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NATURE OF Na^+-INDEPENDENT STIMULATION OF RENAL TRANSPORT OF *p*-AMINOHIPPURATE BY EXOGENOUS METABOLITES

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Abstract—The relationship between *p*-aminohippurate (PAH) and Na^+ transport in rabbit kidney cortical slices was examined under optimal metabolic conditions. Addition of lactate, pyruvate and acetate to the incubation medium stimulated PAH transport and accumulation, but had no effect on active Na^+ efflux from the slices. Conversely, small concentration of F^-, in the presence of acetate, decreased PAH transport and accumulation, but had no effect on Na^+ efflux. These observations constitute evidence that, in addition to Na^+ cotransport [M. I. Sheikh and J. V. Møller *Biochem. J.* **208**, 243 (1982)], PAH uptake is metabolically stimulated by a Na^+-independent pathway. This could occur either by a direct metabolic effect on the PAH carrier, or by exchange with intracellular anions that are generated in the presence of exogenous substrate. In support of the latter possibility the PAH carrier is demonstrated to function as an anion exchanger of PAH and fumurate after preloading of the slices with fumurate under anaerobic conditions.

The renal transport system for organic anions which is responsible for renal tubular secretion of *p*-aminohippurate (PAH) and phenolsulphonphthalein dyes is also involved in the renal excretion of a variety of compounds of pharmacological and physiological importance [1, 2]. Several studies indicate that Na^+ plays an important role in the active tubular secretion of these organic anions. In the case of PAH this is evident from the absence of appreciable PAH accumulation in Na^+-depleted slices [3–7], the correlation between PAH secretion and tubular reabsorption of Na^+ [8–11], and the inhibitory effect of ouabain on PAH transport [6, 12–16]. Studies on PAH uptake by kidney slices uner anaerobic conditions [17] and on preparations of basolateral membrane vesicles of rabbit kidney [18] have indicated that Na^+ cotransport constitutes a significant aspect of active PAH transport. Nevertheless, there are observations which raise the question if extra metabolic energy, independent of Na^+, is needed to achieve maximal rates of transport of PAH and other organic anions by a different mechanism than by establishing a Na^+ gradient across the basolateral membrane of proximal tubule cells. Thus metabolic stimulation with acetate increases PAH transport [15], and low concentrations of metabolic inhibitor reduce PAH transport in the rabbit [19], without changing intracellular levels of Na^+ and K^+. Nikiforov [20] likewise obtained evidence that stimulation of fluorescein transport by acetate in the frog tubules occurs by a Na^+-independent mechanism.

In the present communication we wish to report results which support the view that under certain conditions PAH transport is not correlated with Na^+ transport and the transmembrane electrochemical gradient of Na^+. Our data suggest that metabolic stimulation either activates the PAH carrier directly or, alternatively, that PAH transport may be stimulated by exchange with intracellular anions by a Na^+-independent mechanism.

MATERIALS AND METHODS

The experiments were performed on both male and female rabbits, weighing 2.5–4.0 kg. The general experimental procedures were as previously described [16, 19, 21], and are only briefly summarized here. Thin slices were prepared from kidneys which had been perfused with approximately 50 ml of the Krebs–Ringer phosphate solution via a needle inserted in the renal artery. This procedure was performed to remove plasma albumin from the renal tissue [22, 23].

Aerobic incubation of kidney slices. Slices (200 mg) were preincubated for 1 hr in a medium containing 3 ml 145 mM NaCl, 5 mM KCl, 0.7 mM $CaCl_2$, 1.2 mM $MgSO_4$, 15 mM Tris buffer (pH 7.4), tracer amounts of ^{22}Na, together with various metabolites and metabolic inhibitors in concentrations as indicated in the figure legends. After preincubation, the slices were blotted on a piece of filter paper and transferred to Warburg cups containing 0.075 mM PAH dissolved in the above-mentioned medium without radioactive Na^+. The incubations were carried out in a Warburg respirometer under the following conditions unless otherwise specified: shaker speed, 100 counts/min; gas phase 100% O_2; temperature 25°; pH 7.4; incubation period 0–120 min. The central well contained KOH for CO_2 absorption. After incubation slices were quickly transferred to conical flasks containing 2.5 ml 5% trichloroacetic acid (TCA) and were extracted overnight with continuous shaking. The extracts were then centrifuged for 10 min at 10,000 rev/min in Sorvall centrifuge,

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using the SS-34 rotor. A sample of medium (1 ml) was deproteinized by adding 6 ml 5% TCA. Supernatants obtained by centrifugation of the extracted tissue and media samples were used for the subsequent analyses.

Anaerobic incubation of kidney slices. In these experiments the Warburg vessels were flushed with 100% N_2, and radioactive PAH together with unlabelled PAH was tipped into the medium from the side arm of the Warburg vessels after 10 min N_2 flow. the experiments were performed on Na^+–K^+ depleted slices [16] which had been preincubated under 100% N_2 for 1 hr in a medium containing 2 mM fumurate and 146 mM choline chloride instead of Na^+ and K^+ (for further details see text to Fig. 4). The incubation was carried out either in the same medium plus PAH and minus fumarate, or in a medium containing 150 mM NaCl and PAH. The slices were blotted and extracted as described above.

Analytical methods and calculations. The chemical determination of unlabelled PAH in the supernatants obtained from tissue extracts and media was performed by the diazotization method of Bratton and Marshall [24] as modified by Smith *et al.* [25]. Radioactive PAH and ^{22}Na were measured by adding 0.2 ml supernatant to 10 ml of the scintillation fluid described by Bray [26] and counted in a Tri-Carb scintillation counter (Packard). The counts per min were converted to disintegrations per min by the addition of internal standard. Concentrations of Na^+ and K^+ in renal tissue and media were estimated by atomic absorption spectrometry. Inulin space and dry weight were estimated in separate experiments as described by Maxild [27] and Sheikh and Stahl [28]. The inulin space varied from approximately 25 ± 2% in the aerobic experiments to a minimal value of 18 ± 2% in the anaerobic experiments.

The uptake of PAH in the renal tubules was calculated as the difference between the content of this compound in the whole slices and that present in the inulin space. This difference was then divided by the tubular water content (final wet weight of tissue minus inulin space minus dry weight) to obtain the tubular concentration (T) of the compound, from which the accumulation of PAH (T/M_{PAH}) was calculated by division with the medium concentration (M_{PAH}) after the incubation. The tissue concentration of Na^+ and K^+ was calculated as mmole/kg final wet tissue weight.

RESULTS AND DISCUSSION

Previous studies have shown that in the slice system lactate, pyruvate and acetate are the only exogenous metabolites which uniformly cause a rise in PAH accumulation over a wide range of concentrations [2]. Figures 1a and 1b show that PAH uptake by the slices was more than doubled by the presence of lactate or pyruvate, both during the rapid uptake period immediately after addition of PAH, and during the approach to equilibrium (after 60 min). On the other hand, there was no effect of lactate and pyruvate (10 mM) on the efflux of ^{22}Na from the slices. During the first 4–5 min Na^+ efflux can be described as a monoexponential process with rate constant 0.063–0.071 min^{-1}. Furthermore, the intracellular Na^+–K^+ levels (see legend of Fig. 1) were not significantly affected by the presence of lactate or pyruvate ($P > 0.5$). Exactly the same findings were obtained if lactate or pyruvate was added together with PAH, rather than being added together with $^{22}Na^+$ 1 hr before PAH as in Figs 1a and 1b: This variation in experimental procedure did not cause any delay in the PAH uptake curve relative to that of slices which had not been exposed to exogenous metabolite before addition of PAH (not shown).

The same observations were made in the presence of 10 mM acetate (Fig. 2). This metabolite stimulated PAH uptake without any discernible effect on Na^+

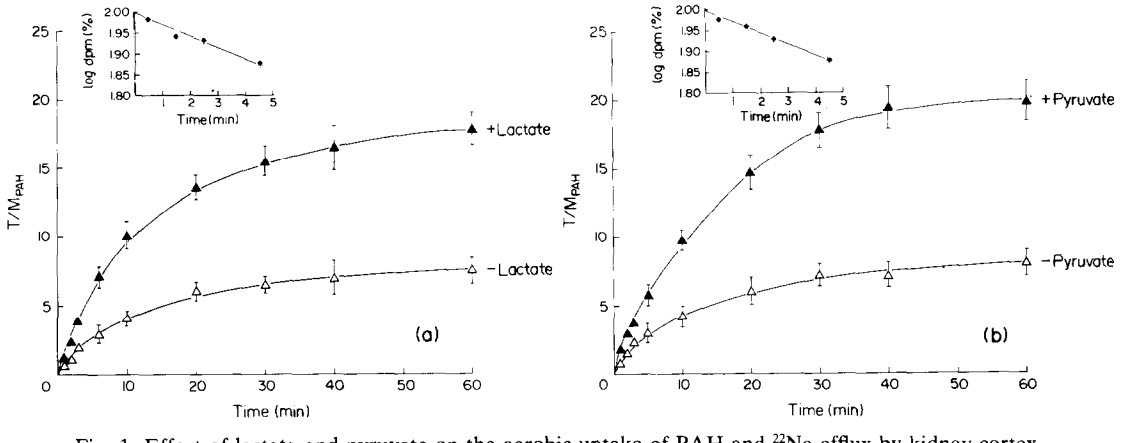

Fig. 1. Effect of lactate and pyruvate on the aerobic uptake of PAH and ^{22}Na efflux by kidney cortex slices. The initial medium concentrations were: PAH, 0.075 mM; lactate or pyruvate, 10 mM. Ordinate: ratio between the concentration of PAH in the tubular water and medium. Abscissa: time of incubation. The inset shows a logarithmic analysis of ^{22}Na efflux (log dpm) vs time, the zero point value (100%) being obtained by extrapolation of the regression line of the experimental points: ◆—◆, in the presence and ◇—◇ in the absence of metabolites. The final tissue concentrations in mmole/kg tissue ± S.D. of five experiments were: Na^+: 71 ± 3 mmoles/kg tissue in the presence of metabolite and 70 ± 4 mmole/kg tissue in the absence of added metabolite. The corresponding values for K^+ were: 62 ± 4 and 64 ± 2 mmole/kg tissue.

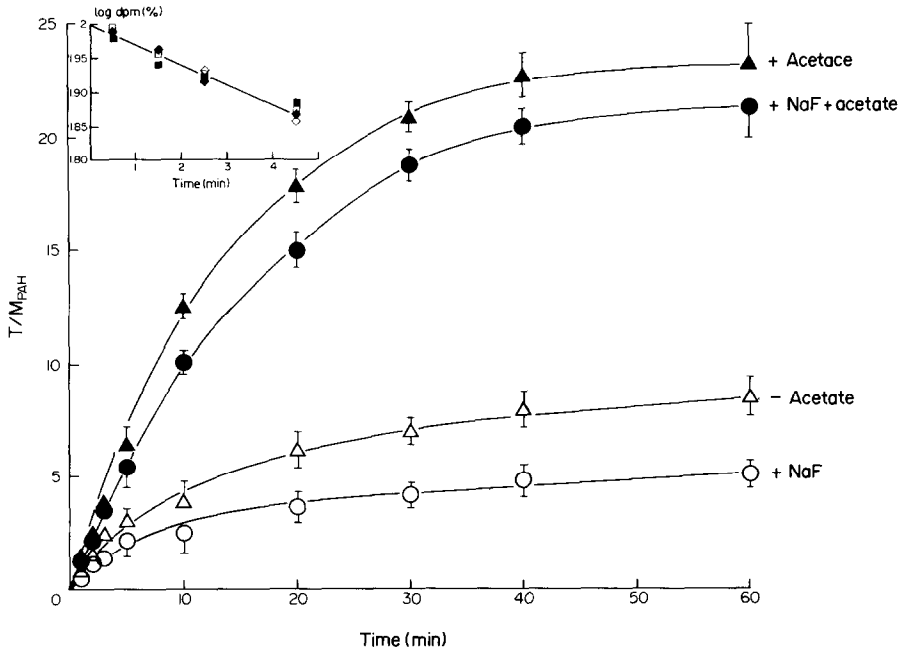

Fig. 2. Effect of acetate and NaF on the aerobic uptake of PAH and ^{22}Na efflux by renal cortical slices. The initial medium concentrations were: PAH, 0.075 mM; F^-, 0.5 mM; and acetate, 10 mM. Ordinate: ratio between the concentration of PAH in the tubular water and medium. Abscissa: time of incubation. The inset shows a logarithmic analysis of ^{22}Na efflux vs time in the presence of acetate (◆—◆) and F^- (■—■), and in the absence of acetate (◇—◇) and F^- (□—□). The final tissue concentrations in mmole/kg tissue ± S.D. of five experiments were: Na^+: 73 ± 6, K^+: 67 ± 3; with F^-: Na^+: 77 ± 5, K^+: 65 ± 2; with acetate: Na^+: 73 ± 5, K^+: 62 ± 3; with acetate and F^-: Na^+: 74 ± 6, K^+: 60 ± 4.

efflux and Na^+–K^+ concentration of the tissue. Addition of low concentrations of F^- as a metabolic inhibitor reduced accumulation, but did not affect Na^+ efflux or tissue Na^+–K^+ concentrations. This is in agreement with previous data showing that low concentrations of various metabolic inhibitors only affect PAH uptake and not the intracellular concentrations of Na^+–K^+ [19]. In the present experiments PAH accumulation after 1 hr increased from 5, in the presence of F^- (0.5 mM), to 23, in the presence of acetate.

In connection with the data of Figs. 1 and 2 the question arises to what extent Na^+ efflux from the slices represents active Na^+ transport. Figure 3 shows the effect of ouabain on the efflux rate of Na^+ at a concentration of the glycoside (0.2 mM) which is capable of blocking active transport of Na^+ in the slices [16]. The efflux rate was reduced about 60% by ouabain, and there was no effect of acetate on the process. The extracellular space as measured with inulin is not significantly changed by ouabain [16], and in agreement with this previous study we found that the intracellular concentration of Na^+ was increased towards the same level as in the medium. Assuming that the ouabain-independent component of Na^+ efflux has diffusional characteristics, an increase in intracellular Na^+ concentration *per se* would not affect the efflux rate. However, the pretreatment with ouabain must lead to depolarization of the basolateral membrane [29], which may be expected to enhance passive efflux of Na^+, relative to that which occurs in the absence of ouabain. Therefore, the major part of the $^{22}Na^+$ efflux in Figs. 1 and 2 may be considered to represent active Na^+ extrusion from the slices as a result of the action of Na^+–K^+-ATPase.

Taken together, our data suggest that PAH accumulation in part depends on metabolic energy by a Na^+-independent mechanism. The only alternative possibility seems to be that metabolite stimulation is accompanied by hyperpolarization of the basolateral membrane so as to increase the electrochemical

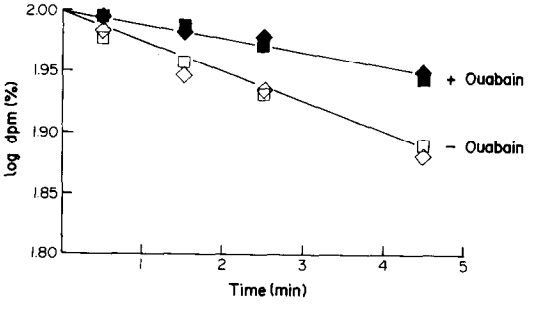

Fig. 3. Effect of ouabain on the efflux rate of ^{22}Na by renal cortical slices. (◆—◆) represents ^{22}Na efflux in the presence of 0.2 mM ouabain and 10 mM acetate. (■—■) represents ^{22}Na efflux in the presence of ouabain without metabolite. Corresponding open symbols indicate ^{22}Na efflux in the absence of ouabain. The final tissue concentrations in mmole/kg tissue ± S.D. of three experiments were: Na^+: 72 ± 6, K^+: 65 ± 3; with ouabain: Na^+: 141 ± 3, K^+: 22 ± 3; with acetate: Na^+: 74 ± 5, K^+: 64 ± 4; with acetate and ouabain: Na^+: 138 ± 5, K^+: 18 ± 7.

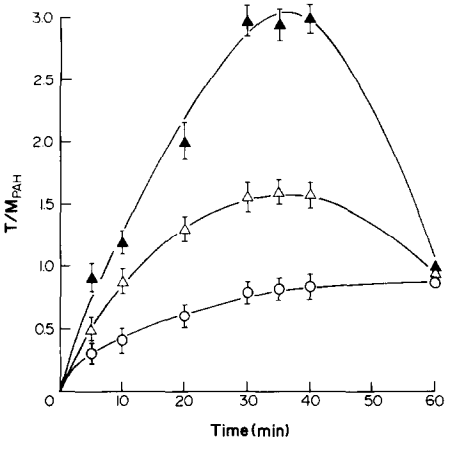

Fig. 4. Anaerobic uptake of PAH by kidney cortex slices under various experimental conditions. (○—○) presents anaerobic uptake of PAH without NaCl gradient. In the case of (△—△) and (▲—▲), slices were depleted of Na^+ and K^+ by pre-incubating in 150 mM choline chloride under 100% N_2 atmosphere at 2°. Then the slices were 'loaded' by incubating in the medium containing 150 mM choline chloride and 2 mM fumarate under anaerobic conditions for 1 hr at 25°. (△—△) represents anaerobic uptake of PAH by Na^+–K^+-depleted and fumarate-'loaded' slices in the medium containing 150 mM choline chloride instead of NaCl. (▲—▲) indicates anaerobic uptake of PAH by Na^+–K^+-depleted and fumarate-'loaded' slices in the presence of 150 mM NaCL. The values given are the means ± S.D. of five experiments.

Na^+ gradient across the basolateral membrane. However, in the presence of unchanged intracellular Na^+–K^+ levels and Na^+ transport, it is difficult to envisage how appreciable changes in the potential difference of the basolateral membrane could arise by the addition of metabolites.

The present findings are complementary to previous observations showing that low concentrations of various metabolic inhibitors may inhibit PAH transport without any discernible effect on Na^+–K^+ transport [19]. Nikiforov [20] has suggested that exogenous metabolites stimulate organic anion secretion by increasing the number of active carriers via an effect on the NADH/NAD^+ couple. However, we would like to point out that the effect could equally well be mediated by an anion-exchange mechanism. In luminal membrane preparations an 'overshoot' of PAH in the presence of a pH gradient has been attributed to exchange with OH^-/HCO_3^- [30]. Hong *et al.* [31] have reported that SITS (4-acetamido-4′-isothiocyano-2,2′-disulphonic stilbene), which is a potent inhibitor of anion exchange in the erythrocyte membrane [32], also has a strong inhibitory effect of PAH accumulation by rabbit cortical slices. Previously, 2,4-dinitrophenol and CCCP were found to inhibit PAH accumulation more strongly in the absence than in the presence of exogenous substrate [33]. Since these uncouplers of oxidative metabolism dissipate pH gradients across the cellular membranes, these observations argue against a role of OH^- in an anion-exchange process across the basolateral membrane of the proximal tubule, which is the site of active PAH transport [2]. Alternatively, intracellular HCO_3^- could function as the exchanging anion. However, more likely candidates for an anion-exchange mechanism include many of the intermediates of the citrate cycle which inhibit PAH transport when added to the medium at a high concentration [22, 28, 34]. Figure 4 demonstrates the effect of intracellular fumarate on PAH uptake. Before measurement of PAH transport the slices were depleted of Na^+ by preincubation in a choline chloride medium under anaerobic conditions, with or without fumarate. The preloading with fumarate resulted in transient accumulation of PAH under anaerobic conditions, when the slices subsequently were transferred to a medium containing PAH, but no fumarate. The highest accumulation of PAH (around 3) was observed when the PAH medium contained NaCl instead of the basic choline chloride medium, i.e. when uptake proceeded in the presence of an extracellular to intracellular Na^+ gradient. The accumulation obtained by the combined fumarate and Na^+ gradient was higher than that previously observed [17] for rabbit kidney slices in the presence of a Na^+ gradient alone. It is obvious that only limited accumulation like that observed here is expected to occur under anaerobic conditions due to the collapse of the transmembrane concentration gradients with time.

In conclusion, the anaerobic experiments demonstrate that the PAH carrier can operate as an anion exchanger, independent of Na^+. Oppositely directed concentration gradients of fumurate and Na^+ seem to activate PAH transport by independent mechanisms. It seems probable that both processes are operating within the same cells, since Woodhall *et al.* [14] in microperfusion studies of rabbit kidney tubules found that PAH transport in each of the S_1–S_3 subdivisions of the proximal tubule is sensitive to ouabain. It is therefore a distinct possibility that the exogenous metabolites examined here stimulate PAH transport by increasing the cytosolic concentration of citrate cycle intermediates as a result of their metabolic utilization. The further evaluation of this hypothesis requires an examination of the generation and release of citrate cycle intermediates from the slices in the presence of exogenous substrate.

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REFERENCES

1. J. M. Irish and J. J. Grantham, in *The Kidney* (Eds. B. M. Brenner and F. C. Rector), pp. 1619–1649. W. B. Saunders (1981).
2. J. V. Møller and M. I. Sheikh, *Pharmac. Rev.* **34**, 315 (1983).
3. S. T. Chung, Y. S. Park and S. K. Hong, *Am. J. Physiol.* **219**, 30 (1970).
4. G. A. Gerencser, Y. S. Park and S. K. Hong, *Proc. Soc. exp. Biol. Med.* **144**, 440 (1973).
5. G. A. Gerencser and S. K. Hong, *Biochim. biophys. Acta* **406**, 108 (1975).
6. R. A. Podevin and E. F. Boumendil-Podevin, *Am. J. Physiol.* **232**, F239 (1977).

7. J. Maxild, J. V. Møller and M. I. Sheikh, *Biochim. biophys. Acta* **601**, 490 (1980).
8. G. Vogel and W. Kröger, *Pflügers Archs* **286**, 317 (1965).
9. G. Vogel and W. Kröger, *Pflügers Archs* **288**, 342 (1966).
10. D. Häberle, *Kidney Int.* **7**, 385 (1975).
11. Y. Kikuta and T. Hoshi, *Biochim. biophys. Acta* **553**, 404 (1979).
12. M. B. Burg and J. Orloff, *Am. J. Physiol.* **203**, 327 (1962).
13. W. H. Dantzler, *Am. J. Physiol.* **217**, 1510 (1969).
14. P. B. Woodhall, C. C. Tischer, C. A. Simonton and R. K. Robinson, *J. clin. Invest.* **61**, 1320 (1978).
15. A. M. Spencer, J. Sack and S. K. Hong, *Am. J. Physiol.* **236**, F126 (1979).
16. J. Maxild, J. V. Møller and M. I. Sheikh, *J. Physiol.* **315**, 189 (1981).
17. R. A. Podevin, E. F. Boumendil-Podevin and C. Priol, *Am. J. Physiol.* **235**, F278 (1978).
18. M. I. Sheikh and J. V. Møller, *Biochem. J.* **208**, 243 (1982).
19. J. Maxild, J. V. Møller and M. I. Sheikh, *J. Physiol.* **310**, 273 (1981).
20. A. A. Nikiforov, *Biochim. biophys. Acta* **686**, 36 (1982).
21. M. I. Sheikh, J. Maxild and J. V. Møller, *Biochem. Pharmac.* **30**, 2141 (1981).
22. M. I. Sheikh, *J. Physiol.* **227**, 565 (1972).
23. M. I. Sheikh, *J. Physiol.* **256**, 175 (1976).
24. A. C. Bratton and E. K. Marshall, *J. biol. Chem.* **128**, 537 (1939).
25. H. W. Smith, H. Finkelstein, L. Aliminosa, B. Crawford and M. Graber, *J. clin. Invest.* **24**, 388 (1945).
26. G. A. Bray, *Analyt. Biochem.* **1**, 279 (1960).
27. J. Maxild, *Archs int. Physiol. Biochim.* **86**, 509 (1978).
28. M. I. Sheikh and M. Stahl, *Am. J. Physiol.* **232**, F513 (1977).
29. E. Frömter, *J. Physiol.* **288**, (1979).
30. J. W. Blomstedt and P. S. Aronson, *J. clin. Invest.* **65**, 931 (1980).
31. S. K. Hong, J. M. Goldinger, Y. K. Song, F. J. Koschier and S. H. Lee, Am. J. Physiol. **234**, F302 (1978).
32. Z. I. Cabantchik and A. Rothstein, *J. Membr. Biol.* **10**, 311 (1970).
33. J. Maxild, *Archs int. Physiol. Biochim.* **81**, 501 (1973).
34. I. Kippen and J. R. Klinenberg, *Am. J. Physiol.* **235**, F137 (1978).