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Choline Kinase in *Cuscuta reflexa*

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1. Choline kinase is a mitochondrial enzyme in *Cuscuta reflexa*. It can be solubilized from the particles by treatment with 350mM-sodium chloride, or by freezing and thawing. 2. Choline kinase of *C. reflexa* was purified by starting from the crude mitochondrial fraction. A 33–52% recovery of the enzyme, on the basis of the activity in the original homogenate, in 1200–2250-fold enrichment, was effected. 3. The purified preparation of choline kinase had a sigmoid saturation curve with respect to choline, with a Hill number of 2.3, and was inhibited by ADP (competitive in nature and allosteric in binding, with a Hill number of 2.7) and by phosphorylcholine (non-competitive and non-allosteric). The kinetic characteristics of the enzyme were consistent with the *K* type allosteric model of Monod *et al.* (1965). 4. The enzyme was desensitized, with respect to choline regulation, by prolonged storage in the cold, was activated significantly on warming before assay and was inactivated by high concentrations of sodium chloride. 5. The significance of allostery in choline kinase in relation to the intracellular regulation of phospholipid synthesis is discussed.

Although choline kinase (EC 2.7.1.32) has an extensive distribution, relatively few studies exist on the localization of the enzyme, its purification and properties. It has been reported to be essentially a soluble enzyme in rabbit brain (McCaman & Cook, 1966), Ehrlich ascites cells (Sung & Johnstone, 1967) and onion stem (Morre *et al.*, 1970). Whereas there was no evidence in these experiments that the microsomal fraction was a site for choline phosphorylation, the results with animal tissue (McCaman & Cook, 1966; Sung & Johnstone, 1967) did not exclude the mitochondria as an additional, though much less active, site.

The enzyme has been purified from yeast (Wittenberg & Kornberg, 1953), rape seed (Ramasarma & Wetter, 1957) and spinach (Tanaka *et al.*, 1966), and from rabbit brain (McCaman, 1962). Only limited enrichment was achieved by these workers.

Our interest in *Cuscuta* as a source of choline kinase arose from the fact that the filaments of the parasite contained a high concentration of phospholipid (Setty *et al.*, 1969). The present paper relates to the localization of choline kinase in *Cuscuta*, its purification and properties. A preliminary report on some of these aspects has appeared (Setty & Krishnan, 1970).

Materials and Methods

Plant source

The filaments of *Cuscuta reflexa* Roxb. were harvested fresh from growth on *Lantana camara* Linn.

Subcellular fractionation by differential centrifugation

The parasite tissue (usually 30g) was ground in a chilled porcelain mortar containing 50mM-cysteine hydrochloride, freshly neutralized, 10mM-tris–HCl buffer, pH9.0, 15mM-$MgCl_2$ and 500mM-mannitol, the suspension squeezed through four thicknesses of muslin and the filtrate diluted with fresh medium to 30% (w/v) concentration. The homogenate was centrifuged at 1000*g* for 20min in an International model PR-2 refrigerated centrifuge and the residue (which was devoid of choline kinase activity) discarded. The supernatant was centrifuged at 5000*g* for 20min and the residue (crude mitochondrial fraction I) dispersed in 5ml of the above medium and resedimented at 5000*g* for 20min. The first 5000*g* supernatant, mixed with the wash fluid, was centrifuged at 12000*g* for 20min and the residue (crude mitochondrial fraction II) dispersed in 5ml of the medium and resedimented at 12000*g* for 20min. Centrifugations at 5000 and 12000*g* were done in the PR-2 machine with a no. 296 multispeed attachment. The supernatant, mixed with the wash, was centrifuged at 105000*g* for 60min in the no. 40 rotor of a Beckman Model L ultracentrifuge to sediment the microsomal fraction, and the supernatant was decanted off. A hand-operated Potter–Elvehjem homogenizer was used for resuspending the particulate fractions during the washing operation. Each washed particulate fraction, sedimented at 5000 or 12000*g*, was resuspended in 5ml of the medium used for cell dispersion; a 10ml volume was used for the 105000*g* particulate fraction.

In other experiments, the total mitochondrial

particles were collected as a single fraction by centrifugation at 16000*g* for 20min, after the 1600*g*-20min fraction had been discarded.

Biochemical characterization of the particulate fractions by the use of markers

Succinate dehydrogenase (EC 1.3.99.1) activity was measured by the method of Ells (1959), as modified by Hiatt (1961). Cytochrome *c* oxidase (EC 1.9.3.1) activity in the 5000*g* and 12000*g* particulate fractions was measured manometrically as described by Lieberman (1960). The method of Hers (1964) was used for the glucose 6-phosphatase (EC 3.1.3.9) assay.

RNA was determined colorimetrically as phosphorus (Fiske & SubbaRow, 1925) in the fractions isolated by the technique of Schmidt & Thannhauser (1945).

Solubilization of choline kinase

The isolated mitochondrial preparation was mixed with NaCl (final concn. 350mM) and kept dispersed with the aid of a Potter–Elvehjem homogenizer at 0°C for 15–20min. At the end of the treatment, the suspension was centrifuged at 16000*g* for 20min. The techniques of freezing and thawing and of detergent treatment were also tested for enzyme solubilization.

Enzyme enrichment

In a typical experiment, 90g of filaments was ground in a mortar with the supplemented mannitol medium to give 300ml of a homogenate (fraction I). A crude mitochondrial fraction (fraction II) was isolated between 1600 and 16000*g*. In later experiments the collection of the mitochondrial fraction was between 1000 and 5000*g*, though about 25–33% of the total enzyme activity was lost in the 5000*g* supernatant. The mitochondrial preparation was treated with 350mM-NaCl and the extract (fraction III) collected by centrifugation at 16000*g* for 20min. Fraction III was heated at 60°C for 1min, chilled and the suspension centrifuged at 16000*g* for 20min, and the supernatant (fraction IV) was kept.

Powdered analytical-grade $(NH_4)_2SO_4$ was added to fraction IV at 0–4°C, with constant stirring to 45% (277g/litre) saturation. After storage overnight at 0–4°C, the precipitated protein was collected by centrifugation at 16000*g* for 30–40min and taken up in 5ml of 50mM-cysteine hydrochloride–5mM-$MgCl_2$. The suspension was dialysed against a total of 200vol. of water containing 10mM-cysteine hydrochloride – 5mB-$MgCl_2$ for 6h at 0–4°C, in two changes. The resulting suspension was centrifuged at 16000*g* for 30min and the supernatant (fraction V) kept.

Fraction V (5ml, containing approx. 4mg of protein) was mixed with 1.7ml of 300mM-KH_2PO_4–K_2HPO_4 buffer, pH7.2, to give a final concn. of 10mM, pH7.2, and loaded on a DEAE-cellulose column (12cm×1cm), equilibrated with 10mM-KH_2PO_4–K_2HPO_4 buffer, pH7.2, and adjusted to a flow rate of 40ml/h. Protein fractions were eluted from the column batchwise by using 30ml each of 50, 100, 200 and 300mM-KH_2PO_4 – K_2HPO_4 buffer, pH7.2, and collected in 10ml fractions. The active fraction, eluted with 100mM-KH_2PO_4 – K_2HPO_4 buffer, pH7.2, was designated as fraction VI.

Determination of choline kinase activity

The assay mixture for determination of choline kinase activity in fractions I–V consisted, in a total volume of 0.375ml, of 7.5μmol of $MgCl_2$, 29μmol of tris–HCl buffer, pH9.0, 1.5μmol of ATP (Sigma Chemical Co., St. Louis, Mo., U.S.A.), 12.5μmol of cysteine, 1.25μmol of choline chloride (E. Merck A.G., Darmstadt, Germany), 0.5μmol of NaF and 0.1ml of appropriately diluted enzyme preparation. The reaction was started by adding choline chloride and, after incubation for 60min at 30°C, was terminated by the addition of 0.4ml of 6% (w/v) $HClO_4$. The residual choline was determined by the periodide precipitation method of Appleton *et al.* (1953).

In the purification schedule, fraction VI was assayed as described above, but for study of the properties of the purified enzyme modifications were necessitated. Cysteine and NaF were omitted, $MgCl_2$ and ATP were used at 1.5μmol each and tris–HCl buffer, pH8.5, was used. The incubation period was shortened to 30min.

Unit of activity and specific activity. One unit of enzyme activity was the disappearance of 1μmol of choline/h at 30°C under the conditions of assay. Specific activity was expressed in units/mg of protein in enzyme preparation.

Determination of protein. Protein in the subcellular fractions obtained in the localization study and in fractions I–V during enzyme enrichment was determined by precipitating with trichloroacetic acid and treating with the Folin–Ciocalteu reagent (Lowry *et al.*, 1951), with modifications described by Khanna *et al.* (1969). A dry sample of bovine serum albumin (Sigma) was used as standard. Protein was determined in fraction VI by spectrophotometry as described by Kalckar (1947), without precipitation with trichloroacetic acid.

Results

Localization of choline kinase activity

Some 80% or more of the enzyme activity of the homogenate was associated with the particles sedimenting at 5000*g*, with 5–7-fold enrichment. One-

Table 1. *Localization of choline kinase in C. reflexa grown on L. camara*

A homogenate in buffered and suitably supplemented mannitol medium was subjected to differential centrifugation as indicated in the Table. The particulate fractions were resuspended in fresh medium and assayed without any special treatment. Incubation was for 60min at 30°C in the presence of 1.3mM-NaF.

		Protein		Choline kinase activity		
	Total volume (ml)	Total protein (mg)	Recovery (%)	Total activity (units)	Specific activity (units/mg of protein)	Recovery (%)
Whole homogenate, 30% (w/v)	100	185	(100)	471	2.5	(100)
5000*g*, 20min, residue	5	32.5	17.4	412	12.7	87.5
12000*g*, 20min, residue	5	10.4	5.4	118	11	25
105000*g*, 20min, residue	10	20.0	10.8	Nil		
105000*g*, 20min, supernatant	110	97.8	52.3	Nil		

quarter to one-third of the activity of the homogenate was recovered from the 12000*g* particulate fraction, in which the enzyme had the same specific activity as in the 5000*g* fraction. It is not clear whether the slightly higher recovery of the enzymic activity from the particles as compared with the homogenate is attributable to the presence of an inhibitor in the 12000*g* or 105000*g* supernatant. When particles were collected between 1600 and 16000*g*, 70–100% of the activity of the homogenate was recovered in this fraction. The particulate fractions sedimenting at 1000 or 1600*g* or between 12000 and 105000*g*, were devoid of choline kinase activity. No activity was demonstrable also in the 105000*g*, 16000*g* or 12000*g* supernatants. Results obtained in a typical experiment are recorded in Table 1.

Characterization of the particulate fractions

Results obtained in a typical experiment are recorded in Table 2.

Succinate dehydrogenase. The 12000*g* supernatant lacked this activity. The activity recovered from the particulate fractions was nearly equally distributed between the 5000 and 12000*g* fractions, with the specific activity twice as high in the latter as in the former fraction.

Cytochrome c oxidase. Of the cytochrome *c* oxidase activity recovered from the particulate fractions 57–60% was associated with the 5000*g* fraction; the specific activity was 20–30% higher in the 12000*g* particles.

Glucose 6-phosphatase. Of the glucose 6-phosphatase activity recovered from the fractions, the major part was associated with the 12000*g* supernatant.

RNA. Of the RNA recovered from the subcellular fractions, 80% was in the 12000*g* supernatant. RNA recovered in the 5000*g* particulate fraction was more than twice that in the 12000*g* particulate fraction. However, the RNA P/protein ratio among the subcellular fractions was lowest in the 5000*g* (0.096) and highest in the 12000*g* (0.26) particulate fractions. The RNA P/protein ratio in the 12000*g* supernatant fraction (0.219) was slightly higher in the homogenate (0.188) and about twice that in the 5000*g* particulate fraction.

Solubilization of choline kinase from particles

Treatment with sodium chloride. Some 85–100% of the activity associated with the 16000*g* particles passed into the supernatant obtained at 16000*g*, after treatment with NaCl. The residual fraction was devoid of enzymic activity. To test whether a true solubilization had occurred, the NaCl extract of 5000*g* particles obtained in an experiment was centrifuged at 105000*g* for 60min. The entire choline kinase activity associated with the original extract passed into the high-speed supernatant fraction, suggesting the presence of the enzyme in true solution.

Freezing and thawing. A suspension of the 5000*g* particles in the homogenizing medium was kept frozen for 2h at −18°C and thawed in contact with cracked ice (about 20–25min). Immediately after thawing, the suspension, which had almost the same activity as the fresh preparation, was centrifuged at 1600*g* for 20–25min, when the entire activity passed into the supernatant.

Treatment with detergents. Triton X-100, Tween 20, 40, 60 and 80, sodium deoxycholate and cetyltrimethylammonium bromide, tested at concentrations 0.5–1%, interfered in the enzyme assay by giving rise to coloured precipitates with the periodide reagent, the colour being extractable with ethylene dichloride.

Enzyme purification. The enzyme was purified from about a dozen batches of the parasite filaments, from either the 16000 or 5000*g* mitochondrial fraction. The 16000*g* mitochondrial fraction represented, on

Table 2. *Characterization of the subcellular fractions of C. reflexa*

The details of analysis in the various fractions were as reported in the text. A unit of succinate dehydrogenase activity was the conversion of 1 μmol of substrate/min under assay conditions. A unit of cytochrome *c* oxidase activity was the conversion of 1 μmol of substrate/h under assay conditions. A unit of glucose 6-phosphatase activity was the liberation of 1 μmol of phosphate/min under assay conditions.

	Succinate dehydrogenase				Cytochrome *c* oxidase			
	Total volume (ml)	Total activity (units)	Specific activity (unit/mg of protein)	Recovery (%)	Total volume (ml)	Activity (unit/g fresh weight)	Specific activity (unit/mg of protein)	Distribution between particles (%)
Whole homogenate, 30% (w/v)	50	1.13	0.013	(100)	50			
5000*g*, 20min, residue	3	0.648	0.032	60	5	0.101	0.200	60
12000*g*, 20min, residue	3	0.576	0.063	50	5	0.060	0.236	40
12000*g*, 20min, supernatant	50	Nil	Nil	Nil	50			

	Glucose 6-phosphatase				Total RNA P			
	Total volume (ml)	Total activity (units)	Specific activity (unit/mg of protein)	Recovery (%)	Total volume (ml)	(μg/g of fresh tissue)	(μg/μg of protein)	Recovery (%)
Whole homogenate, 30% (w/v)	100	10.9	0.04	(100)	100	51.88	0.14	(100)
5000*g*, 20min, residue	5	2.4	0.07	22	5	6.44	0.09	12.4
12000*g*, 20min, residue	5	4.3	0.34	40	5	2.68	0.26	5.1
12000*g*, 20min, supernatant	110	11.8	0.05	108	110	37.35	0.22	72

the average, a sixfold enrichment and a 80–100% recovery of the activity. With the 5000*g* fraction, enzyme activity was enriched by about sevenfold, with a recovery of about 85%. On treatment with 350mM-NaCl, 86–98% of the enzymic activity in the particles passed into the extract in a fourfold further enriched form.

Heat treatment of the NaCl extract led to 50–70% increase in the specific activity of the enzyme, with 98–123% recovery. In trial experiments, heating for 3min at 60°C caused significant loss of enzymic activity, without contributing any marked enrichment.

In most of the purification trials, the enzyme activity in the mitochondrial extract was largely recovered in the protein fraction obtained on 45% saturation with $(NH_4)_2SO_4$. When the supernatant fraction from 45% saturation with $(NH_4)_2SO_4$ was raised to 60% saturation, the protein precipitated was devoid of choline kinase activity.

On dialysis, the resulting suspension generally retained the entire enzymic activity. On clarification of the suspension by centrifugation at 16000*g* for 20–30min, the enzymic activity passed into the supernatant, usually enriched threefold. The recovery of the enzyme ranged from 94 to 132% of the activity in the homogenate.

Dialysis of the solution against water before chromatography on DEAE-cellulose did not alter the enzymic activity. After adsorption on the DEAE-cellulose column, choline kinase was eluted sharply as a single peak by 100mM-potassium phosphate buffer, pH 7.2, in a 5–9-fold further enriched form and in 50% recovery.

The overall purification was of the order of 1200–2250-fold; the recovery varied from 33 to 52% of the activity in the homogenate.

The results obtained in an experiment with the 5000*g* particles are recorded in Table 3.

Properties of choline kinase

The following experiments were performed with the enzyme fraction eluted from the DEAE-cellulose column. The preparations were stored in cracked ice.

Optimum pH. In tris–HCl buffer in the range pH 7.5–9.0, choline kinase from *Cuscuta* exhibited a sharp pH optimum at 8.5 (Fig. 1).

Effect of thiol group-containing and -blocking agents. The enzyme was preincubated with cysteine (1 and 10mM), β-mercaptoethanol (20mM) and dithiothreitol (50mM) at 30°C for 25min. On assay of fractions, at intervals or at the end of the period, no activation was found. In other experiments, incubation of the enzyme with *p*-chloromercuribenzoate and *N*-ethylmaleimide (0.5 and 50mM respectively) had no effect on the enzymic activity.

It was concluded that choline kinase of *Cuscuta* does not have functional thiol groups.

Test for specificity of nucleotide requirement. To

Table. 3. *Purification of choline kinase from C. reflexa*

The enzyme assay was carried out as for Table 1. Since the assay involved incubation for 60min it was apparent that the optimum activity of the purified enzyme was not evaluated and that the true specific activity of the enzyme was somewhat higher than the value recorded.

Fraction	Total volume (ml)	Total activity (units)	Total protein (mg)	Specific activity (units/mg of protein)	Enrichment	Recovery (%)
Whole homogenate (fraction I)	300	309	639	0.48	(1)	(100)
Particles sedimented at 5000*g* for 20min after discarding the 1000*g* particles (fraction II)	11.0	267	69.6	3.8	7.9	86
NaCl extract of the particles (fraction III)	31.3	303	12.0	25.2	52.5	98
Heat treated and centrifuged clear (fraction IV)	24.8	321	9.46	34.0	70.8	101
Precipitated with $(NH_4)_2SO_4$ at 45% saturation and dialysed and clarified by centrifugation (fraction V)	6.1	320	3.48	91.8	191.2	101
Adsorbed on DEAE-cellulose column and eluted batchwise with phosphate buffer, pH 7.2, of increasing molarity: eluate with 100mM (fraction VI)	12.2	101	0.094	1074	2238	33

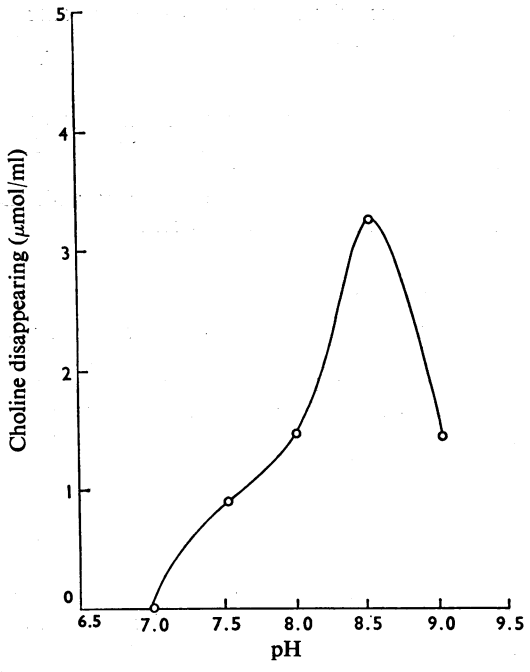

Fig. 1. *pH–activity profile of choline kinase in* 200 mM-*tris–HCl buffer*

For experimental details see the text.

test for the specificity of the phosphate donor in the kinase-catalysed reaction, UTP and GTP (both Sigma products) were substituted for ATP in equimolar concentrations. GTP gave 50% of the activity with ATP and UTP 20%.

Influence of substrate concentration. (*a*) ATP. With 3.3 mM-choline and 20 mM-Mg^{2+}, various concentrations of ATP (0.6–8.0 mM) were incorporated into the assay mixture. A rectangular hyperbola was obtained on plotting ATP concentration against reaction velocity (Fig. 2).

The Michaelis constant arrived at by a Lineweaver–Burk plot was 5 mM and the $V_{max.}$ 0.28 μmol of choline disappearing/h per μg of protein. The optimum concentration of ATP in the assay system was 3.2–4.0 mM. At 4.9 mM- and 8.0 mM-ATP there was about 30% inhibition.

(*b*) Choline. With choline in the range 0.33–9.9 mM in an assay system with 4 mM-ATP and 20 mM-Mg^{2+} a sigmoid Michaelis curve was obtained (Fig. 3).

The K_m value was 2.5 mM and the $V_{max.}$ 1.42 μmol of choline disappearing/h per μg of protein, calculated from the Lineweaver–Burk plot. Concentrations of choline higher than 3.0–4.9 mM appeared to give slight inhibition of the enzymic activity. In other assays, ATP and Mg^{2+} were both employed at 4 mM; the maximum choline concentration tested was 3.3 mM. A sigmoid response was observed also in this experiment.

Thus choline kinase belongs to the class of allosteric enzymes, as shown by the regulation of its activity by low concentration of substrate.

Product inhibition. (*a*) ADP. (i) Nature of inhibition and of binding. The reaction velocity was determined with inhibitor (ADP) in the range 0.52–3.12 mM, the ATP concentration being kept at the subsaturating value of 2 mM and saturating value of 4 mM, with 4 mM-Mg^{2+} and 3.3 mM-choline. The results are plotted in Fig. 4.

A sigmoid curve was obtained, establishing the co-operativity in the inhibitor binding, the sigmoidicity persisting at the saturating concentration of ATP. (The same type of plot was observed in another experiment in which ADP concentration was varied in the range 0.26–2.08 mM, with ATP and Mg^{2+} at 4 mM.)

To define the nature of the inhibition, $1/v$ was plotted against [ADP] in the two sets of experiments (see Dixon & Webb, 1964). Extrapolation of the tangents of the curved lines showed that the inhibition by ADP was of the competitive type with respect to ATP. The K_i value read from the graph was 2.0 mM (50% inhibition was caused by 1.7 mM), suggesting that ADP was a powerful inhibitor of the enzyme activity.

(ii) Influence of ADP on binding of choline and Hill number. A plot of the choline saturation curve in the presence of 1.3 mM-ADP, with 4 mM-ATP and 4 mM-Mg^{2+}, revealed a slight accentuation of the sigmoidicity in the absence of inhibitor. The influence of ADP on the co-operativity in choline binding was evaluated by the Hill plot ($\log[v/(V_{max.}-v)]$ against $\log[S]$), as shown in Fig. 5.

Straight lines, tending to be curved at low concentrations of choline, were obtained. The Hill number was 2.3 in the absence of ADP and altered to 2.7 in the presence of ADP.

K_m, determined as the $[S]_{(0.5)}$, defined as half saturating concentration of substrate, from the Hill plot, was 3.3 mM in the absence of effector and increased to 4.0 mM in its presence. From the Lineweaver–Burk plot, the apparent K_m increased from 2.9 to 3.7 mM; the $V_{max.}$ remained unchanged at 1.28 μmol of choline disappearing/h per μg of protein.

(iii) Measurement of the binding of ADP. When the velocity function in the presence of various concentrations of ADP (0.52–3.12 mM), with 2 mM-ATP, was plotted by the method of Taketa & Pogell (1965) ($\log[(v_0-v)/v]$ against $\log[I]$, where v_0 and v represent the reaction velocity in respectively the absence and presence of inhibitor, and [I] the concentration of inhibitor), the slope of the straight-line plot, that is,

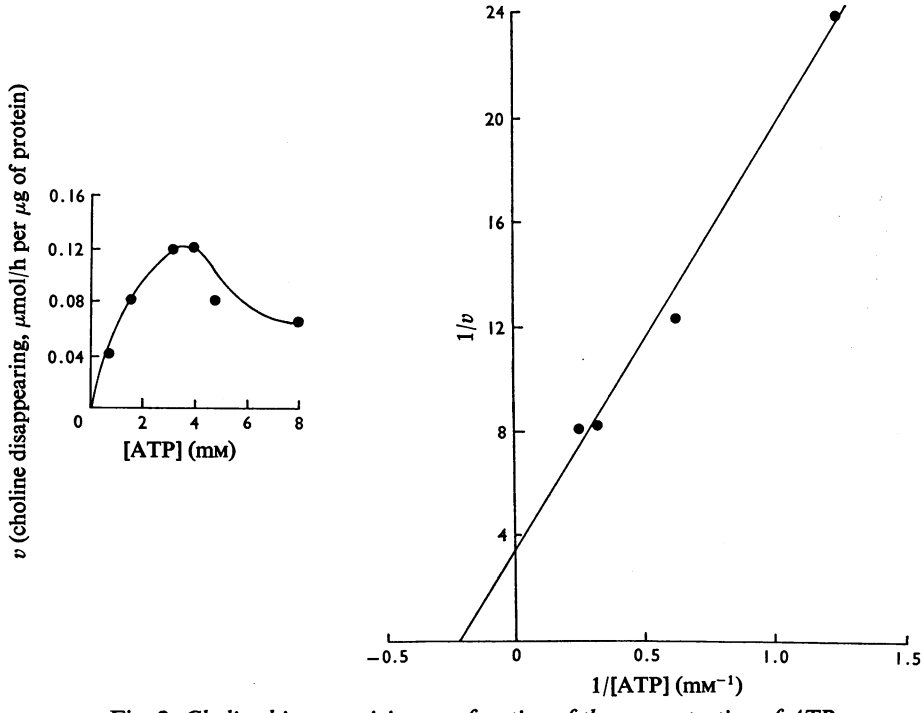

Fig. 2. *Choline kinase activity as a function of the concentration of ATP*

The enzyme activity was determined as described in the Materials and Methods section.

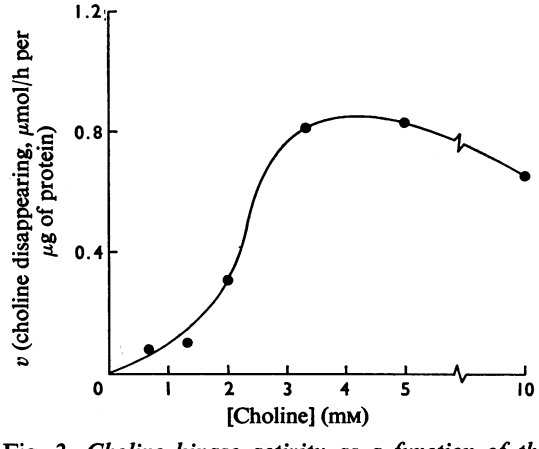

Fig. 3. *Choline kinase activity as a function of the concentration of choline*

The enzyme activity was determined as described in the Materials and Methods section.

the Hill number for ADP binding, was 4.8 (Fig. 6). This revealed a high order of co-operativity in the binding of ADP. With 4mM-ATP, a similar plot gave the Hill number as 2.6. It is known that at saturating concentration of substrate, the binding of effector is decreased.

(*b*) Phosphorylcholine. Phosphorylcholine was varied from 0.27–1.89mM in the assay system with two fixed concentrations of choline, 3.3 and 1.5mM, with 4mM-ATP and 4mM-Mg^{2+}. The plot of reaction velocity against inhibitor concentration did not reveal any co-operativity in the binding (Fig. 7).

The Dixon & Webb (1964) plot revealed that the inhibition was non-competitive with respect to choline. The K_i value was 0.62mM, indicating that phosphorylcholine inhibited the enzyme more powerfully than did ADP.

Desensitization by storage in the cold. When the purified preparation was stored in the cold and assayed at weekly intervals, it was found that the sigmoid characteristic of the choline-saturation curve was retained for 2 weeks, but was subsequently replaced by the classical Michaelis–Menten curve, as shown in Fig. 8. In these experiments 20mM-Mg^{2+} and 2mM-ATP were used.

Cold-sensitivity of enzyme. A sample of the purified preparation was kept at 20°C and another at room temperature (31°C) for 3h, after which the samples were assayed, with 4mM-Mg^{2+} and 4mM-ATP. The preparation held at 20°C showed no change

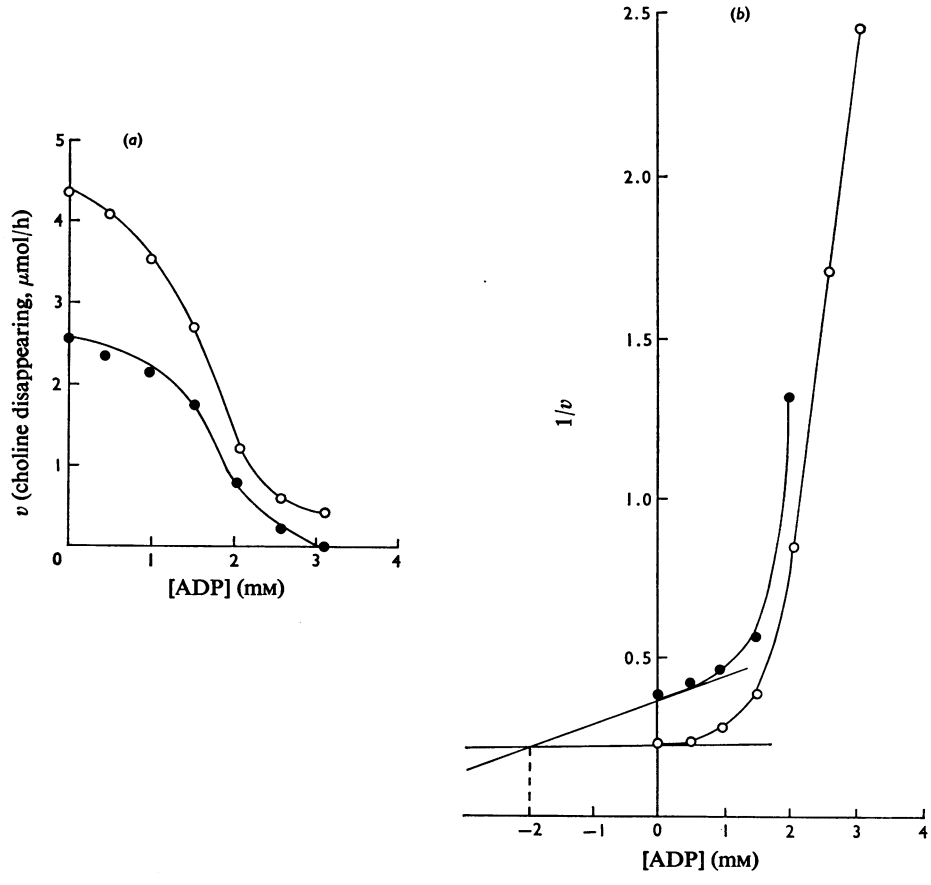

Fig. 4. *Inhibition of choline kinase activity by ADP*

(*a*) The enzyme activity was determined at saturating (4mM, ○) and subsaturating (2mM, ●) concentrations of ATP, choline being at the optimum concentration (3.3mM) in both cases; (*b*) graphical plot showing nature of inhibition by ADP (Dixon & Webb, 1964); data derived from (*a*). The extrapolated tangents give a K_i value of 2mM for ADP.

in activity, but that at 31°C had 80% higher activity. When another portion was kept frozen for 3h at −18°C and thawed (15min, in contact with ice) and assayed, no activity was found. Such inactive preparations could not be reactivated.

To test if the heat-activation was reversible, the enzyme preparation maintained at 31°C for 2h was returned to the ice bath. This treatment resulted in a progressive reversal of the activation. After 30min the activation was decreased to 59% and after 2h to 7%.

Effect of change of pH. Kinetic changes caused by pH alteration have been noticed in some allosteric enzymes and attributed to changes in the state of aggregation of the enzyme molecule (Stadtman, 1966). When the choline-saturation curve of the kinase was determined at pH7.5 (tris–HCl buffer), which was significantly removed from the optimum pH of 8.5, as shown by the fact that the activity was only 27%, the sigmoid response was not altered.

Influence of serine and methionine. Serine and methionine proved to be inhibitors. Methionine (2mM) caused a 25% decrease in enzyme activity, and 2mM-serine, 30%. The enzyme activity in the presence of 6mM-methionine or -serine was respectively one-third and one-half of that in the control samples. In these experiments, 3.3mM-choline, 4mM-Mg^{2+} and 4mM-ATP were used. The nature of the inhibition was not determined.

Influence of sodium chloride. Incorporation of NaCl (8.7–35mM) in the assay system led to a slight activation (10–20%) of the enzyme, but concentra-

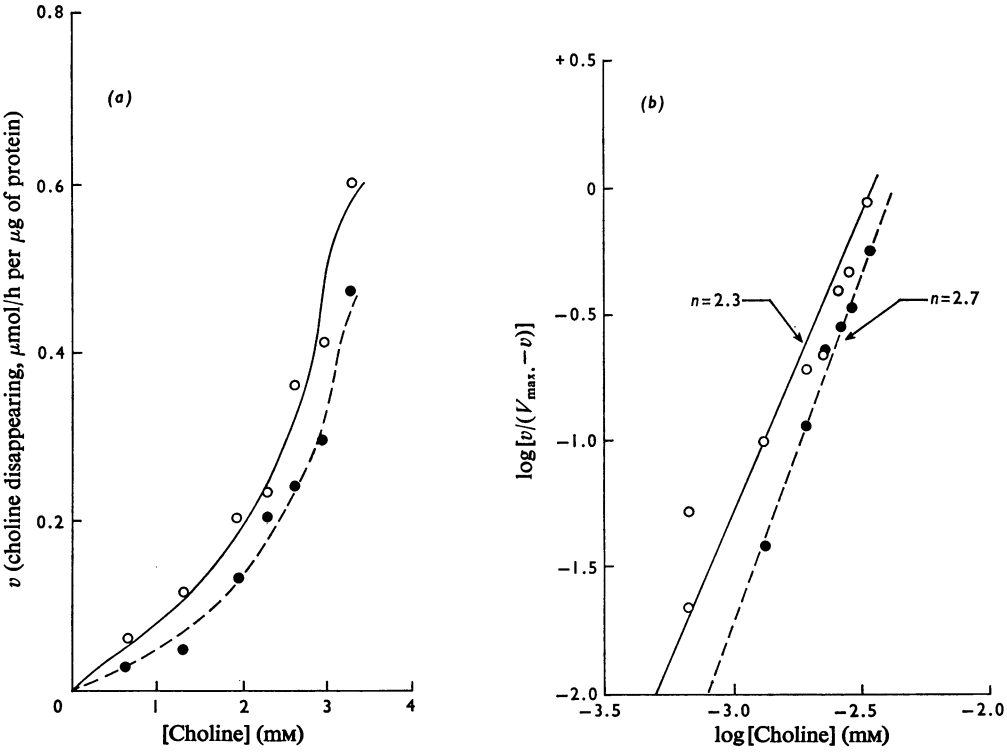

Fig. 5. *Choline kinase activity as a function of the concentration of choline in the absence and in the presence of ADP*

(*a*) Plot of enzyme activity as a function of the concentration of choline in the absence (○) and in the presence (●) of 1.3 mM-ADP. (*b*) Hill plot showing the extent of co-operativity in choline binding in the absence (○) and in the presence (●) of ADP; data derived from (*a*).

tions of 70 mM and above resulted in the inactivation of the enzyme.

Discussion

Some of the experiments with labelled precursors have pointed to the ability of mitochondria to synthesize *de novo* phosphatidylcholine and other phospholipids (see Bygrave & Kaiser, 1968; Bygrave & Bucher, 1968) although other experiments have cast serious doubts on such a synthesis (see Jungalwala & Dawson, 1970).

The results of subcellular location of choline kinase in *Cuscuta* assume considerable significance in the light of utilization of choline by mitochondria for the possible elaboration of choline-containing phospholipids. In the absence of electron-microscopic examination and purification of particles by density-gradient centrifugation, no claim can be made as to the homogeneity of the isolated fractions. The subcellular distribution of succinate dehydrogenase, cytochrome *c* oxidase and glucose 6-phosphatase activities and of RNA P considered together suggested that the 5000 and 12000*g* particulate fractions from homogenates of the parasite tissue were essentially mitochondrial, with possibly some contamination with the microsomal fraction. Since the filaments of *Cuscuta* are known to contain only low concentrations of chlorophyll (MacLeod, 1962), and since the 1600*g* sediment from homogenates was inactive, the chloroplast may be excluded as a site of localization of choline kinase. Since the 12000*g* and 16000*g* supernatants of homogenates were devoid of choline kinase activity, it was doubtful whether the enzyme was present in the microsomal or soluble fractions. Most of the choline kinase in *Cuscuta* filament was therefore associated with the mitochondria.

The ready solubilization of choline kinase by treatment of the isolated particles with NaCl, or by incorporation of the salt in the dispersion medium for the tissue, suggested that the mitochondrial enzyme was associated with the external membrane. This fitted in with the studies by Stoffel & Schiefer (1968) and Kaiser & Bygrave (1968). A possible location of

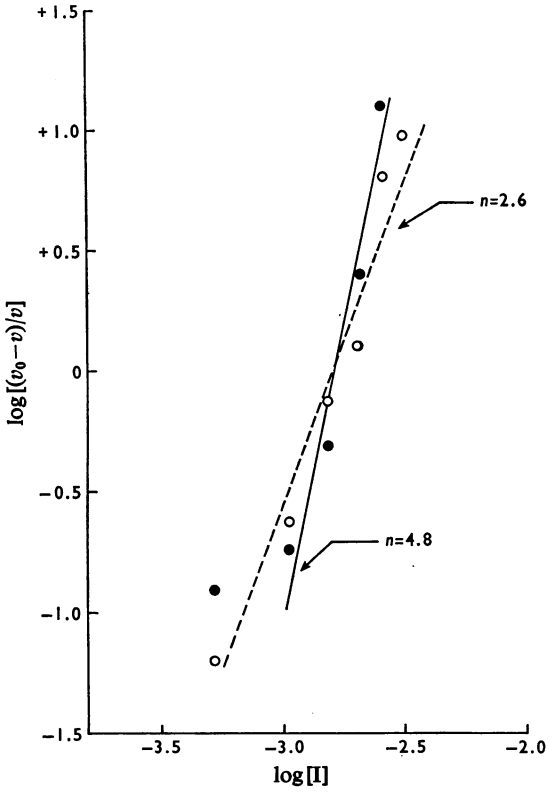

Fig. 6. *Hill plot to determine the extent of co-operativity in ADP binding at saturating and sub-saturating concentrations of ATP*

○, Saturating concentration of ATP (4mM); sub-saturating concentration of ATP (2mM); data derived from Fig. 4(*a*).

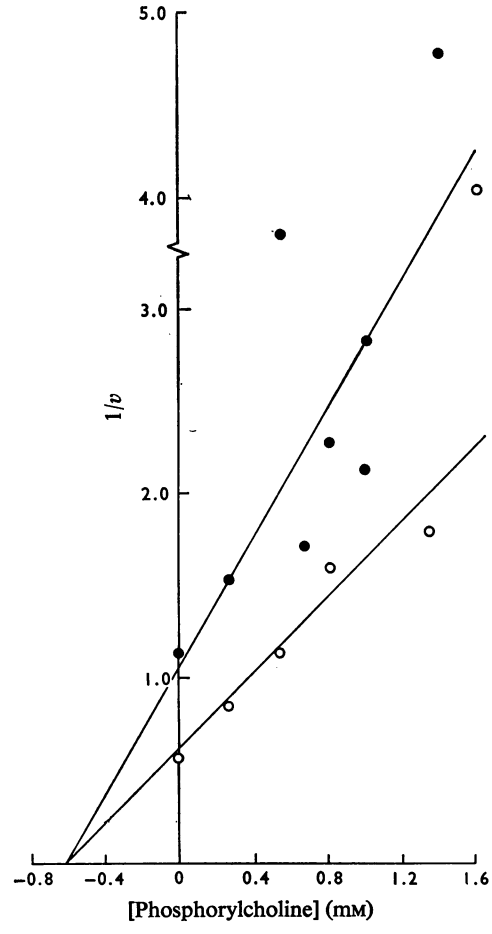

Fig. 7. *Graphical plot (Dixon & Webb, 1964) showing the nature of the inhibition of phosphorylcholine*

The enzyme activity was determined at saturating (3.3mM, ○) and sub-saturating (1.6mM, ●) choline concentrations. The extrapolated tangents give a K_i value of 0.62mM for phosphorylcholine.

choline kinase in the outer membrane would obviate the difficulty arising from permeability restriction for the entry of choline into mitochondria (Williams, 1960). The ready solubilization of choline kinase can be understood also it if were to be a matrix enzyme (Green, 1961; Kaiser & Bygrave, 1968).

Enzymes functioning at branch points of metabolic pathways and catalysing unidirectional reactions are generally more likely to be allosteric in nature. Allostery may be anticipated in choline kinase, since it catalyses an irreversible reaction and since choline can be channelled into at least one alternative route of metabolism in the mitochondria, namely oxidation by choline oxidase (see Kensler & Langemann, 1951). Other possible, but by no means proven, routes in mitochondria for choline utilization are the formation of acetylcholine (see Hebb & Whittaker, 1958) and choline sulphate (see Kaji & McElroy, 1958).

Our present results revealed that choline kinase is a regulatory enzyme in *Cuscuta*. Earlier workers failed to detect allostery in choline kinase derived from micro-organisms, higher-plant or animal tissue, tested as partially purified preparation or as tissue homogenate. Either there is a distinction between the enzyme from different sources [see Taketa & Pogell (1965); Preiss *et al.* (1967); Behrisch & Hochachka (1969*a*,*b*) for fructose diphosphatase (EC 3.1.3.11); Schuberth (1966) and Potter *et al.* (1968) for choline acetyltransferase (EC 2.3.1.6); White & Kaplan (1969) for glycerol 3-phosphate dehydrogenase (EC 1.1.1.8)], or the allostery of the enzyme was

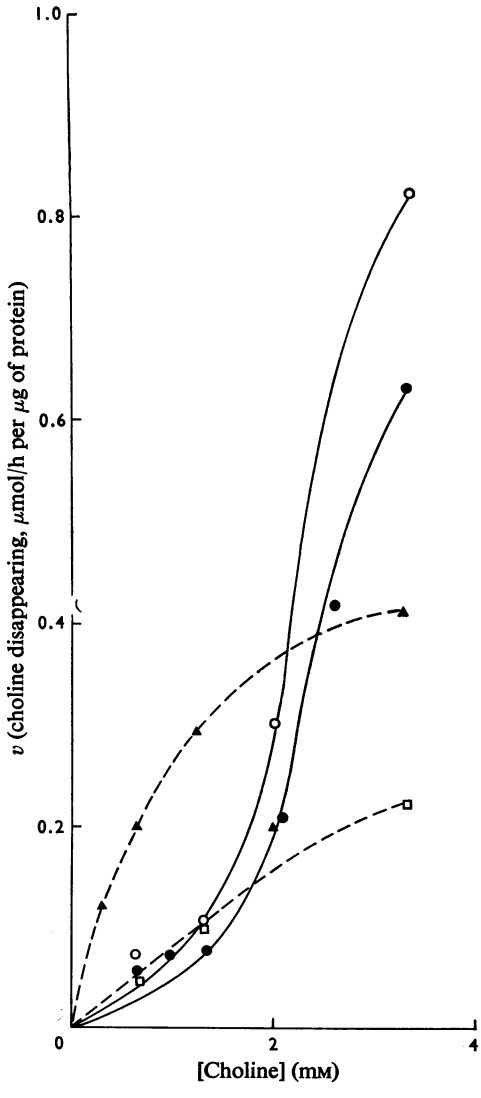

Fig. 8. *Desensitization of choline kinase on storage at 0–4°C*

The enzymic activity was determined after storage for 1 week (○), 2 weeks (●), 3 weeks (▲) and 4 weeks (□); the enzyme samples taken for analysis contained, respectively, 3.25, 3.82, 5.34 and 10.83 μg of protein/ml.

missed by earlier workers owing to unfavourable conditions of assay (see Atkinson, 1966; Preiss & Greenberg, 1969) or their enzyme preparation had suffered desensitization (see Stadtman, 1966).

ADP has close structural similarity to ATP and should have been expected to be a steric inhibitor. The saturation curve for ADP binding suggests that the nucleotide bound at an allosteric site. The intramitochondrial ATP/ADP ratio would be an effective means of regulation of choline kinase activity. In turn, the action of choline kinase can regulate the ATP/ADP ratio.

The *K* system (Monod *et al.*, 1965) of allosteric enzymes show sigmoid substrate-saturation curves and are characterized by the fact that negative effectors increase the apparent K_m value without altering the $V_{max.}$. In these respects, choline kinase qualifies itself for grouping under the *K* system. The large value of *n* was suggestive of strong interaction among the substrate-binding sites. It is, however, necessary to point out that when there is more than one participant in the reaction, as in choline kinase action, it is not the effector molecule alone that influences the enzyme–substrate interaction, but also the other participants.

Some of the properties of choline kinase were compatible with it being a protein molecule made up of subunits. The quantitative evaluation of the Hill number for choline binding pointed to the enzyme being a polymer made up of subunits. The desensitization of choline kinase on prolonged storage in the cold was analogous to the desensitization of other enzymes (see Martin, 1963; Caskey *et al.*, 1964). The cold-sensitivity observed for choline kinase has been reported for some other allosteric enzymes (Cohen, 1965; Holmes & Levinson, 1970), the cold-inactivation usually being accompanied by association or dissociation of the enzymes. The complete loss in activity of choline kinase in the presence of 70mM-NaCl was probably associated with structural disruption.

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