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The Intramitochondrial Location of the Glutaminase Isoenzymes of Pig Kidney

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1. The glutaminase activity of pig kidney is located almost entirely in the cortex. 2. Pig renal cortex contains two glutaminases, one phosphate-dependent and one phosphate-independent. Both isoenzymes are localized exclusively in the mitochondria. 3. After sonication of the mitochondria, the phosphate-dependent isoenzyme is entirely soluble, whereas approximately half the phosphate-independent isoenzyme is associated with the membranes. 4. In intact mitochondria, the activities of both isoenzymes respond to changes in the pH of the intramitochondrial compartment. 5. It is concluded that both glutaminase isoenzymes are situated in the intramitochondrial compartment, and that the phosphate-independent glutaminase may be bound to the inside of the inner mitochondrial membrane.

Glutaminase (L-glutamine amidohydrolase, EC 3.5.1.2) catalyses the deamidation of glutamine to yield ammonium glutamate. Glutaminase has been purified from kidney, liver and brain of the rat, and is reported to exist as two isoenzymes in each tissue (Katunuma *et al.*, 1967). One isoenzyme displays a requirement for a high concentration of phosphate when isolated (at least 75mM-phosphate is needed for maximum activity), whereas the other isoenzyme does not show this dependency; accordingly, the isoenzymes are referred to as P_i-dependent glutaminase and P_i-independent glutaminase.

The properties of the two isoenzymes in each tissue are quite distinct. In rat kidney, the P_i-dependent glutaminase has a high K_m for glutamine (40mM), a pH optimum of 8.5 and is inhibited by glutamate. The P_i-independent isoenzyme has a relatively low K_m for glutamine (4mM), a pH optimum of 7.5 and is not inhibited by glutamate (Katunuma *et al.*, 1967). This P_i-dependent glutaminase may be identified with the P_i-activated glutaminase studied by several groups of workers. The P_i-dependent glutaminases from the kidneys of the pig (Klingman & Handler, 1958) and the dog (Sayre & Roberts, 1958) are inhibited by glutamate (competitive with phosphate) and ammonia (competitive with glutamine). Thus the regulation of this enzyme is thought to be effected in a feedback manner by the products of the reaction (see Balagura-Baruch, 1971; Lund *et al.*, 1970).

To understand more fully how renal ammonia production by the glutaminases is regulated, it is essential to know the subcellular compartments in which these isoenzymes function. Katunuma *et al.* (1967) have reported that both the P_i-dependent and P_i-independent glutaminases from the kidney, liver and brain of the rat are recovered largely in the mitochondrial fraction. Also, the P_i-dependent glutaminases of pig kidney (Klingman & Handler, 1958) and guinea-pig liver (Guha, 1961) appear to be localized entirely in the mitochondria. However, since the inner mitochondrial membrane exhibits selective permeability properties towards metabolites, and encloses an intramitochondrial pool of metabolites and enzymes quite distinct from the extramitochondrial, or cytoplasmic, pool, it is important to establish the precise location of the glutaminase isoenzymes in the mitochondria. This information should enable the metabolite changes in the environment of the isoenzymes, and the possible implications of metabolite transport on the glutaminase activities, to be discussed with more certainty. The present paper deals with the location of the P_i-dependent and P_i-independent glutaminases in pig kidney, and evidence is presented that both isoenzymes are situated in the intramitochondrial compartment, and that the P_i-independent glutaminase may be bound to the inside of the inner mitochondrial membrane.

Methods

Isolation of pig kidney mitochondria

Kidneys were collected from pigs about 30min after slaughter and were immediately sliced and chilled in ice-cold extraction medium: 0.25M-sucrose,

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3mM-Tris–HCl, 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], final pH7.4. The mitochondria were prepared from the chilled tissue as described by Chappell & Hansford (1969), and suspended finally in extraction medium (approx. 100mg of mitochondrial protein/ml). The respiratory control ratio (Lardy & Copenhaver, 1954), used as an index of the integrity of the mitochondria, was measured with an oxygen electrode (Chappell, 1964) in a reaction medium containing 100mM-choline chloride, 45mM-Tris–HCl, 5mM-potassium phosphate, bovine serum albumin (0.05%, w/v) and 0.5mM-EDTA, final pH7.4; the value of this ratio varied between 4 and 6 with succinate as substrate.

Fractionation

Preparation of cytoplasmic fractions. Cytoplasmic fraction refers to the supernatant remaining after sedimentation of the mitochondria (see the paragraph above). This supernatant was centrifuged once more to sediment residual mitochondria.

Preparation of membrane and soluble fractions of pig kidney mitochondria. Mitochondria (3.0ml, containing about 300mg of protein) were diluted with 17ml of 100mM-Tris–HCl, pH8.0, containing 20mM-Tris–borate, which prevents inactivation of the P_i-dependent glutaminase (Klingman & Handler, 1958), and sonicated for 2min in an MSE ultrasonic disintegrator no. 1, set at 1.7A, with a 1cm-diameter probe; the sonication was carried out with the vessel containing the mitochondrial suspension in ice and with intermittent cooling periods to ensure that the sonicate remained cold. The sonicate was centrifuged for 1h at 4°C and 66000*g* ($r_{av.}$ 3.69cm) and the pellet (designated the membrane fraction) and the supernatant (soluble fraction) were separated and retained.

Enzyme assays and mitochondrial measurements

Assay of glutaminase. Glutaminase activity was measured by continuously monitoring the formation of NH_3 with an ion-selective electrode (type GKN 33; Electronic Instruments Ltd., Richmond, Surrey, U.K.). The electrode was calibrated in each experiment by the addition of a known amount of NH_4Cl.

Assay of marker enzymes. Malate dehydrogenase activity was measured as described by Ochoa (1955). Glutamate dehydrogenase was assayed in the forward direction as described by Strecker (1955). Rotenone-insensitive NADH–cytochrome *c* reductase was measured as described by King & Howard (1967), with the addition of rotenone (1μg/mg of mitochondrial protein). Cytochrome $a+a_3$ was determined in a dual-wavelength spectrophotometer by measuring the extinction change at 605nm minus 630nm after the addition of dithionite to a suspension of the fraction in 50mM-Tris–HCl, pH7.5, containing rotenone (1μg/ml).

Measurement of mitochondrial swelling. This was done with a Hilger–Watts recording spectrophotometer by measuring changes in E_{610} as described by Chappell & Crofts (1966).

Determination of mitochondrial protein. Protein was measured by the method of Gornall *et al.* (1949), with bovine plasma albumin (Sigma Chemical Co.) as standard.

Results

Location of glutaminase in pig kidney

Table 1 shows that glutaminase was recovered only in the mitochondrial fraction. No glutaminase was detected in the cytoplasmic fractions from either cortex or medulla.

The specific glutaminase activities of mitochondria isolated from cortex and from medulla are presented in line (*b*). The highest specific activity is in the mitochondria from cortex. The cortex also contains the highest concentrations of mitochondria (line *c*). Hence, the glutaminase activity per g wet wt. of tissue (line *a*) is very much higher in the cortex than in the medulla. The low activity recovered in the medulla is located in the outer zone, and may have resulted from a small contamination of the medulla samples by pieces of cortex. Experiments were also carried out to assess the distribution of glutaminase throughout the cortex. The activity was measured in mitochondria prepared from different parts of the cortex, i.e. the outer, middle and inner regions. Approximately the same specific glutaminase activity (i.e. 2μmol of NH_3/min per g fresh wt. at pH7.5; 6μmol of NH_3/min per g fresh wt. at pH8.5) was detected in each of the three regions, indicating that glutaminase is quite uniformly distributed throughout the cortex. The pH values, 7.5 and 8.5, are close to the pH optima of the glutaminase isoenzymes (Katunuma *et al.*, 1967), and the purpose of assaying at these pH values was to discover whether the separation of kidney tissue into the various regions of cortex and medulla produced any separation of the two isoenzymes. The results show approximately the same ratio of activities at pH7.5 and pH8.5 in the different regions of cortex and medulla, which indicates a similar gross distribution of the isoenzymes in pig kidney.

Glutaminase activity of the membrane and soluble fractions of sonicated pig renal-cortex mitochondria

Pig renal-cortex mitochondria were separated into membrane and soluble fractions. The recoveries of the glutaminases after sonication were determined by measuring the activity of intact mitochondria in

Table 1. *Glutaminase activity of the mitochondrial and cytoplasmic fractions from different regions of pig kidney*

The reaction medium contained choline chloride (80mM), Tris–HCl (40mM), Tris–phosphate (30mM), ADP (5mM), KCl (50μM) and glutamine (10mM). The amount of tissue extract used corresponded to about 0.5g of fresh kidney tissue. The final volume was 8.0ml and the temperature was 25°C.

	Mitochondrial fraction						Cytoplasmic fraction			
	Cortex		Medulla: Outer		Medulla: Inner		Cortex		Medulla	
pH ...	7.5	8.5	7.5	8.5	7.5	8.5	7.5	8.5	7.5	8.5
Glutaminase activity										
(*a*) NH_3 produced (μmol/min per g fresh wt.)	2.0	6.2	0.13	0.44	<0.01	<0.01	<0.2	<0.2	<0.2	<0.2
(*b*) NH_3 produced (nmol/min per mg of mitochondrial protein)	109	327	19	65	<3	<3				
(*c*) Mitochondrial protein (mg/g fresh wt.)	18.8		6.5		3.0					

Table 2. *Activity of marker enzymes in the membrane and soluble fractions of sonicated pig renal-cortex mitochondria*

The assays were carried out at 25°C as described in the Methods section.

	Proportion of total activity recovered (%)			
	Malate dehydrogenase	Glutamate dehydrogenase	Cytochrome $a+a_3$	Rotenone-insensitive NADH–cytochrome *c* reductase
Membrane fraction	4.4	4.7	85.4	82.8
Soluble fraction	94.2	96.3	4.1	8.6

30mM-Tris–phosphate (which is sufficient to activate the P_i-dependent glutaminase maximally in intact mitochondria; Crompton & Chappell, 1973) and the activity of the total sonicate in 300mM-Tris–phosphate; the recovery was 69% at pH8.6 and 87% at pH7.3.

These two fractions were assayed for glutaminase under various conditions designed to distinguish between the activities of the glutaminase isoenzymes. The purity of the fractions was estimated by assaying for certain marker enzymes (see Ernster & Kuylenstierna, 1970). Thus malate dehydrogenase and glutamate dehydrogenase were used as markers for the soluble fraction, cytochrome $a+a_3$ was used to detect the presence of the inner mitochondrial membrane, and rotenone-insensitive NADH–cytochrome *c* reductase was used as an indicator for the outer membrane. The separation of the marker enzymes by the fractionation procedure is given in Table 2. The soluble fraction contained 94–97% of the soluble marker activity and 4–9% of the membrane marker activity. The membrane fraction contained most of the inner-membrane (85%) and outer-membrane (83%) material, and 4–5% of the soluble marker activity, as judged by the criteria set out above.

Fig. 1 shows the glutaminase activity of the membrane and soluble fractions in the presence and the absence of added phosphate over a range of pH values. In the absence of phosphate, both fractions displayed maximum activity at about pH7.5. In the presence of phosphate the activity of both fractions was increased, and maximum activity was observed at about pH8.2–8.6. However, the inclusion of phosphate produced a much larger increase in the activity of the soluble fraction than for the membrane fraction. Specifically, the increases in glutaminase activity produced by the addition of phosphate to the soluble and membrane fractions were 5-fold and 0.25-fold respectively at pH7.5, and 19-fold and 2.5-fold respectively at pH8.4. Thus approx. 95% (calculated from values at pH8.4) of the P_i-dependent activity was recovered in the soluble fraction of the mitochondrial sonicate. The 5% of total soluble

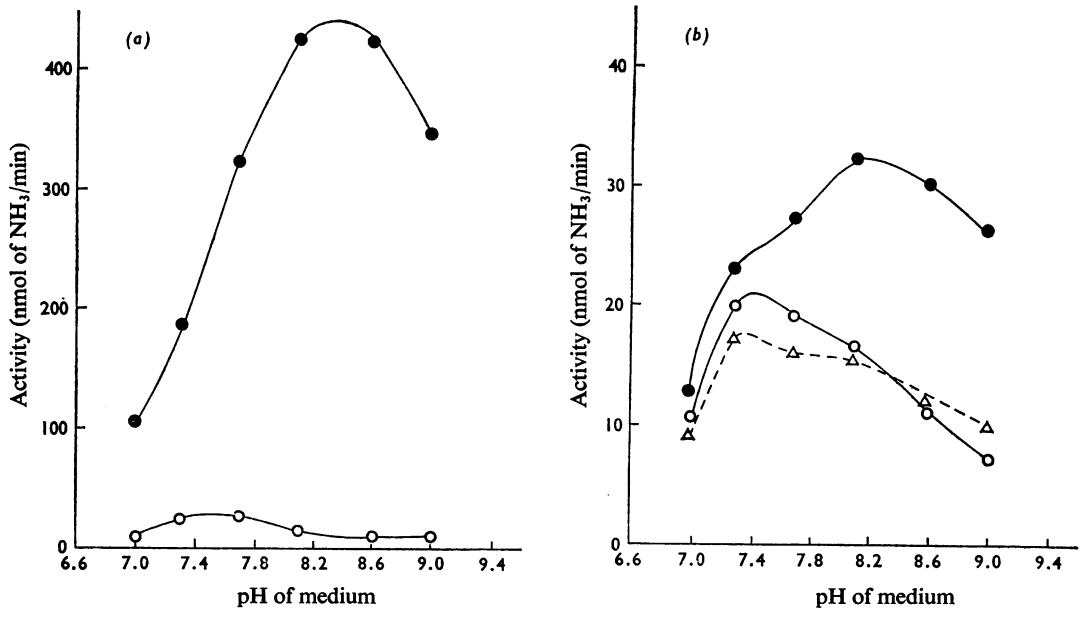


Fig. 1. *Glutaminase activity of the soluble (a) and membrane (b) fractions of sonicated pig renal-cortex mitochondria in the presence and absence of phosphate*

The reaction medium contained Tris–HCl (50mM) and glutamine (10mM), with and without tris–phosphate (300mM). The amount of fraction used corresponded to about 5mg of mitochondrial protein before sonication. The activities are expressed per mg of mitochondrial protein before sonication. The final volume was 8.0ml and the temperature was 25°C. ●, Plus phosphate; ○, minus phosphate; △---△, glutaminase activity in the presence of phosphate corrected for the phosphate-dependent activity (see the text).

activity remaining in the membrane fraction may be accounted for by contamination of membrane material by soluble protein, since about 4.5% of the total activity of glutamate dehydrogenase and malate dehydrogenase was also recovered in the membrane fraction (Table 2). If this contamination is taken into account, and the activity of the membrane fraction in the presence of phosphate is corrected for the presence of soluble activity (calculated to be 4.5%), then the corrected activity agrees reasonably well with the membrane activity in the absence of phosphate (Fig. 1, broken line). This indicates that the glutaminase activity associated with the membrane in sonicated mitochondria is solely P_i-independent. The P_i-dependent activity is entirely soluble and not bound to the membranes. Although the membrane activity is wholly P_i-independent, an equal amount of P_i-independent activity was also recovered in the soluble fraction. This cannot be attributed to membrane contamination of the soluble fraction, which was only about 4–9% as judged by the distribution of rotenone-insensitive NADH–cytochrome *c* reductase and cytochrome $a+a_3$.

The degree to which phosphate activates the glutaminase activities of the membrane and soluble fractions of sonicated rat kidney mitochondria has been measured by McDermott & O'Donovan (1971). These workers observed that the addition of phosphate (170mM) stimulated the soluble activity threefold that of the membrane activity, which is consistent with the results reported here.

Fig. 1 shows that the glutaminase activity of pig renal-cortex mitochondria is largely P_i-dependent. If the values for the P_i-dependent and P_i-independent activities are calculated at pH8.4 and 7.5 respectively, then about 95% of the total glutaminase activity is represented by the P_i-dependent form. This compares well with the value of 98% in whole pig kidney obtained by Katunuma *et al.* (1966).

Fig. 2 shows reciprocal plots (Lineweaver & Burk, 1934) for the glutaminase activity of the membrane and soluble fractions. Both fractions were assayed at pH7.7. The K_m for glutamine of the soluble fraction in the presence of phosphate was approx. 5mM. The K_m for glutamine of the membrane fraction with no phosphate added was approx. 0.4mM. The reciprocal plot for the membrane activity in the presence of

phosphate was not linear, since at high glutamine concentrations the curve diverged from the values obtained in the absence of phosphate. Presumably this was due to contamination of the membrane fraction by soluble activity with a higher K_m for glutamine. The K_m for the P_i-dependent activity (5mM) is identical with the value reported for the P_i-dependent glutaminase purified from pig kidney by Klingman & Handler (1958) and from dog kidney by Sayre & Roberts (1958). The K_m values for glutamine of the P_i-dependent and P_i-independent glutaminase from rat kidney are 40mM and 4mM respectively (Katunuma *et al.*, 1967), i.e. the K_m values are both about tenfold those of the corresponding constants for the pig kidney activities.

Fig. 3 shows the inhibition of the membrane and soluble glutaminase activities by glutamate. The soluble activity is inhibited markedly by glutamate, whereas the membrane activity is inhibited only slightly. Several groups of workers have shown that

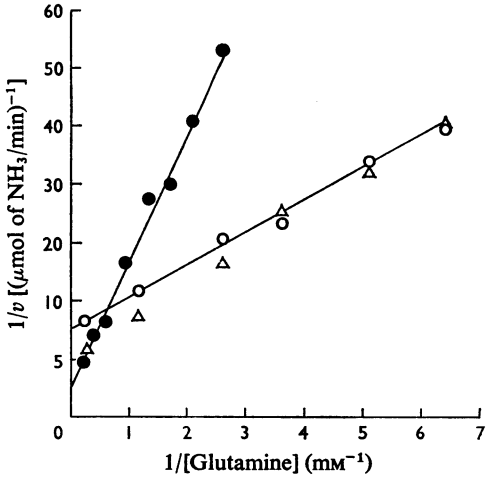


Fig. 2. *Reciprocal plots of the glutaminase activity of the membrane and soluble fractions of sonicated pig renal-cortex mitochondria*

The reaction medium contained Tris–HCl, pH7.7 (50mM), with and without Tris–phosphate, pH7.7 (300mM). The amount of fraction used corresponded to about 10mg of mitochondrial protein before sonication. The reaction rates are expressed per mg of mitochondrial protein before sonication. Final volume, 8.0ml; temperature, 25°C. The reciprocals of the membrane activities have been multiplied by 10^{-1}. ●, Soluble glutaminase in the presence of phosphate; ○, membrane glutaminase in the absence of phosphate; △, membrane glutaminase in the presence of phosphate.

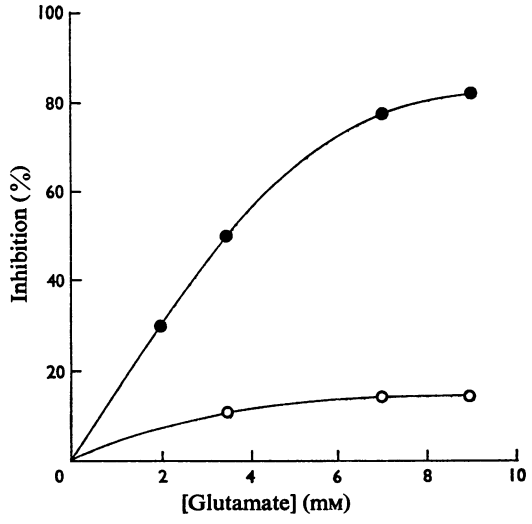


Fig. 3. *Inhibition by glutamate of the glutaminase activity of the membrane and soluble fraction of sonicated pig renal-cortex mitochondria*

The reaction medium contained Tris–phosphate, pH7.7 (50mM), and glutamine (10mM). The amount of fraction used was equivalent to about 5mg of mitochondrial protein before sonication. Final volume, 8.0ml; temperature, 25°C. ●, Soluble glutaminase; ○, membrane glutaminase.

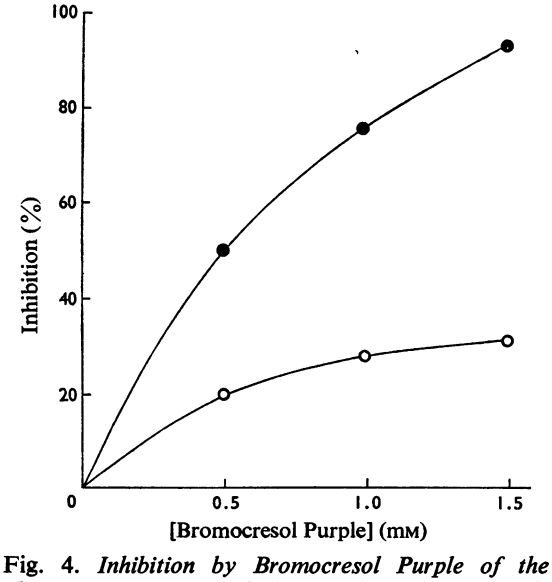


Fig. 4. *Inhibition by Bromocresol Purple of the glutaminase activity of the membrane and soluble fractions of sonicated pig renal-cortex mitochondria*

The assay conditions were as described for Fig. 3. ●, Soluble glutaminase; ○, membrane glutaminase.

the purified P_i-dependent glutaminase from kidney tissue is inhibited by glutamate (e.g. Katunuma *et al.*, 1967; Klingman & Handler, 1958), and the inhibition is competitive with phosphate (Sayre & Roberts, 1958). The P_i-independent glutaminase of kidney, however, is not inhibited by glutamate (Katunuma *et al.*, 1967).

Fig. 4 shows the inhibition of the soluble and membrane glutaminase activities by Bromocresol Purple. It is evident that the soluble activity is much more sensitive to inhibition than the membrane activity. The inhibition of the purified P_i-dependent glutaminase from dog kidney by Bromocresol Purple and other phthalein dyes has been reported by Sayre & Roberts (1958). These authors showed that the inhibition is competitive with phosphate, and it is likely therefore that the difference in the degree of inhibition by Bromocresol Purple of the membrane and soluble activities results from their different requirements for phosphate.

Response of the glutaminase activities of pig renal-cortex mitochondria to pH changes in the intramitochondrial and extramitochondrial compartments

Because of the selectively permeable properties of the inner mitochondrial membrane the most important single question about the location of the glutaminase isoenzymes is whether they are located on the inside or the outside of the inner mitochondrial membrane. This question was approached by observing whether the activities of the two isoenzymes responded to changes in the pH of the intra- or extra-mitochondrial compartment.

The glutaminase activity of intact mitochondria was measured in the presence of rotenone and antimycin to inhibit respiration. Under these conditions the oxidation of glutamate is prevented, and the glutamine that disappears can be accounted for entirely by the accumulation of glutamate in the external medium (Crompton & Chappell, 1973). Thus the only enzyme involved in this process is

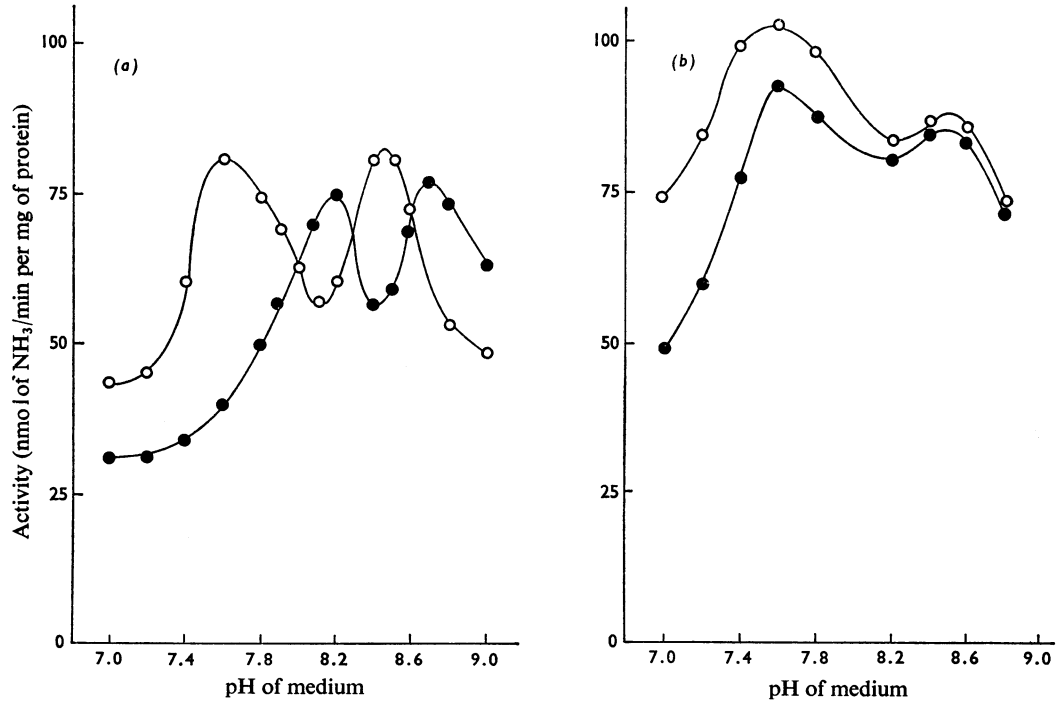


Fig. 5. *Glutaminase activity of intact (a) and sonicated (b) mitochondria from pig kidney cortex in the presence of uncoupler and the presence and absence of tris–nitrate*

The reaction medium contained choline chloride (100mM), Tris–HCl (50mM), rotenone (2μg), antimycin (2μg), carbonyl cyanide phenylhydrazone (5μg), glutamine (1mM) and 12mg of mitochondrial protein. Final volume, 7.0ml; temperature, 25°C. Additions: o, none; •, Tris–nitrate (20mM) adjusted to the pH of the reaction medium.

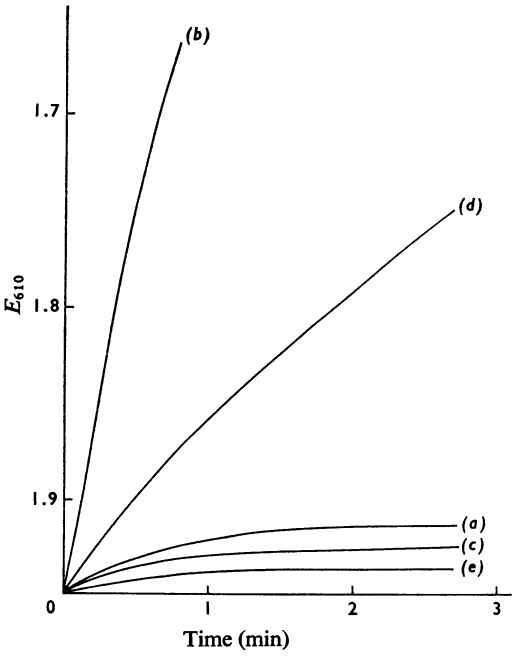
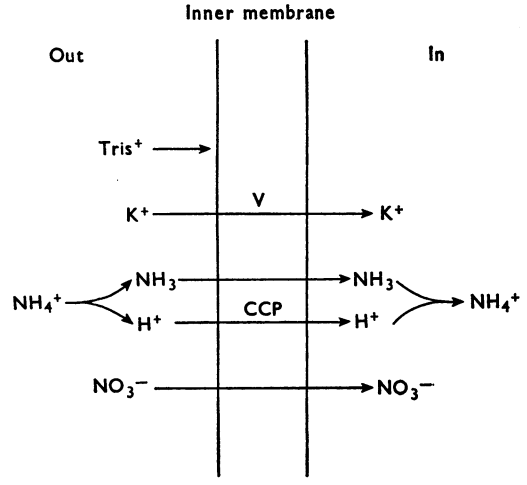


Fig. 6. *Swelling of mitochondria in iso-osmotic solutions* (150 mM) *of nitrate salts*

The incubation medium, pH 7.5, contained 3 mg of mitochondrial protein, rotenone (2 μg) and (*a*) KNO_3, (*b*) KNO_3 and valinomycin (1 μg), (*c*) NH_4NO_3, (*d*) NH_4NO_3 and carbonyl cyanide phenylhydrazone (5 μg) or (*e*) Tris–nitrate. Final volume, 2.0 ml; temperature, 25°C.



Scheme 1. *Permeation of nitrate salts through the inner mitochondrial membrane*

Valinomycin (V) and carbonyl cyanide phenylhydrazone (CCP) catalyse the permeation of K^+ and H^+ respectively.

glutaminase. The glutaminase activity of respiration-inhibited mitochondria in the presence of the uncoupler carbonyl cyanide phenylhydrazone is shown as a function of pH in Fig. 5(*a*). The activities of both glutaminase isoenzymes, pH optima 7.6 (P_i-independent) and 8.4 (P_i-dependent), were detected. The experimental conditions (1 mM-glutamine and no added phosphate) were chosen to limit the P_i-dependent activity to a low value. The P_i-dependent activity observed may be attributed to the endogenous phosphate content of isolated mitochondria (about 5 mM: McGivan & Klingenberg, 1971).

The effect of changing the pH of the intramitochondrial compartment relative to the pH of the external medium was investigated by adding tris–nitrate to the reaction medium. The inner mitochondrial membrane is permeable to NO_3^-, but impermeable to the tris cation. This is demonstrated in Fig. 6, where the rate of influx of various nitrate salts was followed by mitochondrial swelling in iso-osmotic nitrate solutions. Potassium nitrate enters rapidly in the presence of valinomycin, which permits rapid electrogenic movement of K^+ across the inner mitochondrial membrane (Henderson *et al.*, 1969). Ammonium nitrate enters rapidly only in the presence of the uncoupler, carbonyl cyanide phenylhydrazone, which allows rapid, electrogenic permeation of protons (Mitchell, 1965). Tris–nitrate, however, permeates very slowly. Thus an electrogenic influx of cations is accompanied by an influx of NO_3^-, which indicates that NO_3^- permeates the inner mitochondrial membrane electrogenically. Tris–nitrate influx is slow because the Tris cation permeates slowly. These ion movements are shown in Scheme 1.

Because of the large difference in the permeability of the inner membrane to the Tris cation and NO_3^-, the addition of Tris–nitrate to the reaction medium would be expected to produce a diffusion potential (positive outside) across the inner mitochondrial membrane; the inclusion of carbonyl cyanide phenylhydrazone allows an influx of protons down the electrical gradient. That is, there is a net influx of HNO_3 and the matrix pH is decreased relative to the external pH.

Fig. 5(*a*) shows that the addition of Tris–nitrate to the reaction medium produces an apparent alkaline shift in the pH optima of both the P_i-independent and P_i-dependent activities in intact mitochondria. However, Tris–nitrate does not change the pH optima of either the P_i-independent or the P_i-dependent activity in broken mitochondria (Fig. 5*b*), although

the addition of Tris–nitrate does seem partially to inhibit the P_i-independent activity (pH optimum 7.6). These results may be interpreted to indicate that both the P_i-independent and P_i-dependent glutaminase activities are sensitive to pH changes occurring in the mitochondrial matrix, and that both activities are located in the intramitochondrial compartment.

Discussion

Pig renal-cortex mitochondria contain two types of glutaminase activity. On the basis of their requirements for phosphate, pH optima, K_m values for glutamine, and degree of inhibition by glutamate and Bromocresol Purple, the two types of activity may be identified with the P_i-dependent and P_i-independent glutaminase isoenzymes purified from kidney tissue by other workers.

After sonication of pig kidney mitochondria, the P_i-dependent glutaminase is recovered almost entirely in the soluble fraction. Further, the activity of this isoenzyme in intact mitochondria responds to variations in the pH of the intramitochondrial compartment. It is therefore proposed that the P_i-dependent glutaminase in pig kidney is a soluble enzyme localized in the matrix compartment of the mitochondrion.

The activity of the P_i-independent glutaminase in intact mitochondria is dependent on the pH of the matrix compartment, which indicates that this isoenzyme also is situated in the intramitochondrial compartment. The evidence relating to the precise location of the P_i-independent glutaminase within the matrix compartment is difficult to interpret, since approximately equal activities were recovered in the membrane and soluble fraction after sonication. This may mean that in unbroken mitochondria the enzyme exists in two states, i.e. partially bound to the inner membrane and partially free in the matrix. However, in view of the degree of mitochondrial disruption brought about during sonication, it is possible that the P_i-dependent glutaminase is wholly bound to the inner membrane but becomes partially dissociated from the membrane during sonication. The reverse situation, that this isoenzyme is entirely soluble in intact mitochondria and binds to the membrane as a result of sonication, seems less likely. On the basis of these considerations it is tentatively suggested that the P_i-independent glutaminase may be entirely bound to the inside of the inner mitochondrial membrane *in vivo*.

An important point, which merits re-emphasis, is that since both isoenzymes are situated in the inner mitochondrial compartment, the glutaminase activity *in vivo* will be subject to any restrictions imposed by the permeability of the inner mitochondrial membrane towards the substrate and products of the glutaminase reaction, and will be influenced by changes in the intramitochondrial, rather than cytoplasmic, concentrations of regulatory metabolites. The activity of the glutaminase isoenzymes in relation to the permeation of glutamine and glutamate across the inner membrane is the subject of the following paper (Crompton & Chappell, 1973).

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