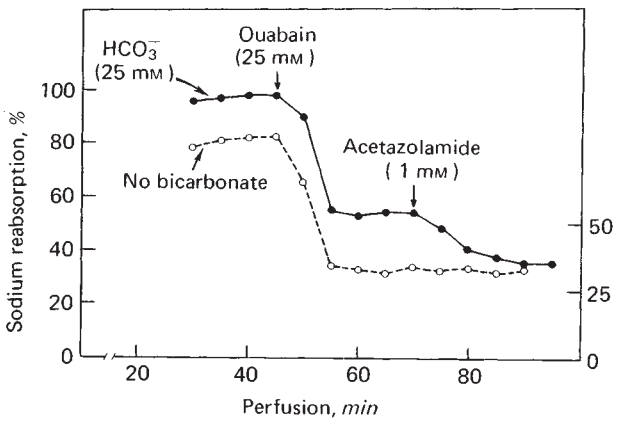

Fig. 3. *Effects of ouabain and acetazolamide on sodium reabsorption in the presence or absence of bicarbonate.*

from 95.2 ± 1.3 to 57.9 ± 2.5% (Table 1). The effect was seen immediately, was fully marked after ten minutes and persisted at the same level thereafter (Fig. 2). There was a concomitant reduction in GFR from 0.64 ± 0.05 to 0.33 ± 0.02 ml/min. Urinary flow increased with V/GFR rising sevenfold from 6.1 ± 1.3 to 45.9 ± 2.4%.

The subsequent addition of 1 mM acetazolamide to kidneys already perfused with ouabain produced a further decrease in sodium reabsorption from 57.9 ± 2.5 to 37.2 ± 3.5%. Glomerular filtration rate declined further to 0.22 ± 0.02 ml/min, while urinary flow increased to 69 ± 3.7% of the GFR. The residual fractional reabsorption of approximately 35% of the filtered load of sodium, remaining after both ouabain and acetazolamide were added to the perfusion medium, was not reduced further by omitting glucose from the medium. This moiety of sodium transport is therefore not dependent on the co-transport of glucose or on glucose as a substrate.

Effect of ouabain and acetazolamide in the absence of bicarbonate (Table 2 and Figs. 3 and 4). Fractional reabsorption of sodium by the perfused rat kidney was approximately 80% when bicarbonate was omitted from the perfusion medium and replaced by chloride. Addition of ouabain resulted in a decrease

Table 2. Effect of ouabain and acetazolamide on sodium reabsorption in the isolated perfused rat kidney in the presence and absence of bicarbonate[a]

	No bicarbonate ($N = 9$)	P	Ouabain, 25 mM ($N = 9$)	P	Ouabain, 25 mM + bicarbonate, 25 mM ($N = 4$)	P	Ouabain, 25 mM + acetazolamide, 1 mM ($N = 4$)
Fractional sodium reabsorption × 100	82.1 ± 1.5	<0.01	29.6 ± 1.3	<0.01	53.1 ± 1.0	<0.01	34.1 ± 1.2
GFR, *ml/min*	0.41 ± 0.01	<0.01	0.16 ± 0.01	<0.05	0.23 ± 0.07	NS	0.23 ± 0.08
$\dot{V}$, *ml/min*	0.079 ± 0.005	<0.01	0.119 ± 0.007	NS	0.114 ± 0.018	NS	0.163 ± 0.018

[a]Values are mean ± SEM. P values represent calculated probability figures between adjacent columns.

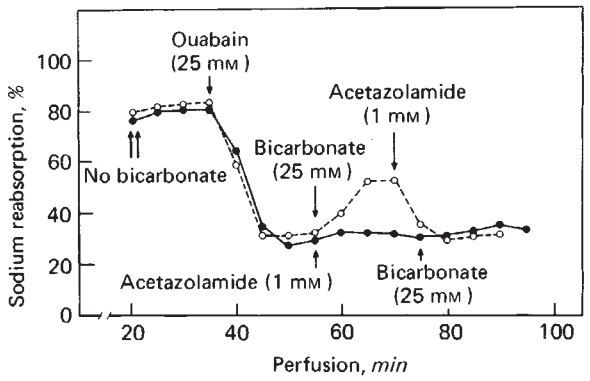

Fig. 4. *Role of bicarbonate in sodium reabsorption.* The addition of bicarbonate to the perfusion medium increased fractional sodium reabsorption even though ouabain was present, indicating that the reabsorption of $NaHCO_3$ does not depend on Na-K-ATPase.

in sodium reabsorption from 82.1 ± 1.5 to 29.6 ± 1.3% (Fig. 3). Restoration of the bicarbonate concentration of the perfusate to 25 mM (Fig. 4) increased the reabsorption of sodium to 53.1 ± 1.0%, a value similar to that found after the addition of ouabain to a medium containing normal amounts of bicarbonate. The further addition of acetazolamide was followed by the reverse effect, sodium reabsorption decreasing to 34.1 ± 1.2%. Bicarbonate reabsorption, therefore, accounted for the tubular transport of about 20% of filtered sodium. This could be inhibited either by giving acetazolamide or by substituting chloride for bicarbonate in the perfusion medium, and the magnitude of the effect was roughly the same whether or not renal Na-K-ATPase was inhibited.

Effects of ethacrynic acid and furosemide (Tables 3 and 4). Both ethacrynic acid and furosemide produce a sodium diuresis in the isolated perfused rat kidney, reducing fractional sodium reabsorption from about 94% to approximately 80% of the filtered load. When given after ouabain, however, there was only a small additional diuretic effect. The average fractional sodium reabsorption after ouabain plus ethacrynic acid was 53.0 ± 1.8% and after ouabain plus furosemide was 54.5 ± 1.8%, as compared with 57.9 ± 2.5% after ouabain alone. The residual sodium reabsorption remaining after the kidney had been exposed to ouabain and acetazolamide (37.2 ± 3.5%) did not differ significantly from that after exposure to ouabain and acetazolamide plus ethacrynic acid (35.6 ± 1.8%) or furosemide (39.8 ± 2.4%). In two experiments, ethacrynic acid-cysteine (2 mM) did not produce any more pronounced effects than ethacrynic acid alone. Neither ethacrynic acid nor furosemide, therefore, appeared to inhibit the residual pump mechanisms responsible for sodium transport in the absence of both Na-K-ATPase and carbonic anhydrase activity.

Effect of cooling on sodium reabsorption (Fig. 5). The effect of temperature on sodium transport activity was studied next. The perfusion medium could be conveniently and reversibly cooled by the use of a jacketed glass lung. In four experiments in which the kidney was cooled to 11 to 16°C, GFR fell to 0.28 ± 0.09 ml/min and fractional sodium reabsorption to 0.17 ± 0.04%. In the representative experiment shown in Fig. 3, sodium reabsorption fell to 12% of the filtered load at 11°C. When the kidney was warmed to 37°C, fractional sodium reabsorption returned to 95%. The addition of ouabain and acetazolamide had no further inhibitory effect in the cold. Cooling a kidney that had been treated with ouabain and acetazolamide produced a urine to plasma inulin ratio of 1.1, reducing residual sodium reabsorption to 10 to 12% of the filtered load. Rewarming the kidney to 37°C after the addition of both inhibitors restored sodium reabsorption to 30%, close to the value seen when both ouabain and acetazolamide were given to a kidney perfused constantly at 37°C.

Discussion

The existence of more than one mechanism for sodium transport by the kidney has been suggested

Table 3. Effect of ethacrynic acid on residual sodium reabsorption in the isolated perfused rat kidney[a]

	Control	*P*	Ethacrynic acid, 2 mM	*P*	Ethacrynic acid, 2 mM + acetazolamide, 1 mM	*P*	Ethacrynic acid, 2 mM + ouabain, 25 mM	*P*	Ethacrynic acid, 2 mM + ouabain, 25 mM + acetazolamide, 1 mM
Fractional sodium absorption × 100	94.7 ±1.0	<0.01	81.1 ± 2.4	<0.02	67.0 ± 3.2	<0.02	53.0 ±1.8	<0.01	35.6 ± 1.8
GFR, *ml/min*	0.830 ± 0.05	<0.01	0.580 ± 0.03	<0.01	0.320 ± 0.030	NS	0.250 ± 0.030	NS	0.220 ± 0.060
V, *ml/min*	0.055 ± 5.0	<0.01	0.139 ± 0.009	NS	0.124 ± 0.008	NS	0.120 ± 0.013	NS	0.132 ± 0.016
N	10		3		6		4		10

[a] Figures are mean ± SEM. *P* values represent calculated probability between adjacent columns.

Table 4. Effect of furosemide on residual sodium reabsorption in the isolated perfused rat kidney[a]

	Control	*P*	Furosemide, 2 mM	*P*	Furosemide, 2 mM + acetazolamide, 1 mM	*P*	Furosemide, 2 mM + ouabain, 25 mM	*P*	Furosemide, 2 mM + ouabain, 25 mM + acetazolamide, 1 mM
Fractional sodium reabsorption × 100	92.4 ±1.4	<0.01	78.3 ± 2.1	<0.05	68.7 ± 2.4	<0.01	54.5 ± 1.8	<0.01	39.8 ± 2.4
GFR, *ml/min*	0.560 ± 0.080	NS	0.370 ± 0.060	NS	0.260 ± 0.030	NS	0.370 ± 0.02	<0.02	0.210 ± 0.020
V, *ml/min*	0.049 ± 0.008	<0.02	0.092 ± 0.010	NS	0.097 ± 0.009	<0.05	0.156 ± 0.019	NS	0.125 ± 0.016
N	9		5		5		5		5

[a] Figures are mean ± SEM. *P* values represent calculated probability between adjacent columns.

and speculated about for many years. Since the time of Cushny, for example, physiologists have suggested that salt and water filtered by the glomerulus could in part be reabsorbed from the tubule into the postglomerular capillaries by physical forces (oncotic and capillary pressures) deriving ultimately from the energy imparted to the circulation by the heart. Studies of kidney cortex slices have suggested the presence of at least two chemical pumps for extruding sodium from kidney cells, one dependent on Na-K-activated ATPase and sensitive to ouabain, and the other inhibited by ethacrynic acid [6]. Analogous studies of sodium reabsorption by the intact kidney may be more pertinent, but are more difficult to perform because it is difficult or impossible to inhibit transport mechanisms completely by doses of inhibitors that can be used safely *in vivo* in the intact animal. The development of an isolated perfused rat kidney has permitted a closer examination of the various mechanisms responsible for sodium reabsorption since, in the perfused kidney, hydrostatic pressure and flow can be controlled, chemical inhibitors can be used in a dosage known to inhibit enzyme reactions completely in the test tube and the composition of the perfusion medium can be kept constant or changed at will. It should be noted that changes in the distribution of regional capillary flow and glomerular filtration within the perfused kidney have not been measured and since these might conceivably alter fractional sodium reabsorption, data from whole kidney perfusion experiments like these must be interpreted with caution.

Previous experiments with the perfused kidney of the rat have demonstrated that approximately one-half of the sodium normally reabsorbed by the tubules is transported via Na-K-ATPase while the remainder continues to be reabsorbed even in the presence of maximally inhibitory concentrations of ouabain. (Although the GFR usually falls when ouabain is added, a comparable drop in sodium reabsorption is seen even when a fall in GFR is avoided [3, 7].) The present results show that the residual absorption of sodium can be divided into two portions. The first moiety, comprising about 15 to 20% of the filtered load of sodium, evidently depends on the reabsorption of bicarbonate via the action of carbonic anhydrase. Presumably the mechanism involves the secretion of H^+ in exchange for Na^+. It is noteworthy that the magnitude of this bicarbonate-dependent fraction of sodium is roughly the same whether or not ouabain is present in the perfusion medium [8]. Reabsorption of sodium associated with bicarbonate is therefore not dependent on an intact Na-K-ATPase system. Finally, sodium reabsorption remaining after the inhibition of both Na-K-ATPase and carbonic anhydrase is largely eliminated by cooling the preparation.

An important question was whether this residual sodium reabsorption could be inhibited by the powerful diuretics, ethacrynic acid and furosemide. Whittembury and Proverbio proposed the existence of an electrogenic pump in renal tubular cells that is inhib-

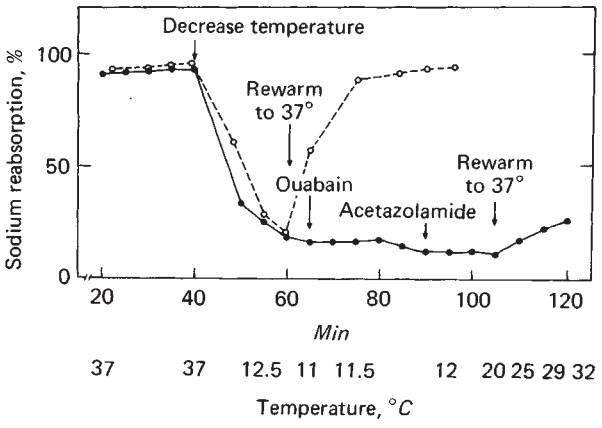

Fig. 5. *Effect of temperature on fractional sodium reabsorption.*

ited by cold, extrudes sodium with chloride and water and is sensitive to ethacrynic acid, but not to ouabain [6]. The present experiments indicate that, at least in the rat kidney, both ethacrynic acid and furosemide have little additional diuretic action in the kidney blocked with ouabain, and none when ouabain and acetazolamide have been given. This is consonant with other experiments *in vivo* in intact dogs [1, 9–11]. The failure of ethacrynic acid or furosemide to block substantial amounts of sodium reabsorption in a kidney already inhibited by ouabain does not mean that these drugs exert their effect through inhibition of Na-K-ATPase, but merely that the pathway that they do block is, in the rat, either dependent at some stage on that enzyme or sensitive to the intracellular concentration of potassium, which may be expected to fall when Na-K-ATPase is inhibited. It should be recalled that both furosemide and ethacrynic acid inhibit chloride transport when applied to the luminal suface of the thick ascending limb of the rabbit nephron, but that this process is also blocked by ouabain, even though Na-K-ATPase is not thought to be directly involved in the transport of anions [12]. Furosemide was reported by Bowman, Dolgin and Coulson to inhibit reabsorption in the perfused rat kidney even after the administration of ouabain [13], but in those experiments smaller concentrations of ouabain were used than in the present ones, and renal Na-K-ATPase was probably not completely inhibited. It would appear that, whatever ion pumps are responsible in the rat kidney for the residual sodium transport that remains after Na-K-ATPase and carbonic anhydrase have been inhibited, they are not sensitive to high concentrations of ethacrynic acid or furosemide.

Is the residual sodium reabsorption that continues when both Na-K-ATPase and carbonic anhydrase have been inhibited mediated by metabolic or by physical factors? If it is mediated by a metabolic pump, it should be inhibited at low temperatures. If it is mediated by physical forces, such as the difference in oncotic pressure between tubular and capillary fluid, little change should be noted when the kidney is cooled. Initial experiments with the perfused kidney indicated that sodium reabsorption could be reduced almost to zero by blocking oxidative metabolism with cyanide, if glycolysis was also eliminated by adding iodoacetate or omitting glucose from the perfusion [3]. These earlier experiments were complicated by a profound reduction in the GFR to less than 0.1 ml/min, and it was therefore deemed desirable to investigate the problem by reducing renal metabolism in another way.

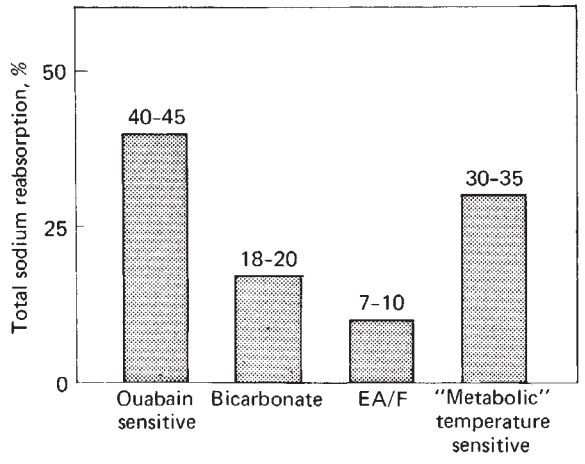

Fig. 6. *Relative contribution of various pumps to sodium reabsorption by the isolated perfused rat kidney.*

Inhibiting the metabolism of the kidney by any means appears to decrease GFR, but though this was also observed in the cooling experiments reported in the present paper, the fall in GFR was not nearly so marked as seen previously with cyanide and iodoacetate. Furthermore, the diuresis induced by cooling cannot be ascribed simply to a reduction in GFR since, as the present data indicate, fractional sodium reabsorption by the perfused rat kidney is increased rather than decreased by lowering glomerular filtration through a reduction in perfusion pressure. The residual fractional reabsorption of sodium, present after ouabain and acetazolamide have been given, is almost completely inhibited in the cold. This is strong evidence that all of the glomerular filtrate that is reabsorbed is actively transported through the expenditure of metabolic energy, rather than from kinetic energy derived from the pumping action of the heart.

The data therefore suggest that in the perfused rat kidney there are at least three separate mechanisms for the reabsorption of sodium (Fig. 6): *1*) an ouabain-sensitive mechanism, dependent on Na-K-ATPase and responsible for the reabsorption of about 50% of the filtered load; *2*) a second pathway related to the reabsorption of bicarbonate, inhibited by acetazolamide, and contributing about 20% of sodium reabsorption; and *3*) a third process sensitive to temperature, that is responsible for the bulk of the residual reabsorption of filtered salt and water.

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