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Transport of Glutamine and Glutamate in Kidney Mitochondria in Relation to Glutamine Deamidation

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1. In the absence of added ADP glutamine is transformed by pig kidney mitochondria to ammonium glutamate, which appears in the external medium. This reaction is stimulated only slightly by the addition of ADP, but under these conditions about 20% of the glutamate is oxidized to aspartate. 2. Externally added glutamate is oxidized to aspartate, and at about the same rate as glutamine. 3. The net rates of glutamine and glutamate influx into the intramitochondrial compartment are very slow. 4. The phosphate-dependent glutaminase activity of intact mitochondria is stimulated by the provision of energy. 5. The provision of energy also decreases the concentration of glutamate and increases the concentration of glutamine in the intramitochondrial compartment. These energy-linked changes in the glutamine and glutamate concentrations are of equal magnitude. 6. It is suggested that transport of glutamine and glutamate across the inner membrane of kidney mitochondria occurs by an obligatory exchange between the two metabolites, and is electrogenic. The existence of an electrogenic glutamine–glutamate anti-porter is proposed.

The glutaminase activity of pig renal-cortex mitochondria is due to the existence of two glutaminases, a P_i-dependent and a P_i-independent isoenzyme, both of which are localized in the intramitochondrial compartment (Crompton *et al.*, 1973). The two isoenzymes have widely different K_m values for glutamine (the P_i-dependent isoenzyme has a relatively high K_m value), and differ markedly in their degree of inhibition by glutamate (the P_i-dependent isoenzyme is inhibited strongly). The relative activities of the two glutaminases therefore may well depend on the relative concentration of glutamine and glutamate in the mitochondrial matrix. In pig kidney, which lacks glutamine synthetase (Wu, 1963*a*), the source of the glutamine is the extrarenal tissues, and it is evident that glutamine must permeate the inner mitochondrial membrane to gain access to the glutaminases. The situation is probably similar in kidney tissues that possess glutamine synthetase (e.g. in the rat), since the synthetase activity is largely associated with the endoplasmic reticulum (Wu, 1963*b*). Glutamine is rapidly deamidated and oxidized by isolated kidney mitochondria (Kovačević *et al.*, 1970; Hird & Marginson, 1968), and it is clear therefore that glutamine is a highly permeable substrate. However, there is no evidence in the literature to suggest that glutamine permeation is carrier-mediated, and there are no recognized parameters regulating the influx of glutamine into the mitochondrial matrix. Glutamate has been recognized for some time as a potential feedback regulator of glutaminase activity in kidney (see Lund *et al.*, 1970) and, in this regard, the permeability of the inner membrane to glutamate must be considered as a possible factor influencing the glutamate concentration in the vicinity of the glutaminases. An important question is whether a limiting rate of glutamate oxidation does cause an accumulation of glutamate in the intramitochondrial compartment or whether, under these conditions, the glutamate formed from glutamine is rapidly transported into the cytoplasm. The problem is further complicated by the existence (*in vitro*) of two pathways of glutamate oxidation: via glutamate dehydrogenase (Braunstein, 1957) and via glutamate–oxaloacetate transaminase. The latter pathway has been designated the transaminase pathway (Borst, 1962), and is generally considered to be the principal route of glutamate oxidation in mitochondria from liver (De Haan *et al.*, 1967), kidney (Krebs & Bellamy, 1960) and heart (Borst & Slater, 1960).

The present paper examines the mechanism of the permeation of glutamine and glutamate in pig renal-cortex mitochondria, and relates this information to the pathway of glutamine oxidation in this tissue.

Methods

Isolation of mitochondria

Pig renal-cortex mitochondria were prepared as described in the preceding paper (Crompton *et al.*,

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1973) in a medium containing 0.25M-sucrose, 3mM-Tris-HCl and 1mM-EGTA [ethanedioxybis(ethylamine)tetra-acetic acid], pH7.4. The final mitochondrial pellet was suspended in preparation medium (approx. 100mg of mitochondrial protein/ml).

Measurement of O_2 consumption

The rate of O_2 uptake by isolated mitochondria was measured with an oxygen electrode (Chappell, 1964).

Measurement of NH_3 formation

The rate of NH_3 production, used to monitor the glutaminase activity, was measured by using an NH_4^+-selective electrode as described in the preceding paper (Crompton *et al.*, 1973).

Determination of the end products of the metabolism of glutamine and glutamate

Mitochondria (containing about 10mg of protein) were incubated at 25°C in the presence of either glutamine (10mM) or glutamate (10mM) in the following reaction medium: choline chloride (100mM), Tris-HCl (40mM), EDTA (0.5mM), Tris-phosphate (10mM) and ADP (8mM), final pH7.5; the final volume was 10ml. After an incubation period of 3–12min, a portion (1.5ml) of the mixture was withdrawn, and the mitochondria were sedimented in an Eppendorf bench centrifuge (model 3200) operating at 15000rev./min for 2min. The supernatant was removed and rapidly mixed with 0.05ml of 6M-$HClO_4$. The precipitated protein was removed by centrifugation, and the solution was neutralized with 1M-K_3PO_4. After the mixture had been chilled in ice for 30min, the $KClO_4$ was removed by centrifugation. The supernatant was assayed for certain metabolites as indicated below. Blank values were obtained by centrifuging a portion of the incubation mixture at the beginning of the incubation period.

Metabolites were assayed as described by the following authors: α-oxoglutarate, Bergmeyer & Bernt (1963); malate, Hohorst (1963); oxaloacetate, Hohorst & Reim (1963); aspartate, Pfleiderer (1963); glutamate, Bernt & Bergmeyer (1963). Glutamine was assayed by conversion into glutamate. Samples (0.1ml) were incubated with 50mM-potassium acetate, pH5.0 (1.0ml), and 0.2mg of glutaminase (Sigma Chemical Co., St. Louis, Mo., U.S.A.) for 30min at 25°C. After this time the solutions were neutralized (approximately) with 3M-KOH, and the glutamate contents were determined.

Measurement of the distribution of labelled metabolites across the inner mitochondrial membrane

The rate of influx of metabolites into the mitochondrial matrix and the steady-state concentrations of these metabolites in the matrix compartment, under various conditions, were measured by using labelled compounds. The method used is described below.

Mitochondria (100μl, containing about 10mg of protein) were incubated at 25°C in a medium (500μl) containing choline chloride (50mM), Tris-HCl (100mM), Tris-phosphate (10mM), rotenone (1μg) and the U-^{14}C-labelled metabolite (approx. 1μCi). After an incubation period, reactions were stopped by sedimenting the mitochondria from the reaction medium into a fixation layer. This was carried out in glass centrifuge tubes (Fig. 1) containing the following layers: layer A (50μl), i.e. the fixation medium, contained sucrose (10%, w/v) and $HClO_4$ (20mM); layer B (24μl) was prepared from reaction medium (20μl; the same composition as used in the incubation mixtures) plus mitochondrial suspension medium (4μl; see 'Isolation of mitochondria'). The composition of layer B was identical therefore with the composition of the incubation mixtures, except that layer B did not contain mitochondria. Layer C was a sample (50μl) of the incubation mixture. The procedure entailed preparing a number of centrifuge tubes containing layers A and B in advance, and

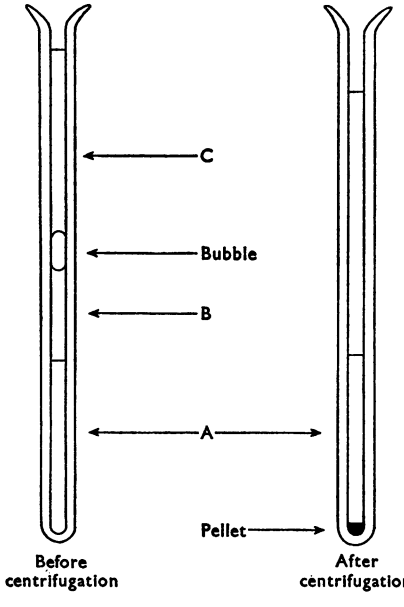


Fig. 1. *Centrifuge tubes used to sediment mitochondria into a fixation layer*

introducing the samples of incubation mixture into the tubes with a small bubble separating the sample layer, i.e. C, from layer B. After this, the tubes were immediately centrifuged in a Beckman Microfuge (model 152) at 12000rev./min for 2min; this caused the bubble to rise and mix the layers B and C. The incubation times reported in the results refer to the time-period from initiation of the reaction by addition of the mitochondria to the reaction medium and the start of the centrifugation. The stated incubation times are an underestimate therefore of the true incubation times, which would include the time taken for the mitochondria to sediment into the fixation layer. In fact, experiments showed that about 95% of the mitochondria had penetrated the fixation layer after centrifugation for 25s.

After the centrifugation, the upper layer was carefully removed with a micro-syringe, and the radioactivity content was determined by liquid-scintillation spectrometry, in a scintillation solution prepared as follows: 1200ml of toluene, 800ml of 2-methoxyethanol, 160g of naphthalene and 12g of butyl-PBD [5-(4-biphenylyl)-2-(4-t-butylphenyl)-1-oxa-3,4-diazole; Koch-Light Laboratories Ltd., Colnbrook, Bucks., U.K.]. The mitochondrial pellet was resuspended in the fixation layer after the addition of a solution (5μl) containing the unlabelled metabolite (10mM) to act as carrier. The mitochondrial precipitate was sedimented again, and the radioactivity content of the supernatant was counted in a Packard Tri-Carb liquid-scintillation spectrometer, model 3380.

Parallel experiments were carried out with ^3H_2O and [^{14}C]sucrose to determine the total water-permeable space and the extramitochondrial space sedimented with the mitochondria (Chappell, 1968). It is important to note that the extramitochondrial space includes the space between the inner and outer membranes. Knowledge of these spaces permitted the amount of labelled anion sedimented in the extramitochondrial space to be accounted for and the matrix volume to be calculated.

The method outlined above is essentially the centrifuge technique, pioneered by Werkheiser & Bartley (1956) and thereafter used by a number of workers (e.g. Klingenberg & Pfaff, 1967), which employs an intermediate layer of silicone between the incubation and fixation layers. However, in our attempts to use methods involving a silicone layer, the matrix space sedimented was only 10–15% of the total permeable space. With the method described and careful preparation of the layers, the matrix space comprised 45–50% of the total permeable space. It is important to note that the internal diameter of the centrifuge tubes was small (0.19cm). The use of tubes of larger diameter resulted in some mixing of the fixation and upper layers during centrifugation.

To measure the steady-state concentrations of glutamine and glutamate in the matrix, the radioactive samples were subjected to paper electrophoresis in pyridine–acetic acid–water (1:4:1000, by vol.), pH3.4, at 1000V for 2h (Peterson, 1968). The separated glutamate and glutamine spots were eluted with water and the radioactivity contents measured.

Measurement of mitochondrial swelling and protein

Measurement of swelling was done by recording changes in E_{610} as described by Chappell & Crofts (1966), with a Hilger–Watts recording spectrophotometer. Protein was measured by the method of Gornall *et al.* (1949).

Results

End products of the oxidation of glutamine and glutamate

In the presence of ADP and P_i, pig renal-cortex mitochondria convert added glutamate quantitatively into aspartate (Fig. 2). The fate of added glutamine is shown in Fig. 3. In the absence of added ADP there is a quantitative conversion of glutamine into glutamate, which accumulates in the external medium. On the addition of ADP, glutamine is

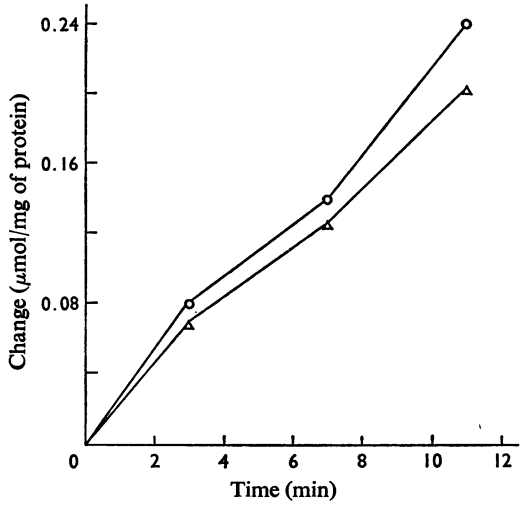


Fig. 2. *Formation of aspartate by kidney mitochondria during the oxidation of glutamate*

Mitochondria were incubated at 25°C and pH7.5 with glutamate (10mM), phosphate and ADP as described in the Methods section. The metabolite changes in the incubation medium were measured. o, Glutamate decrease; △, aspartate increase.

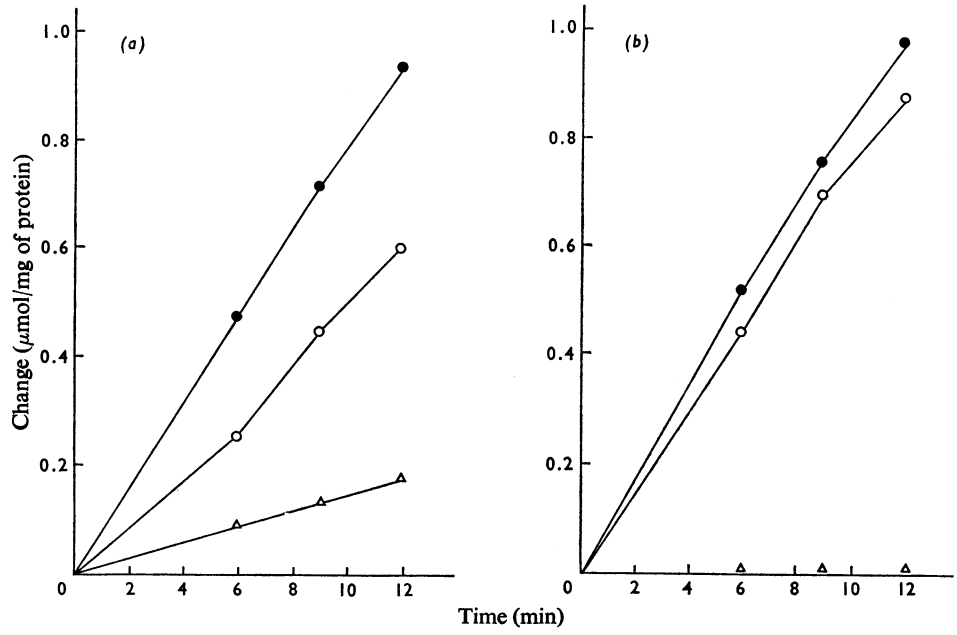


Fig. 3. *Formation of glutamate and aspartate from glutamine by kidney mitochondria in the presence (a) and absence (b) of added ADP*

Mitochondria were incubated at 25°C and pH7.5 with glutamine (10mM) and phosphate in the presence and absence of added ADP as described in the Methods section. The metabolite changes in the incubation medium were measured. ●, Glutamine decrease; ○, glutamate increase; △, aspartate increase.

transformed at about the same rate, but less glutamate accumulates and some aspartate is produced. There was no detectable formation of oxoglutarate, oxaloacetate or malate with either glutamate or glutamine as substrate. These results indicate that, under these experimental conditions, both glutamine and glutamate are oxidized predominantly by the glutamate–oxaloacetate transaminase pathway rather than via glutamate dehydrogenase. The conversion of glutamine into glutamate in pig renal-cortex mitochondria is catalysed by two glutaminase isoenzymes (Crompton *et al.*, 1973). The proposed route of the oxidation of glutamine and glutamate is presented below.

Glutamine → glutamate + NH_3
Glutamate + oxaloacetate → oxoglutarate + aspartate
Oxoglutarate + 3 [O] → oxaloacetate + CO_2 + H_2O

If this pathway is assumed to operate, the rates of oxygen consumption during the oxidation of glutamine and glutamate may be converted into rates of substrate utilization (Table 1). It is evident that the oxidation of glutamate (15nmol/min per mg) produces relatively little NH_3 (about 3nmol/min per mg), which indicates that little glutamate is oxidized by glutamate dehydrogenase, and is consistent with the transaminase pathway providing the principal route of glutamate oxidation as indicated by Fig. 2. Table 1 also shows that the rate of production of NH_3 from glutamine (96nmol/min per mg) is about fivefold that of the rate of glutamine oxidation (18nmol/min per mg), which suggests that the rate of glutamate production from glutamine is about fivefold faster than the rate of glutamate oxidation. This conclusion is in agreement with the relative amounts of glutamate and aspartate recovered after various periods of glutamine oxidation (Fig. 3*a*).

If glutaminase is assayed by measuring the disappearance of glutamine (Fig. 3), no effect on the activity is detected by adding ADP to the incubation medium. However, this technique would not be expected to reveal small changes in glutaminase activity. An ADP effect is detected if the activity is monitored continuously by the rate of production of NH_3. Thus Table 1 shows a small stimulation of glutaminase activity (about 14%) on addition of ADP; in five separate experiments of this kind the stimulation by ADP varied between 8 and 15%. This

Table 1. *Rates of ammonia production and oxygen consumption during oxidation of glutamine and glutamate*

Mitochondria (5.3 mg of protein) were incubated at 25° C in a reaction medium (final volume, 8.0 ml) containing choline chloride (100 mM), Tris–HCl (40 mM), Tris–phosphate (10 mM), EDTA (0.5 mM), bovine plasma albumin (0.1%, w/v) and KCl (50 μM). The final pH was 7.5. Additions: ADP (Tris salt; 1 mM), glutamine (10 mM) and glutamate (Tris salt, 10 mM).

	Successive additions							
Production of NH_3 (nmol/min per mg of protein)	None	16			None	16		
	Glutamine	100			Glutamate	18		
	ADP	112			ADP	19		
Production of NH_3 (nmol/min per mg of protein for added substrate)		96				3		
Uptake of O_2 (nmol/min per mg of protein)	None	1	None	1	None	1	None	1
	Glutamine	4	ADP	3	Glutamate	3	ADP	3
	ADP	30	Glutamine	28	ADP	25	Glutamate	25
Uptake of O_2 (nmol/min per mg of protein for added substrate)				25				22
Substrate oxidation (nmol/min per mg of protein)				17				15

stimulation is very small in comparison with the sevenfold stimulation of the rate of glutamate oxidation observed after addition of ADP under the same experimental conditions (Table 1). It is clear therefore that a large increase in the rate of glutamate oxidation results in only a very small increase in glutaminase activity.

Mitochondrial swelling in glutamine and glutamate

During glutamine deamidation, ammonium glutamate permeates rapidly from the intramitochondrial compartment into the external medium (Fig. 3). The inner mitochondrial membrane is very permeable to NH_3 (Chappell & Haarhoff, 1967); with regard to the permeation of glutamic acid, there seemed to be two obvious possibilities: (*a*) an electroneutral exchange of the glutamate anion for hydroxyl ion, or (*b*) an active efflux of protons (driven by respiration) accompanied by a charge-compensating electrogenic (electrophoretic) efflux of the glutamate anion. The rate of glutamate–hydroxyl ion exchange was examined by observing the rate of mitochondrial swelling in iso-osmotic ammonium glutamate (Chappell & Haarhoff, 1967). The possibility of an electrogenic permeation of the glutamate anion was evaluated in two ways: first, by measuring the rate of swelling in iso-osmotic potassium glutamate in the presence of valinomycin, which permits a rapid electrogenic influx of K^+ (Henderson *et al.*, 1969); secondly, by monitoring the rate of swelling in iso-osmotic ammonium glutamate in the presence of carbonyl cyanide phenylhydrazone, which allows electrogenic proton permeation down the proton gradient created by the influx of NH_3. The ion movements involved in these two techniques have been reported more fully in the preceding paper (Crompton *et al.*, 1973), demonstrating the electrogenic nature of nitrate influx.

Fig. 4 shows the swelling of kidney mitochondria in iso-osmotic glutamate solutions under the conditions described above. It is evident that, in all cases, the rate of mitochondrial swelling was very slow, indicating that glutamate does not permeate at an appreciable rate either by exchange with hydroxyl ion or by electrogenic uniport of the glutamate anion.

Kidney mitochondria swell moderately rapidly when suspended in 180 mM-glutamine, i.e. approaching iso-osmotic conditions (Fig. 4). However, a small decrease in glutamine concentration produces a relatively large decrease in the rate and extent of swelling. The disproportionate nature of this effect renders interpretation of the results very difficult; it is possible that extremely high concentrations of glutamine have a deleterious effect on the integrity of the inner mitochondrial membrane.

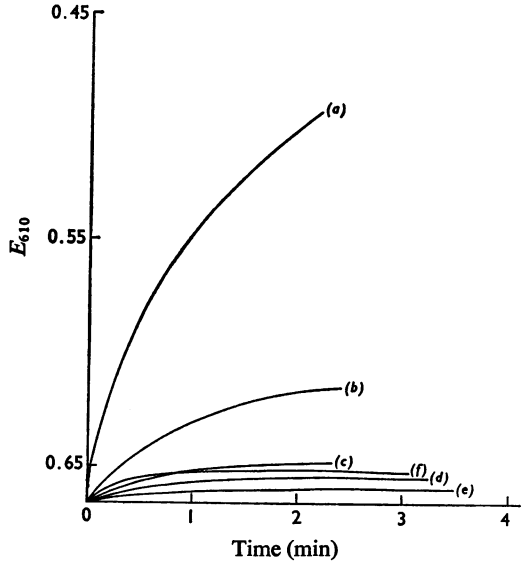


Fig. 4. *Swelling of kidney mitochondria in glutamine and glutamate*

The incubation medium contained (*a*) glutamine (0.18M), (*b*) glutamine (0.15M), (*c*) glutamine (0.13M), (*d*) ammonium glutamate (0.10M), (*e*) ammonium glutamate (0.10M) and carbonyl cyanide phenylhydrazone (3μg) or (*f*) potassium glutamate (0.10M) and valinomycin (1μg). The incubation mixtures (final volume, 2.5ml) contained 1.1mg of mitochondrial protein, and the final solute concentration was adjusted to 0.25 osmolar by the addition of 0.125M-Tris–HCl, pH 7.4. The temperature was 25° C.

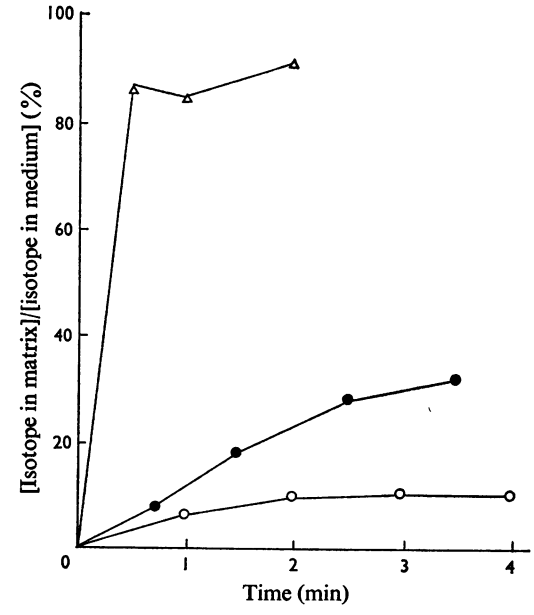


Fig. 5. *Influx of labelled glutamine, glutamate and acetate into kidney mitochondria*

Mitochondria were incubated with 10mM-ammonium [U-^{14}C]acetate (△), or 10mM-ammonium [U-^{14}C]-glutamate (○) or 10mM-[U-^{14}C]glutamine (●) in the presence of rotenone (1μg) and 10mM-phosphate, as described in the Methods section, and sedimented into a fixation layer after the period of incubation indicated. The total radioactivity content of the intramitochondrial compartment was determined as described in the Methods section.

Net rate of influx of ^{14}C from external [U-^{14}C]-glutamine and [U-^{14}C]glutamate

At pH 7.5 in the presence of rotenone, 10mM-tris–phosphate and succinate as respiratory substrate, the glutaminase activity is approx. 110nmol/min per mg of mitochondrial protein, and this is decreased to about 40nmol/min per mg in the absence of succinate (Fig. 6), i.e. the rate of glutamine utilization is decreased by about 70nmol/min per mg. The endogenous concentration of glutamine plus glutamate in isolated pig kidney mitochondria is very low (less than 1mM, estimated by M. Crompton & J. B. Chappell, unpublished work). Therefore, if glutamine permeation is assumed to occur by simple diffusion or by facilitated diffusion not dependent on exchange (i.e. uniport or monoport; Mitchell, 1963), the initial rate of glutamine influx from external 10mM-glutamine in the presence of rotenone but with no added succinate would be predicted to be at least 40nmol/min per mg. Fig. 5 shows the rate of influx of labelled glutamine under these conditions. The intramitochondrial volume of pig kidney mitochondria is about 1.4μl/mg of mitochondrial protein (determined as described in the Methods section), and from this value the initial rate of glutamine influx is about 1.8nmol/min per mg. Thus the rates of glutamine influx, as shown by the rates of glutamine deamidation, are much greater than the rates of glutamine permeation according to a uniport model. Fig. 5 shows that the net rate of permeation of labelled ammonium glutamate is also very slow, which is in agreement with the slow rate of mitochondrial swelling in 100mM-ammonium glutamate (Fig. 4). Ammonium acetate, which is a rapid penetrant (Chappell & Crofts, 1966), was included in these experiments as a control.

Dependence of glutaminase on the energy state of the mitochondria

It was shown by Kovačević *et al.* (1970) that the glutaminase activity of isolated rat kidney mitochondria depends on the energy state of the mito-

chondria. Thus in the presence of rotenone the activity is stimulated two- to three-fold by the addition of succinate, and is inhibited to the original rate by the subsequent addition of an uncoupling agent. In the present study this effect has been investigated with pig renal-cortex mitochondria, by using both production of NH_3 and glutamate formation to monitor the glutaminase activity. The use of the latter technique warrants comment. In the absence of added ADP the amount of glutamine deamidated may be accounted for almost entirely by the glutamate that accumulates in the external medium (Fig. 3*b*). The same stoicheiometric relationship between glutamine disappearance and glutamate accumulation is observed in the presence of rotenone; this experiment was carried out as reported in Fig. 3(*b*), except that rotenone was included in the incubation medium.

As shown in Figs. 6 and 7, the addition of rotenone inhibits the glutaminase activity in intact mitochondria. The subsequent addition of succinate restores the activity to its original value, but the further addition of antimycin or the uncoupling agent carbonyl cyanide phenylhydrazone abolishes the stimulation by succinate.

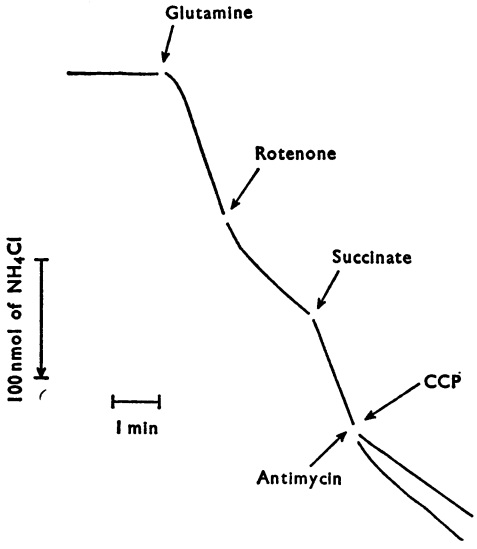


Fig. 6. *Formation of ammonia from glutamine by kidney mitochondria under various conditions*

Mitochondria (about 5mg of protein) were incubated at 25°C and pH7.5 in a medium containing choline chloride(100mM),Tris–HCl(40mM),EDTA(0.5mM), Tris–phosphate (10mM) and KCl (0.05mM); final volume, 8.0ml. Additions: glutamine, 10mM; rotenone, 5μg; succinate, 2mM; carbonyl cyanide phenylhydrazone (CCP), 3μg; antimycin, 3μg.

These effects are observed over a wide range of pH values (Fig. 8). If no additions are made to the standard reaction medium (under these conditions, glutamate, produced from glutamine, may be oxidized), or if rotenone and succinate are present, i.e. in the presence of energy, the glutaminase activity is high and displays an optimum pH of about 8.6. In the presence of rotenone alone, rotenone plus carbonyl cyanide phenylhydrazone, rotenone plus succinate and carbonyl cyanide phenylhydrazone, or rotenone plus succinate and antimycin, i.e. in the absence of energy, the glutaminase activity is low and there are two pH optima; one peak of activity occurs at about pH8.6, as under conditions allowing high glutaminase activity, and another at about pH7.5.

These pH values may be correlated with the pH optima of the P_i-dependent (pH8.5) and P_i-independent (pH7.5) glutaminase isoenzymes in pig renal-cortex mitochondria (Crompton *et al.*, 1973). It is evident that the transition to conditions resulting in low glutaminase activity produces the greatest inhibition at high pH values, suggesting that the P_i-dependent glutaminase becomes inhibited to a

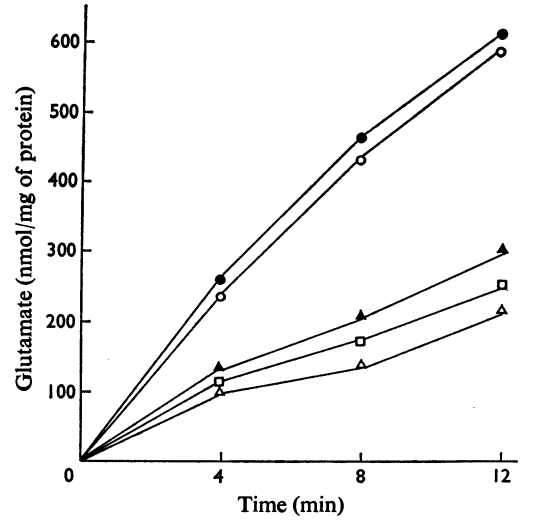


Fig. 7. *Formation of glutamate from glutamine by kidney mitochondria under various conditions*

Mitochondria were incubated at 25°C and pH7.5 with glutamine (10mM) in the absence of added ADP as described in the Methods section. The glutamate content of the incubation medium was measured. Additions; ●, none; □, rotenone (5μg); ○, rotenone (5μg) and succinate (2mM); △, rotenone (5μg), succinate (2mM) and carbonyl cyanide phenylhydrazone (3μg); ▲, rotenone (5μg), succinate (2mM) and antimycin (5μg).

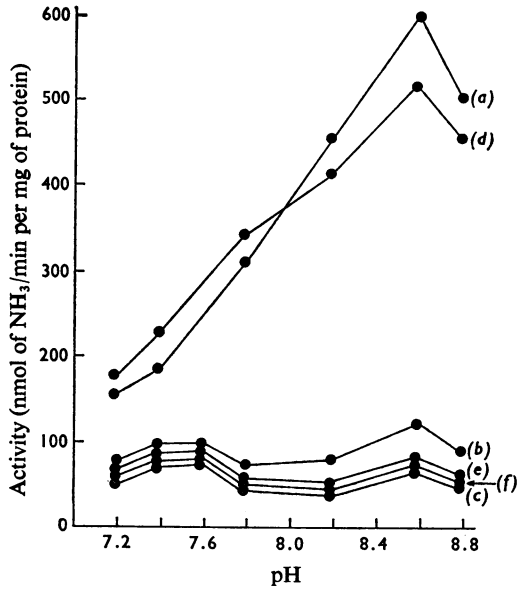


Fig. 8. *pH-dependence of the glutaminase activity of kidney mitochondria under various conditions*

Mitochondria (4mg of protein) were incubated at 25°C in a reaction medium (final volume, 8.0ml) containing choline chloride (90mM), Tris–HCl (50mM), Tris–phosphate (10mM), EDTA (0.5mM), KCl (0.05mM) and glutamine (5mM). Additions: (*a*) none; (*b*) 5μg of rotenone; (*c*) 5μg of rotenone and 3μg of carbonyl cyanide phenylhydrazone; (*d*) 5μg of rotenone and 2mM-succinate; (*e*) 5μg of rotenone, 2mM-succinate and 5μg of antimycin; (*f*) 5μg of rotenone, 2mM-succinate and 3μg of carbonyl cyanide phenylhydrazone.

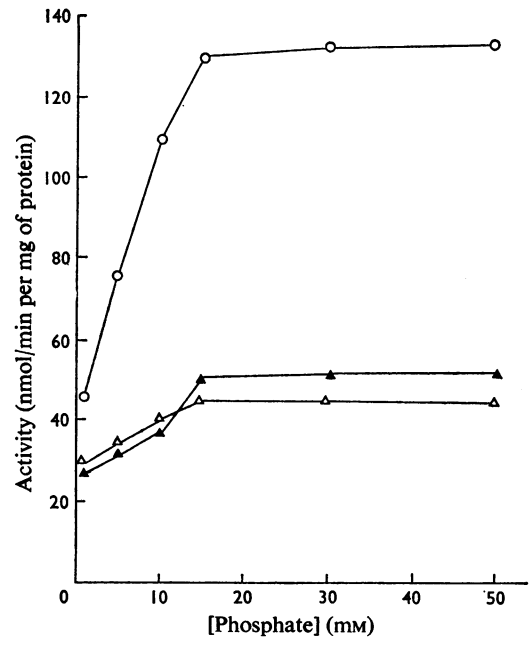


Fig. 9. *Effect of phosphate on the glutaminase activity of kidney mitochondria*

Mitochondria (5mg of protein) were incubated at 25°C and pH7.5 in a reaction medium (final volume, 8.0ml) containing choline chloride (120mM), Tris–HCl (20mM), EDTA (0.5mM), KCl (0.05mM), rotenone (5μg) and various concentrations of phosphate. Additions: △, none; ○, succinate (2mM); ▲, succinate (2mM) and antimycin (5μg).

much greater extent than the P_i-independent isoenzyme when an energy source is removed.

The seemingly greater stimulation of the P_i-dependent glutaminase by the provision of an energy source suggested that this effect might be due to an energy-linked accumulation of phosphate (McGivan & Klingenberg, 1971), since in broken mitochondria the P_i-dependent glutaminase requires about 300mM-phosphate for full activity (Klingman & Handler, 1958). Fig. 9 shows that in intact mitochondria maximum stimulation is effected by about 30mM-phosphate, in both the presence and the absence of an energy source. Thus the inhibition of glutaminase activity produced by the inclusion of respiratory-chain inhibitors or an uncoupling agent is not relieved by an increased concentration of phosphate. It seems therefore that the energy-linked stimulation of glutaminase activity is not due to an accumulation of phosphate. Kovačević *et al.* (1970), working with rat kidney mitochondria, have presented results that support this conclusion. In the presence of rotenone, succinate and 30mM-phosphate, the glutaminase activity was inhibited 80% by the addition of an uncoupling agent. Under similar conditions the intramitochondrial phosphate concentration decreased by only 10%.

Dependence of intramitochondrial concentrations of glutamine and glutamate on the energy state of the mitochondria

The conversion of glutamine into ammonium glutamate in pig renal-cortex mitochondria takes place in the intramitochondrial compartment (Crompton *et al.*, 1973). The rate of this reaction at pH7.5 in the presence of rotenone is about 100nmol/min per mg of mitochondrial protein. The total content of glutamine and glutamate in isolated pig renal-cortex mitochondria is less than 1nmol/mg of

mitochondrial protein (M. Crompton & J. B. Chappell, unpublished work). Under these conditions it is reasonable to assume that the ^{14}C content of labelled glutamine added to the external medium would equilibrate rapidly with the intramitochondrial pools of glutamine and glutamate. In the following determinations it has therefore been assumed that after an incubation period of 30s the specific radioactivity contents of the intramitochondrial and extramitochondrial pools of glutamine and glutamate are equal. Since the specific radioactivity content of the added glutamine and glutamate is known, this assumption enables the intramitochondrial content of glutamine and glutamate to be estimated by measuring the amount of radioactivity in the glutamine and glutamate of the intramitochondrial compartment.

Fig. 10 shows the intramitochondrial concentrations of glutamine and glutamate during glutamine deamidation in the presence of rotenone, and in the presence and absence of a source of energy. With succinate added (i.e. energy source present), the glutamate concentration in the mitochondrial matrix is about 0.38–0.45mM (Fig. 10*a*). In the absence of added succinate and with antimycin present (to inhibit the oxidation of endogenous succinate), or if succinate oxidation is uncoupled by carbonyl cyanide phenylhydrazone (i.e. energy source absent), the glutamate concentration in the matrix is increased by about 53–66%. The corresponding changes in the intramitochondrial glutamine concentration are shown in Fig. 10(*b*). The glutamine concentration in the presence of succinate was about 0.4–0.6 mM. However, the addition of carbonyl cyanide phenylhydrazone or the replacement of added succinate by antimycin resulted in a decrease in the glutamine concentration of about 47–58%. Thus the provision of energy by succinate oxidation causes the matrix glutamine to increase and the matrix glutamate to decrease. Further, the increase in the amount of intramitochondrial glutamine is approximately equal to the decrease in intramitochondrial glutamate; this is evident from Fig. 10(*c*), which shows that the total concentration of glutamine and glutamate in the matrix compartment is about the same in the presence and absence of energy.

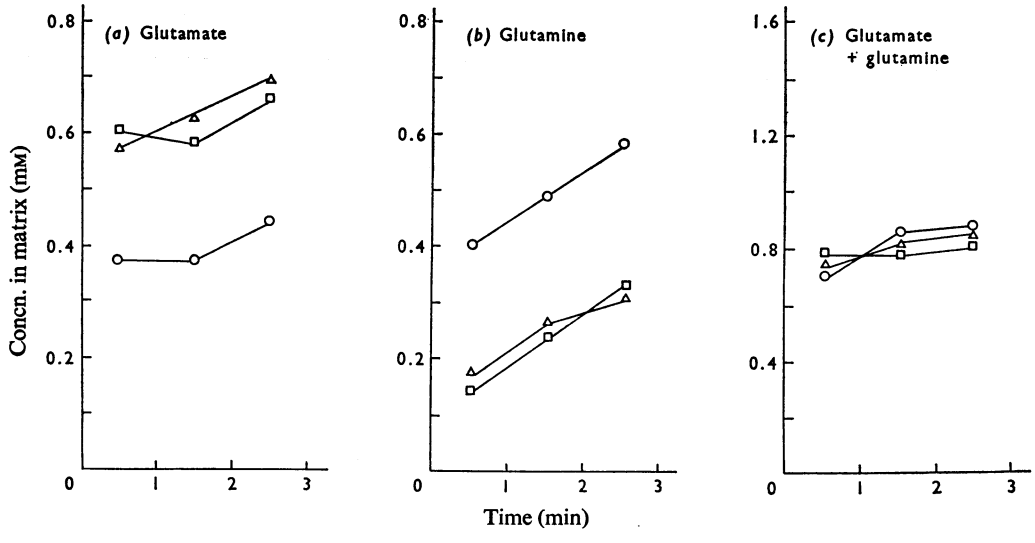


Fig. 10. *Concentrations of glutamine and glutamate in the matrix compartment of respiration-inhibited kidney mitochondria in the presence and absence of an energy source*

Mitochondria were incubated in a reaction medium containing [U-^{14}C]glutamine (1mM, 2μCi/μmol) and [U-^{14}C]glutamate (1mM, 2μCi/μmol), and sedimented into a fixation layer after the period of incubation indicated. Glutamine and glutamate were separated by paper electrophoresis. The radioactivity content of the glutamine and glutamate of the intramitochondrial compartment was determined as described in the Methods section, and these values were used to calculate the intramitochondrial glutamine and glutamate concentrations. Additions: △, antimycin (1μg); ○, succinate (2mM); □, succinate (2mM) and carbonyl cyanide phenylhydrazone (1μg).

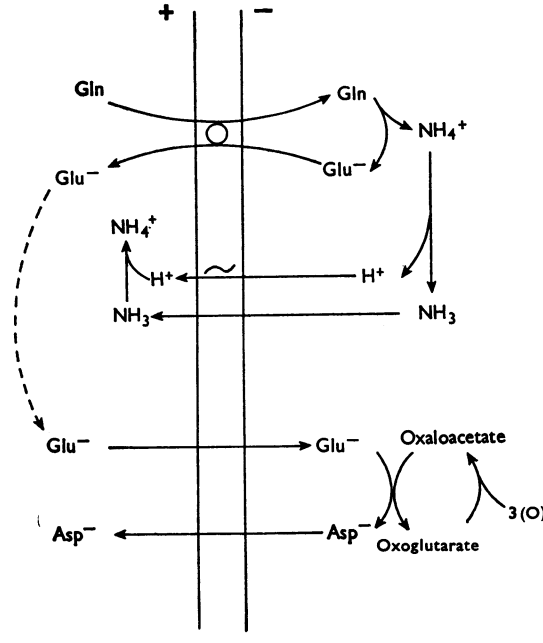
Discussion

At physiological pH values, glutamate carries a net negative charge. To preserve electroneutrality, the efflux of the glutamate anion during glutamine oxidation requires a parallel movement of protons (or hydroxyl ions in the opposite direction), either as an integral element of the transport process (e.g. hydroxyl ion–glutamate exchange) or independently (e.g. respiration-dependent proton efflux) with the glutamate anion moving down a pre-existing gradient of electrical potential. However, experiments designed to measure the movement of glutamate according to either of these mechanisms detected only an extremely slow net rate of permeation. Further, the net rate of influx of ^{14}C from externally added labelled glutamine at physiological concentration is very slow, although, since glutamine has no net charge, there is no theoretical requirement for movement of another ion.

When glutamine is presented as substrate for kidney mitochondria, in the absence of added ADP, there is an almost quantitative conversion of glutamine into glutamate, which accumulates in the external medium, i.e. there is an overall exchange of glutamine for glutamate. The observations discussed above suggest that during this exchange the glutamine influx may be strictly coupled to the glutamate efflux. This postulate would account for the inability of kidney mitochondria to show a net uptake of either glutamine or glutamate, in spite of a high capacity for rapid glutamine–glutamate exchange. An obligatory exchange of this nature is most readily visualized in terms of a single carrier (anti-porter; Mitchell, 1963) catalysing an exchange of glutamine for glutamate on a strict 1:1 basis as shown in Scheme 1. Further evidence in support of this hypothesis is discussed below.

The glutaminase activity of pig renal-cortex mitochondria is due mainly to the presence of the phosphate-dependent isoenzyme, which is strongly inhibited by glutamate (Crompton *et al.*, 1973). Thus any appreciable accumulation of glutamate in the intramitochondrial compartment would result in marked inhibition of the P_i-dependent glutaminase. However, the addition of ADP, allowing glutamate oxidation, makes little difference to the glutaminase activity, indicating that a limiting rate of glutamate oxidation by the transaminase pathway does not lead to an accumulation of glutamate, formed from glutamine, in the mitochondrial matrix. This observation is readily explained by the present hypothesis, which maintains that the influx of glutamine and the efflux of glutamate are strictly coupled.

The chemiosmotic hypothesis (Mitchell, 1966) asserts that substrate oxidation by coupled mitochondria produces an electrical potential across the inner membrane, negative with respect to the matrix. The charge difference between glutamine and



Scheme 1. *Proposed pathway of glutamine oxidation in the presence of ADP and phosphate in pig renal-cortex mitochondria*

glutamate at physiological values of pH leads to the prediction that an obligatory exchange between these metabolites would be electrogenic, and, in terms of the chemiosmotic hypothesis, that energy-linked substrate oxidation would cause glutamate expulsion from the matrix and an equivalent influx of glutamine. It is important to stress that the postulated glutamine–glutamate anti-port requires that the sum of the concentrations of glutamine and glutamate in the mitochondrial matrix should remain constant despite variations in the glutamine and the glutamate concentrations. The measurements made of the intramitochondrial glutamine and glutamate concentrations in the presence and absence of an energy source satisfy these predictions, and are therefore readily interpreted in terms of a membrane potential (negative inside) coupled to substrate oxidation and electrogenic glutamine–glutamate anti-port. This type of metabolite exchange is exemplified by the adenine nucleotide translocase (Klingenberg, 1970).

The glutaminase activity of mitochondria from rat kidney and pig kidney is due to the presence of P_i-dependent and P_i-independent isoenzymes (Katunuma *et al.*, 1966; Crompton *et al.*, 1973). Further, the glutaminase activity of intact mitochondria from both sources is dependent on the energy state. The stimulation of glutaminase by the provision of

energy was previously attributed to an energy-dependent accumulation of phosphate and the necessity for an energy-linked expulsion of protons to preserve the pH of the mitochondrial matrix (Kovačević *et al.*, 1970). In the latter case it was assumed that, in the absence of rapid proton efflux, the outward diffusion of NH_3 (from NH_4^+) would decrease the value of the intramitochondrial pH. The present results show, however, that the inhibition of glutaminase in the absence of an energy source is not relieved either by increasing the phosphate concentration or by adding the uncoupling agent carbonyl cyanide phenylhydrazone (Fig. 8; rotenone plus carbonyl cyanide phenylhydrazone), which catalyses rapid proton permeation across the inner membrane (Mitchell, 1966). It is unlikely therefore that changes in either the phosphate concentration or the pH of the intramitochondrial compartment are responsible for the energy-linked variation in glutaminase activity. A more acceptable explanation is provided by the observed effects of energy on the intramitochondrial concentration of glutamine and glutamate (Fig. 10) and the apparent selective inhibition of the P_i-dependent glutaminase in the absence of energy (Fig. 8). Thus, when energy is available, the internal glutamine concentration is relatively high and the glutamate concentration is low. These conditions would favour the P_i-dependent glutaminase, which has a high K_m for glutamine (5mM) and is inhibited strongly by glutamate. In the absence of energy, the internal glutamate concentration increases at the expense of the glutamine concentration, which would inhibit the P_i-dependent glutaminase but cause little decrease in the activity of the P_i-independent glutaminase, since this isoenzyme is not inhibited by glutamate, and has a relatively low K_m for glutamine (0.4mM; Crompton *et al.*, 1973).

An obligatory coupling between the transport of glutamine and glutamate would mean that glutamate formed from added glutamine is necessarily expelled into the external medium. It follows that externally added glutamine and glutamate would be oxidized by the same pathway. In fact, both glutamine and glutamate are oxidized by the transaminase pathways and at approximately the same rate.

In pig kidney mitochondria at pH7.4, the P_i-dependent glutaminase has about fivefold the activity of the P_i-independent isoenzyme (Crompton *et al.*, 1973), and it is reasonable to assume that, during glutamine oxidation, most of the glutamine is deamidated by the P_i-dependent glutaminase. Rat kidney mitochondria, like pig kidney mitochondria, oxidize externally added glutamate predominantly by the transaminase pathway (Kun *et al.*, 1964). However, rat kidney mitochondria oxidize glutamine two to three times as fast as glutamate (Kovačević *et al.*, 1970) and, in spite of this, produce aspartate from glutamine at a slower rate than with glutamate as substrate (Kovačević, 1971). An important distinction between rat kidney and pig kidney mitochondria is that the former contain about tenfold the activity of P_i-independent glutaminase, relative to P_i-dependent glutaminase, in comparison with pig kidney mitochondria (Katunuma *et al.*, 1966). Further, the K_m values for glutamine of the P_i-dependent and P_i-independent glutaminases are 40mM and 4mM respectively, i.e. about tenfold the corresponding K_m values for the pig kidney isoenzymes (Crompton *et al.*, 1973). This suggests that, at physiological concentrations, glutamine is deamidated to a much greater extent by the P_i-independent glutaminase in rat kidney than in pig kidney. On this basis it seems possible that the P_i-independent glutaminase permits access of glutamate to an oxidation pathway other than the transaminase pathway, and which is not available to externally added glutamate. This would imply that the glutamine–glutamate anti-porter operates only with respect to the P_i-dependent glutaminase and the transaminase pathway.

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