# Tubular Transport Maxima of PAH and Diodrast Measured Individually in the Aglomerular Kidney of *Lophius*, and Simultaneously as Competitors Under Conditions of Equimolar Loading

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ABSTRACT The maximal tubular transfer rates ($Tm$) of both *p*-aminohippurate (PAH) and diodrast (3,5-diiodo-4-pyridone-*N*-acetic acid or iodopyracet) were found to be fixed and reproducible when measured separately in *Lophius* (goosefish) under standard laboratory conditions. Expressed on a molar basis $Tm_{PAH}$ was four times $Tm_D$. However, when these transport competitors were presented simultaneously in equimolar concentrations with the plasma levels of each sufficiently high enough to saturate the carrier system, the relative rates of excretion were reversed with the diodrast transfer rate then four times that of PAH. The combined rate of excretion was far below $Tm_{PAH}$ alone, and roughly equal to $Tm_D$. Interaction with a common carrier was indicated by the gradations in degree of inhibition which resulted when plasma concentration ratios of diodrast to PAH were extended from 0.1 to 3.2, and PAH transfer rates expressed as percentage of $Tm_{PAH}$ were correspondingly depressed from 17 to 1.0 per cent respectively. These observations again point up the inverse relationship between transfer rate and competitive effectiveness which exists for members of a series of substances actively transported by a common mechanism. It appears that carrier affinity and dissociation characteristics may be quite different for various compounds in a series, and also that these parameters may vary significantly from species to species.

Although significant differences in maximal transfer rates ($Tm$) have already been shown for various organic acids in the series of actively transported compounds under consideration here (3, 24, 26), it is still sometimes assumed that

the common carrier for such rapidly transported competitors as PAH, diodrast, and penicillin has a more or less fixed flux capacity when expressed on a molar basis, whether these substances are being transferred individually, or with the capacity simultaneously shared by two or more competitors (21). Emphasis more recently has been placed on the differences in maximal transfer rates, and it has been shown that the more slowly excreted compounds are relatively the more effective competitive inhibitors (4, 10, 25), having the tendency to accumulate intracellularly during transit across proximal tubule cells (9, 14). The present study seeks to explore the inhibitory process quantitatively by measuring *Tm* on a molar basis under conditions of maximal loading, first, with diodrast or PAH administered alone, and then with the competitors presented simultaneously in equimolar plasma concentrations.

The aglomerular kidney of *Lophius* is uniquely suited for renal studies on active transport because transfer rates can be measured directly as quantity of substrate excreted per unit time without having to allow for the fraction filtered, a value difficult to obtain because of the differential binding characteristics of these organic acids for plasma proteins. Also, cytologically the aglomerular nephron has an essentially uniform epithelium without the regional differentiations characteristic of fresh water and terrestrial vertebrates (19), which obviates difficulties in interpretation arising from the simultaneous bidirectional reabsorptive and secretory processes which are involved in the movement of these compounds in structurally differentiated glomerular nephrons having only a relatively short portion of their lengths made up of actively secreting "brush border" cells (15). In addition, transport in *Lophius* can be viewed in the light of considerable information previously obtained by direct visualization *in vitro* of organic acids undergoing active transport in isolated nephrons of another marine teleost with similar renal tubules, the flounder, *Pseudopleuronectes* (9–13, 20, 27).

## EXPERIMENTAL METHODS

Methods employed in handling *Lophii* were generally those as described in earlier papers (2, 5, 7, 8). Diodrast was determined titrimetrically with sodium thiosulfate (1), and PAH colorimetrically by a modification of the Bratton and Marshall method (23).

Animals captured by otter trawl were selected for uniform size and minimal damage, and then maintained while aboard boat in cold running water. Upon return to the laboratory, they were transferred to individual tubs with circulating sea water and then allowed to acclimatize overnight before PAH or diodrast was administered into 4 injection sites of the paravertebral muscle mass. For the main series of equimolar competition studies 5 ml of either 35 per cent diodrast or 20 per cent PAH was found to provide slowly falling plasma concentrations, and loads sufficient to saturate *Tm*. At zero time indwelling catheters were inserted and secured, blood

samples taken, bladders emptied, and 3 to 4 hour collection periods begun. After 2 such periods an additional 1 ml booster of the original priming dose was injected intramuscularly plus 5 ml of the competitor. Urine collections made with approximately equimolar plasma concentrations of both the original compound and the inhibitor were begun 3 to 4 hours later, and the experiment was terminated after 3 additional 3 to 4 hour periods. Plasma concentrations of PAH and diodrast corresponding to the midpoints of each urine collection period were obtained by interpolation on a semilogarithmic plot relating values obtained from blood samples taken at the start and end of each urine collection. A complete record of a typical experiment in which PAH was the first of the competing substances administered is provided in Table I.

TABLE I

DETAILED RECORD OF A SINGLE EXPERIMENT

$Tm_{PAH}$ was measured alone in two control periods, followed by three in which transfer rates of both PAH and diodrast were determined with the competitors simultaneously present in plasma in approximately equimolar concentrations.*

| | | Diodrast | | | | PAH | | | |
|---|---|---|---|---|---|---|---|---|---|
| Time‡ | Urine flow | Urine | Plasma | U/P | Transfer rate | Urine | Plasma | U/P | Transfer rate |
| *hrs.* | *ml/kg hr.* | *μmole/ml* | | | *μmole/kg hr.* | *μmole/ml* | | | *μmole/kg hr.* |
| 0–4 | 0.63 | | | | | 35.25 | 1.58 | 22.30 | 22.20 |
| 4–7.2 | 0.75 | | | | | 28.80 | 1.28 | 22.45 | 21.60 |
| 5 ml of 35 per cent diodrast and 1 ml of 20 per cent PAH given intramuscularly at 7.2 hrs. | | | | | | | | | |
| 10.3–13.4 | 0.95 | 4.78 | 1.85 | 2.58 | 4.55 | 1.11 | 1.70 | 0.65 | 1.06 |
| 13.4–16.5 | 0.96 | 4.49 | 1.70 | 2.64 | 4.30 | 0.90 | 1.60 | 0.56 | 0.87 |
| 16.5–20.3 | 0.95 | 4.21 | 1.57 | 2.68 | 4.00 | 0.93 | 1.50 | 0.62 | 0.88 |

* *Lophius* 135, 4.4 kg body weight.

‡ 5 hours before the start of the first collection period 5 ml of 20 per cent PAH was given intramuscularly.

Slight modifications in this general procedure which were used to study competition under extended ranges of PAH and diodrast plasma concentrations and to establish critical plasma levels needed for saturation of the transport system will be described under Results.

## RESULTS

$Tm_{PAH}$ and $Tm_D$ were measured in preliminary experiments to establish plasma concentrations of PAH and diodrast which would ensure saturation of the transfer mechanism when these competitors were administered separately to *Lophii*. Fig. 1 relates excretion rates of diodrast and PAH to their respective plasma levels during individual urine collection periods with either one or the other compound presented over a wide range of plasma concentrations. Above a critical level both exhibited fixed maximal transfer rates which were inde-

pendent of their plasma concentrations, a phenomenon characteristic of active transport in many biological systems, and previously demonstrated for phenol red in the *Lophius* kidney (22). $Tm_{PAH}$ per kg body weight averaged 23.1 μmole/hr., and $Tm_D$ was 5.7. The *Tm* for phenol red, another competitive member of this transport series, appears to be considerably lower than either $Tm_{PAH}$ or $Tm_D$. The data represented in Fig. 1 were taken from control periods of the 6 experiments presented in Fig. 2, and from 2 additional for PAH, and one for diodrast, in which the range of plasma concentrations was deliberately extended by administering the usual 5 ml dose, but with observa-

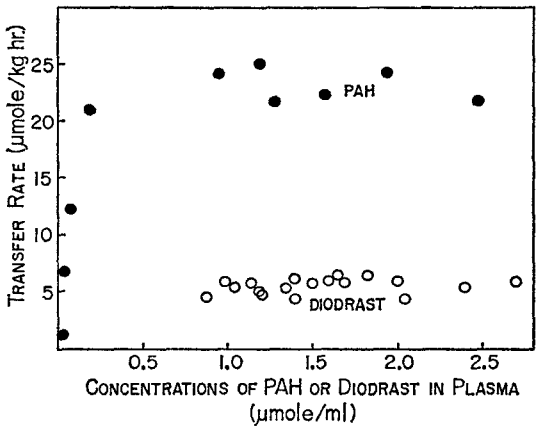


FIGURE 1. Relationship of plasma concentrations to excretion rates; detailed explanation in text.

tions in time prolonged to obtain low values on the falling curve of plasma concentrations. The relatively sluggish rate of diodrast excretion was reflected in the gradual slope of its plasma concentrations. Over 4 days the diodrast concentration in plasma fell only from 2.7 down to 0.88 μmole/ml, the latter value still being above the load needed to saturate the transport mechanism. Saturation of the PAH transfer system occurred when plasma concentrations were approximately 0.3 μmole/ml (6 mg/100 ml), a level close to that needed to achieve phenol red transfer maxima. Assuming the average effective renal plasma flow in *Lophius* per kg to be that arrived at in an earlier study, approximately 100 ml/hr. (5), the PAH load delivered to the kidney at the critical concentration of 0.3 μmole/ml was calculated to be roughly in the range of $Tm_{PAH}$, which per kg averaged 23.1 μmole/hr. in the current study.

In the competition series of experiments PAH was injected first into 3 *Lophii* designated Nos. 134, 137, and 135 in Fig. 2, and weighing respectively 3.0, 5.0, and 4.5 kg. $Tm_{PAH}$ in 2 control periods averaged 22.9, 24.5, and 21.9 μmole/kg hr., and after equimolar loading with diodrast the PAH transfer

rates in 3 periods averaged 1.22, 0.57 and 0.94 μmole/kg hr. respectively. The combined transfer rates of diodrast and PAH were 6.42, 4.10, and 5.22, far below that of PAH alone. The average PAH plasma concentration in the control periods was 1.5 μmole/ml, and 1.81 in the periods with diodrast present simultaneously. Diodrast plasma concentrations in the latter averaged 1.66 μmole/ml. In 3 other *Lophii* designated Nos. 131, 132, and 133 in Fig. 2,

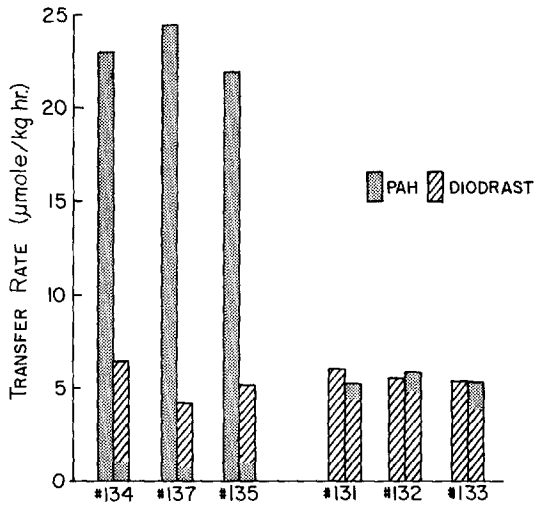


FIGURE 2. Maximal capacity of the transport system measured first while transferring PAH or diodrast alone, and then with both competitors presented in equimolar concentrations. In the first set of three similar experiments diodrast followed the administration of PAH, and in the second series the order was reversed. The first bar for each of the six experiments represents the average of two collection periods during which $Tm_{PAH}$ or $T_D$ was measured individually, and the second bar is the average of three subsequent periods on the same animal after doses of the competitor had been given in addition, with plasma concentrations of each then being high enough to ensure saturation of the tubular transport system.

weighing respectively 2.7, 4.1, and 4.5 kg, diodrast was injected first, and control transfer rates again measured in 2 collection periods on each animal. $Tm_D$ values were 6.05, 5.62, and 5.51 μmole/kg hr. when the respective diodrast concentrations in plasma averaged 1.5 μmole/ml. After PAH was administered to these animals the plasma concentrations of diodrast and PAH were 1.39 and 2.0 μmole/ml respectively, as averaged in 3 competition periods in the 3 animals. PAH replaced only very little of the diodrast being excreted. The combined transfer rates were approximately the same as those of diodrast alone, and similar to combined rates after diodrast had been added to PAH in the first series of competition experiments. The combined transfer rates of PAH and diodrast now were 5.34, 5.91, and 5.50 μmole/kg hr., with those of diodrast being 4.34, 4.81, and 3.88, and PAH 1.0, 1.1, and 1.62 re-

spectively; again expressed as averages of the 3 collection periods for each of the 3 *Lophii* in this experimental series.

Diodrast levels in plasma were extended by varying dosages in several experiments to obtain ratios of diodrast to PAH which were above and below the equimolar relationship used for the competition observations previously mentioned. Six such experiments are represented in Fig. 3 in which $Tm_{PAH}$ was determined first in two control periods, and then varying amounts of diodrast injected to yield ratios extending over a 30-fold range, from approximately 0.1 to 3. Three additional urine collections were then made on each animal with both competitiors present, and the averaged PAH transfer rates plotted as percentages of their control $Tm_{PAH}$. The remarkable competitive effectiveness of diodrast is disclosed by the high degree of $Tm_{PAH}$ inhibition obtained

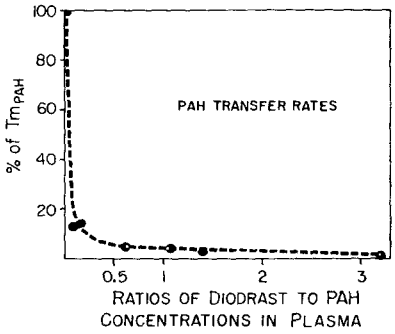


FIGURE 3. Graded inhibitory effect of varying plasma concentrations of diodrast on the active tubular transport of PAH. The curve relating average values obtained from six animals was drawn by visual inspection. Experimental details are included in the text.

(85 per cent) when the diodrast molar concentration in plasma was as low as one-tenth that of PAH. Inhibition was about 99 per cent complete when the molecular concentration of diodrast was 3 times that of PAH. Studies are planned to extend this kind of quantitative approach to the substrate-carrier problem by conducting the reciprocal experiment with variations in plasma PAH concentrations, and also to determine kinetic constants through a systematic study of transfer rates at various plasma PAH and diodrast levels which are far below those required to saturate the transport system.

## DISCUSSION

The carrier concept is a useful hypothesis to explain many of the features which characterize the active transport system involved in the tubular excretion of such organic acids as PAH, diodrast, penicillin, the phenolsulfonephthaleins, etc. Transcellular movement of these compounds in unaltered form occurs against gradients which result in concentration ratios as high as $6 \times 10^3$. Energy dependence is made evident in that transfer can be blocked, or reversed subsequent to intracellular or luminal accumulation, by such general metabolic inhibitors as anaerobiosis, cold, and cyanide, and by

relatively specific inhibitors of Krebs' cycle activity. Furthermore, aerobic phosphorylation and ATP have been implicated as the source of free energy for this work because of the similarly selective inhibitory influence exerted by members in a series of substituted nitrophenols both on the transfer process and on the generation of high energy bonds. The nature of chemical interactions which these organic acids undergo during transit is entirely unknown; certain shared chemical characteristics have been established, and the possibility of chelation and ionic binding explored, but much further study along these lines is needed. Carrier involvement is indicated by limited transport capacity, competitive inhibition of transfer by any one substance of others in the homologous series, and by specificity of interaction with only certain closely related compounds among all those known to be actively secreted by renal tubules. Graded affinities for carrier which apparently are related to dissociation constants of the individual competitors have been indicated in earlier studies which showed that those substances with low *Tm* are the more powerful competitive inhibitors, and also have the greater tendency to accumulate intracellularly during transit across proximal tubule cells. These, and other features of the organic acid transport series, have been discussed in recent reviews (6, 18).

Interpretation of current results in the light of similar observations on transport across such systems as single cell membranes of erythrocytes or accumulation in mammalian kidney slices is made difficult because of the complexity of transcellular movement into tubular urine. The excretory process involves active transfer across two cell membranes, and, in addition, perhaps transient non-specific binding of these organic anions to intracellular proteins, such as to plasma proteins where degree of binding appears to be inversely related to the corresponding p$K$ (16). Furthermore, there is considerable evidence to indicate that these sites may have different kinetic characteristics in proximal tubule cells among various species of vertebrates. For any given concentration of competitor it is to be expected that one of the transfer steps will be rate-limiting, but it is also to be expected that the rate-limiting step may vary with changing concentrations of substrate. Under conditions of maximal loading, as with these experiments on *Lophius*, it is interesting to note the resemblance to conditions existing in red cells where the total amount of sugar entering from a mixture of sugars in equimolar concentration is less than the sum of their individual penetrations rates measured separately (29). As with PAH and diodrast in *Lophius*, two sugars added successively to red cells, rather than simultaneously, show graded effectiveness; the penetration of glucose in the presence of sorbose is practically unchanged, whereas that of sorbose in the presence of glucose is strongly inhibited (17).

In the simplest model of membrane carrier transfer, one would expect a

directly proportional relationship among various competitors between affinity and rate of transport. However, observations reported here, and earlier for the phenolsulfonephthaleins (9, 10), point to a reciprocal relationship with the powerful competitors being transported slowly, and *vice versa*. Kinetic analyses would indicate the predicted relationship to depend on carrier-substrate saturation conditions; the transfer rate with substrate concentration far from saturation being directly proportional to affinity or inversely proportional to dissociation constant, and near saturation being proportional to the dissociation constant and inversely proportional to affinity. Thus, the order of rates for different substances should depend on the degree of substrate-carrier saturation; near saturation the competitor with the highest affinity would be the slowest one transported, and far from saturation it should be the fastest. Transport of sugars in erythrocytes is in agreement with this prediction (28). Our earlier studies on isolated tubules which showed this reciprocal relationship among various phenolsulfonephthaleins perhaps were carried on near saturation levels, if this analysis is applicable to the multiple carrier mechanisms operating in transcellular movement (9, 10, 12, 13).

In a complex transfer system such as that involved here in the movement of organic acids across cells, the compound finally appears in tubular urine in the same free state it possessed initially in plasma on the vascular side of the cell. In proximal tubule cells of cold blooded vertebrates at least two specific membrane carrier sites have been identified, each energy-dependent and subject to competitive inhibition. It would be an oversimplification solely to consider substrate-carrier affinity or association, and not dissociation, until it is clearly demonstrated that one site is definitely rate-limiting, and that in the course of association the complex is raised to a higher energy level through intervention of some exergonic chemical reaction. This may well be the case for certain competitors at certain concentration levels, but at the moment it is not at all clear that this situation prevails in all species and under all saturation conditions for various competitors transported at widely different rates. Suitable refinements of experimental design are needed to subject this transport system to the kind of kinetic analysis which could lead to a characterization of the separate steps known to be involved, and perhaps to some understanding of the chemical nature of the carrier molecules themselves.

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