The Nature and Control of the Tricarboxylate Cycle in Beetle Flight Muscle

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1. The only exogenous substrates oxidized by mitochondria isolated from the flight muscle of the Japanese beetle (*Popillia japonica*) are proline, pyruvate and glycerol 3-phosphate. The highest rate of oxygen consumption is obtained with proline. 2. The oxidation of proline leads to the production of more NH_3 than alanine, indicating a functioning glutamate dehydrogenase (EC 1.4.1.2). 3. Studies of mitochondrial extracts confirm the presence of a very active glutamate dehydrogenase, and this enzyme is found to be activated by ADP and inhibited by ATP. These extracts also show high alanine aminotransferase activity (EC 2.6.1.2) and a uniquely active 'malic' enzyme (EC 1.1.1.39). The 'malic' enzyme is activated by succinate and inhibited by ATP and by pyruvate. 4. It is suggested that the input of tricarboxylate-cycle intermediate from proline oxidation is balanced by the formation of pyruvate from malate, and the complete oxidation of the majority of the pyruvate. 5. Studies of the steady-state concentrations of mitochondrial CoASH and CoA thioesters during proline oxidation show a high succinyl (3-carboxypropionyl)-CoA content which falls on activating respiration with ADP. There is a concomitant rise in CoASH. However, the reverse transition, from state-3 to state-4 respiration, causes only very slight changes in acylation. The reasons for this are discussed. 6. Studies of the mitochondrial content of glutamate, 2-oxoglutarate, malate, pyruvate, citrate and isocitrate during the same phases of proline oxidation give results consistent with control at the level of glutamate dehydrogenase and isocitrate dehydrogenase during proline oxidation, with the possibility of further control at 'malic' enzyme. 7. During the oxidation of pyruvate all of the tricarboxylate-cycle intermediates and NAD(P)H follow the pattern of changes described in the blowfly (Johnson & Hansford, 1975; Hansford, 1974) and isocitrate dehydrogenase is identified as the primary site of control.

The tricarboxylate cycle in blowfly (*Phormia regina*) flight muscle has been well characterized and is extremely simple in that the flux through any one of the reactions is essentially the same as that through any other. This is shown by the assay of steady-state contents of tricarboxylate-cycle intermediates (Johnson & Hansford, 1975) and is a consequence of the impermeability of the mitochondrial inner membrane towards these intermediates. In blowfly flight muscle, NAD-isocitrate dehydrogenase (EC 1.1.1.41) has been identified as the primary locus of control of the cycle (Johnson & Hansford, 1975). The present work extends the study of control to an insect muscle in which the tricarboxylate cycle is more complex. For this purpose a beetle (Coleoptera) was chosen, since de Kort *et al.* (1973) had shown very active proline oxidation in the Colorado beetle (*Leptinotarsa decemlineata*). Combined with the enzyme assays of Crabtree & Newsholme (1970), showing high alanine aminotransferase (EC 2.6.1.2) and low glutamate dehydrogenase (EC 1.4.1.2) activity in another beetle, the cockchafer (*Melolontha melolontha*), this suggested a high flux in the section of the tricarboxylate

cycle from 2-oxoglutarate to oxaloacetate compared with that from oxaloacetate to 2-oxoglutarate. For the present study, the Japanese beetle (*Popillia japonica*) was used, as it is available in large numbers during the summer, and is of considerable economic significance in the Eastern United States. The pre-eminence of proline oxidation shown by de Kort *et al.* (1973) was confirmed. Since the mitochondria in these insects were found to be impermeable to tricarboxylate-cycle intermediates this raised the question of what species left the mitochondria to compensate osmotically for the entry of proline. The finding of an extremely active 'malic' enzyme (EC 1.1.1.39), and of the further metabolism of its product pyruvate, provided some answer. Quite unexpected was the finding that proline oxidation leads to the production of more NH_3 than alanine. This is the first implication of an active glutamate dehydrogenase in insect flight muscle, and means that the model of proline oxidation via alanine aminotransferase, which was shown directly to apply to the tsetse fly (*Glossina morsitans*) (Bursell, 1963, 1965, 1966, 1967) and which was inferred to occur in the cockchafer (Crabtree &

Newsholme, 1970), is not of universal application within the Insecta. The assay of enzymically active fractions from the Japanese-beetle mitochondria showed the presence of an active glutamate dehydrogenase directly, and that the enzyme is very sensitive to control by the energy charge of the adenylate system (in the terminology of Atkinson, 1968). By contrast, the flavoprotein proline dehydrogenase was shown to be only slightly affected by adenine nucleotides, though proline dehydrogenases from other insect flight muscles are sensitive to ADP (Hansford & Sacktor, 1970; Norden & Venturas, 1972). The assay of the steady-state concentrations of glutamate, 2-oxoglutarate, succinyl- (3-carboxypropionyl)-CoA, CoASH, malate, pyruvate, citrate and isocitrate within these mitochondria permitted comment to be made on control at glutamate dehydrogenase, and elsewhere.

Materials and Methods

Preparation and incubation of mitochondria

Japanese beetles (*Popillia japonica*) of both sexes were collected locally over the period 1st July–21st August, after which they were unobtainable. Mitochondria were prepared by the proteinase method described in Hansford (1974), by using flight muscles from 50 beetles and 2 mg of Nagarse.

Rates of oxygen uptake were determined using a Clark-type O₂ electrode in 2 ml of a basal medium comprising 0.12 M-KCl, 20 mM-Tes* (potassium salt) and 10 mM-potassium phosphate, pH 7.2. All incubations were performed at 25°C.

Measurement of mitochondrial intermediates and of alanine and NH₃

CoASH, acetyl-CoA and succinyl-CoA were assayed in HClO₄ extracts of mitochondria as described by Hansford (1974). Mitochondrial citrate, isocitrate, 2-oxoglutarate and malate contents were assayed by the methods of Williamson & Corkey (1969) as described by Johnson & Hansford (1975). Glutamate was measured by the method of Williamson & Corkey (1969), and pyruvate was measured fluorimetrically by using lactate dehydrogenase and by reference to a standardized pyruvate solution. Estimates of mitochondrial contents of pyruvate and glutamate followed from the procedure described for the tricarboxylate-cycle intermediates (Johnson & Hansford, 1975). ATP, ADP and AMP, and mitochondrial NADH, were measured as described by Hansford (1975).

* Abbreviations: Tes, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulphonic acid; Mes, 2-(*N*-morpholino)-ethanesulphonic acid; Hepes, 2-(*N*-2-hydroxyethyl)piperazin-*N'*-yl)ethanesulphonic acid.

For the measurement of NH₃ and alanine during proline oxidation, a 2 ml portion of the complete incubation mixture lacking only proline was added to 1.3 ml of 16% (v/v) HClO₄. Proline was added to this denatured sample to 10 mM, the concentration used in the incubation, to serve as a blank. Further 2 ml samples were withdrawn from the incubation after proline addition and added to HClO₄ as described above. Extracts were adjusted to pH 5.6 with KOH after addition of Mes to 50 mM. The extracts were assayed for NH₃ by the method of Kirsten *et al.* (1963), and for alanine by the method of Bergmeyer & Bernt (1963), allowing the alanine aminotransferase and lactate dehydrogenase reactions to reach equilibrium in the presence of a large amount (200 µg) of transaminase. The results shown are corrected for the blank values.

Ultrasonic disruption of mitochondria

Mitochondrial sonicates were prepared by subjecting mitochondrial suspensions, of concentration 2 mg of protein/ml, to four 15 s periods of ultrasonic irradiation, in a Branson sonifier at power setting 2. The glass tube containing the suspension was surrounded with ice-water and 30 s periods were allowed for cooling. For sonication before proline-ferricyanide reductase determinations, the mitochondria were suspended in 0.12 M-Tes (potassium salt)–10 mM-potassium phosphate, pH 7.2; for glutamate dehydrogenase determinations, in 50 mM-potassium phosphate (pH 7.6)–1 mM-EDTA–1 mM-dithiothreitol–0.1 mM-ADP; for 'malic' enzyme determination, in 50 mM-Hepes (potassium salt) (pH 7.4),–3 mM-dithiothreitol–0.5 mM-MnCl₂.

Measurement of the activity of mitochondrial enzymes

Proline-ferricyanide reductase was measured by adding 100 µl of the supernatant obtained by centrifuging a mitochondrial sonicate at 10000g for 4 min to a cuvette containing 0.8 ml of 0.12 M-KCl–20 mM-Tes (potassium salt), pH 7.2, 10 mM-L-proline, 1 mM-potassium ferricyanide, 2 mM-KCN and 5 µg of oligomycin/ml. The rate of decrease in *E*₄₁₂ was recorded. Where indicated, adenine nucleotide was added to the reaction mixture before the addition of the enzymically active fraction.

Glutamate dehydrogenase was assayed in the direction of glutamate synthesis by the method of Schmidt (1963). The reaction mixture comprised 45 mM-triethanolamine-HCl, pH 8, 70 mM-ammonium acetate, 0.13 mM-NADH, 1.6 mM-EDTA, 1 mM-dithiothreitol, 1 mM-KCN, 0.3 µg of rotenone/ml and 0.02% (v/v) Triton X-100. The reaction was initiated by the addition of 2-oxoglutarate to a final concentration of 6.7 mM.

In the direction of glutamate oxidation the medium comprised 45 mM-potassium phosphate, pH 7.6, 0.2 mM-NAD⁺, 1.6 mM-EDTA, 1 mM-dithiothreitol, 1 mM-KCN, 0.3 μ g of rotenone/ml and 0.03% (v/v) Triton X-100. The reaction was initiated by the addition of L-glutamate, to a final concentration of 40 mM, 1 min after the addition of mitochondria (approx. 0.2 mg of protein/ml). Where 'sonication' is indicated a mitochondrial sonicate was prepared as described above and subjected to centrifugation at 100000g for 30 min. A portion (50 μ l) of the supernatant fraction was used for the assay, and Triton X-100 was omitted.

Alanine aminotransferase was assayed by the method of Bergmeyer & Bernt (1963). The reaction was initiated by adding 10 μ l of mitochondrial suspension (approx. 0.1 mg of protein) to a reaction mixture comprising 57 mM-potassium phosphate, pH 7.4, 0.1% (v/v) Triton X-100, 30 mM-L-alanine, 7.5 mM-2-oxoglutarate, 2 mM-KCN, 0.2 mM-NADH and 0.03 mg of lactate dehydrogenase/ml and the decrease in E_{340} was recorded. The lactate dehydrogenase was extensively dialysed against potassium phosphate, to remove NH₄⁺. Alternatively, mitochondria were added to the reaction mixture minus 2-oxoglutarate, and the reaction was started with the latter substrate. If the reaction was initiated instead with L-alanine, the rates recorded were significantly lower and very variable. Glutamate dehydrogenase does not contribute to the activities recorded, as there was no oxidation of NADH in the absence of L-alanine, and L-alanine was shown not to be an effector of glutamate dehydrogenase.

'Malic' enzyme activity was determined by adding mitochondria to 50 mM-Hepes (potassium salt), pH 7.4, containing 0.1% (v/v) Triton X-100, 10 mM-L-

malate, 0.6 mM-MnCl₂, 3 mM-dithiothreitol, 2 mM-KCN and 0.3 μ g of rotenone/ml. The electron acceptor was either 1 mM-NAD⁺ or 1 mM-NADP⁺, and the progress of the reaction was followed at 340 nm. When mitochondria were disrupted by ultrasound, the suspension was centrifuged for 30 min at 100000g after sonication, and enzyme activity was assayed in the supernatant layer. Triton X-100 was omitted.

Expression of results

In Figs. 3 and 6, results are quoted as means for duplicate experiments, matched carefully with respect to protein concentration and times of sampling. In Fig. 6, the data permitted statistical analysis by Student's *t* test, considering values from state 4 and state 3 (as defined by Chance & Williams, 1956) as independent. However, in Fig. 3 this was not attempted, since the oxidation of proline caused an increase in the total amount of tricarboxylate-cycle intermediate during the course of the experiment, and made grouping as state-4 and state-3 values impossible.

Results

Substrate utilization by Japanese-beetle mitochondria

Table 1 presents the rates of oxygen consumption obtained with various substrates. The highest rate was obtained with proline (0.57 μ g-atom of oxygen/min per mg of protein), a lesser rate being obtained with pyruvate (0.47), even when pyruvate oxidation was potentiated by pyruvate carboxylation in the presence of ATP and KHCO₃. These results closely

Table 1. Substrate utilization by beetle mitochondria

Oxygen uptake was measured as described in the Materials and Methods section. The rates tabulated are for state-3 respiration, obtained by adding ADP to a final concentration of 1 mM. Where a number of determinations are cited, these involved different mitochondrial preparations. Where appropriate, results are given as means $\pm$ S.E.M. of the numbers of determinations in parentheses.

Substrate concn. (mM)	Rate of oxygen uptake (μ g-atom/min per mg of protein)
L-Proline (10)	0.569 $\pm$ 0.079 (10)
L-Proline (0.5)	0.077
L-Proline (0.5)+palmitoyl-L-carnitine (0.01)	0.074
L-Proline (0.25)	0.028
L-Proline (0.25)+palmitate (0.1)+bovine serum albumin (0.014)	0.029
Pyruvate (2.5), ATP (0.5), KHCO ₃ (2.5)	0.465 $\pm$ 0.073 (8)
DL-Glycerol 3-phosphate (20)	0.114, 0.242, 0.295
2-Oxoglutarate (5.0)	0.01 (2)
2-Oxoglutarate (5.0)+L-malate (2.5)	0.01
2-Oxoglutarate (5.0)+L-alanine (5.0)	0.10
L-Glutamate (5)	0.016
Citrate (5.0)+L-malate (2.5)	0.01
Succinate (5.0)	0.01
NADH (0.45)	0.064

Table 2. *Activities of some key enzymes involved in the oxidation of proline*

Assays were carried out at 25°C as described in the Materials and Methods section. The activities are calculated on the basis of the protein in the mitochondrial preparation from which the enzymically active fraction was derived. Activities varied by less than 20% between preparations except where the values are presented separately.

Enzyme activity and conditions	Enzyme activity (nmol/min per mg of mitochondrial protein)
(7) Proline–ferricyanide reductase	
Mitochondrial sonicate	94 (2)
+2mM-ADP	107 (2)
+2mM-ATP	94 (2)
(2) A. Glutamate dehydrogenase (EC 1.4.1.2) operating in the direction of glutamate synthesis	
Mitochondria solubilized with Triton X-100	160, 760, 1200
+0.5mM-ADP	2850, 5860
B. Glutamate dehydrogenase operating in the direction of glutamate oxidation	
Mitochondria solubilized with Triton X-100	4, 9
+0.5mM-ADP	133, 101
Mitochondria disrupted by sonication	
+2.5mM-ADP	132
(3) Alanine aminotransferase (EC 2.6.1.2)	
Reaction initiated with mitochondria	1970
Reaction initiated with 2-oxoglutarate	1790
(4) 'Malic' enzyme (EC 1.1.1.39)	
Mitochondria solubilized with Triton X-100, NAD ⁺ as coenzyme	395 (2)
Mitochondria solubilized with Triton X-100, NADP ⁺ as coenzyme	168
Mitochondria disrupted by sonication, NAD ⁺ as coenzyme	315 (3)

resemble those of de Kort *et al.* (1973) obtained with the Colorado beetle (*Leptinotarsa decemlineata*). The rates of oxygen consumption obtained with glycerol 3-phosphate varied more than expected on the grounds of experimental error. This variation may reflect maturation of the insects, a process described in the Colorado beetle by de Kort & Bartelink (1972), as the higher rates were obtained later in the summer. Respiratory-control ratios with the two prime substrates were high, being 11 for proline oxidation, and essentially infinite for pyruvate oxidation. Nevertheless, some broken mitochondria must have been present, as there is a finite rate of NADH oxidation. The rates obtained in the presence of each of the tricarboxylate-cycle intermediates are exceedingly low (Table 1), suggesting a lack of permeability towards these intermediates. This was shown more directly for citrate, isocitrate, 2-oxoglutarate, glutamate and malate in centrifugation studies reported below. The oxygen consumption in the presence of 2-oxoglutarate and alanine may not be an exception to this rule, as it may involve either a vectorial transaminase, as suggested for the housefly by Van den Bergh (1964), or a transaminase external to the mitochondrial inner membrane, and the penetration of one of the products, pyruvate. The lack of oxidation of 10 μ M-palmitoyl-L-carnitine in the presence of a low concentration of proline (Table 1) and the lack of oxidation of 100 μ M-palmitate in the presence of 14- μ M bovine

serum albumin and low proline would tend to rule out lipid oxidation as a source of energy for flight in this species.

Activities of some key enzymes involved in proline oxidation

Proline dehydrogenase was measured by ferricyanide reduction in mitochondria disrupted by sonication (Table 2). This removes the objection to work involving intact mitochondria and dye reduction, that some of the reduction may be due to NADH generated in later enzymic reactions. However, it also means that the activities are minima, as some mitochondria survive the sonication and are removed by centrifugation. Subsequent work showed that the apparent K_m for proline of proline–ferricyanide reduction is 6mM in the presence of 2mM-ADP and 10mM in the absence of nucleotide. The effect of ADP is thus rather slight. There is a good correlation with the apparent K_m for proline during state-3 respiration by intact mitochondria, this being 6mM in the presence of 2.5mM-ADP. The values presented in Table 2 were obtained at 10mM-proline and thus represent an activity somewhat less than V_{max} .

Glutamate dehydrogenase was initially assayed in the direction of glutamate synthesis (Table 2) to permit comparison of these results with those obtained by Crabtree & Newsholme (1970) from a

variety of insects. Exact comparison is not possible, as flight-muscle protein was used as a reference in the previous work, and mitochondrial protein in the present. However, it is obvious that the activity of glutamate dehydrogenase (in the direction of glutamate synthesis) is much higher in the Japanese beetle than in any of the other species surveyed (Crabtree & Newsholme, 1970), which included a beetle. It is just possible that the difference is trivial, as the homogenization procedure used in the earlier work may not have led to complete mitochondrial disruption, and thus may not have elicited a full expression of glutamate dehydrogenase activity. ADP was found to activate the enzyme markedly (Table 2), and this was true for the reaction of glutamate oxidation also. ATP was inhibitory (not shown). The effect of adenylate energy charge on glutamate oxidation is discussed below.

Alanine aminotransferase (EC 2.6.1.2) was found to be active (Table 2). It was necessary for the reaction mixture to contain alanine when the mitochondria were solubilized with the detergent Triton X-100 if full activity was to be expressed. Addition of alanine some minutes after solubilization resulted in a very poor activity.

The rapid and extensive oxidation of proline demands the formation of some product which can easily leave the mitochondrion, as no extensive mitochondrial swelling is observed. It was surmised that malate might be oxidatively decarboxylated to pyruvate, and mitochondrial extracts were accordingly assayed for 'malic' enzyme (EC 1.1.1.39). It was found to be exceedingly active (Table 2), indeed more than 20 times as active as the same enzyme assayed under identical conditions in mitochondria from mammalian liver, heart, kidney or brain (Hansford & Lehninger, 1973). It is noted that malate dehydrogenase (EC 1.1.1.37) is not a sufficient explanation of the rates of NAD^+ reduction given in Table 2, and elsewhere in the present paper, in that the malate dehydrogenase reaction was at equilibrium before the recording of rate was begun. This was confirmed by the lack of further NAD^+ reduction on adding a large amount of pure malate dehydrogenase. A combination of malate dehydrogenase and oxaloacetate decarboxylase (EC 4.1.1.3) could give the progressive reduction of NAD^+ which is observed, in which case the oxaloacetate decarboxylase is tissue specific and of interest in proline metabolism. However, the apparent K_m for malate, and the inhibition by ATP of the enzyme being described (Fig. 8), make 'malic' enzyme a more likely candidate. The reduction of NADP^+ (Table 2) cannot be explained on the basis of malate dehydrogenase and oxaloacetate decarboxylase, and must be attributed to 'malic' enzyme. Lin & Davis (1974) have purified a protein from heart mitochondria with 'malic' enzyme activity, which

reduces either NAD^+ or NADP^+ but with a lower K_m for NAD^+ , and a higher $V_{\max}$. The activity of 'malic' enzyme recorded in Table 2, which approximates to $V_{\max}$, is in excess of the activities of proline-ferricyanide reductase and glutamate dehydrogenase (operating in the direction of glutamate oxidation) and is in accord with a role in proline oxidation in the beetle.

Pathway of proline oxidation in the beetle

The data presented so far demonstrate that beetle mitochondria have the complement of enzymes necessary for oxidizing glutamate, derived from proline, either via glutamate dehydrogenase or via alanine aminotransferase. In order to discriminate between these two pathways, assays were undertaken of NH_3 , the product of glutamate dehydrogenase, and of alanine, the product of the aminotransferase pathway. Table 3 shows that during both controlled and active (state 3) respiration there was more NH_3 formed than alanine, on a molar basis. Although the enzymic assay for alanine has drawbacks and the results are approximations it is very clear that alanine production is a small fraction of the quantity predicted on the transaminase model, i.e. 1 mol of alanine/5 g-atoms of oxygen. Pyruvate production was measured in a parallel experiment (Table 3b) and was found to be small. It represents the resultant of pyruvate formation, which requires glutamate dehydrogenase and 'malic' enzyme, and pyruvate metabolism via pyruvate dehydrogenase and alanine aminotransferase.

Control of proline oxidation

Fig. 1 presents the results of an experiment in which the state of acylation of mitochondrial CoA was determined during the oxidation of proline. The total of CoASH plus CoA thioester was extremely low (0.3–0.4 nmol/mg of mitochondrial protein) and a modification of the kinetic assay of Allred & Guy (1969) was used, as described by Hansford (1974). The pattern of acylation differed markedly from that found in blowfly flight-muscle mitochondria oxidizing pyruvate (Hansford, 1974) in that in the present study the major component was succinyl-CoA. This fell in concentration on the activation of respiration by ADP, with a concomitant increase in the content of CoASH. These changes scarcely reversed, however, at the end of the phase of activated respiration (Fig. 1). Acetyl-CoA content was low throughout the experiment, suggesting a low activity of pyruvate dehydrogenase relative to that of the tricarboxylate cycle, but by no means ruling out an effective oxidation of pyruvate. The disparity between the succinyl-CoA content, at the beginning and end of the phase of activated respiration, raised the question of whether

Table 3. *Relation between the production of NH₃, alanine and pyruvate and the consumption of oxygen during proline oxidation*

(a) Mitochondria were added to an O₂-saturated basal medium containing either 10mM-proline ('Controlled') or 10mM-proline plus 7mM-ADP ('Active') to give a protein concentration of 0.3 mg/ml. Samples (2ml) were withdrawn at the stated times and analysed for NH₃ and alanine as described in the Materials and Methods section. Oxygen consumption was measured polarographically (see the Materials and Methods section) in a parallel incubation in air-saturated medium. (b) The experiment was performed as in (a) except that air-saturated medium was used. Pyruvate contents of samples were measured as described in the Materials and Methods section. Abbreviations: N.D., not detectable; N.M., not measured.

(a) Respiratory state	Time of sampling (min)	Oxygen (ng-atoms/mg of protein)	NH ₃ (nmol/mg of protein)	Alanine (nmol/mg of protein)
Active (3)	2.47	917	83	N.D.
	4.42	2230	192	80
	5.50	3000	224	56
Controlled	5.55	318	24	N.D.
	8.28	457	25	N.D.
	10.63	576	32	17

(b) Respiratory state	Time of sampling (min)	Oxygen (ng-atoms/mg of protein)	Pyruvate (nmol/mg of protein)
Active (3)	0.58	77	5
	1.47	490	11
	2.48	995	15
	3.52	1550	18
Controlled	1.23	31	N.M.
	2.85	110	N.M.
	4.53	190	2.2
	6.12	265	1.8

Fig. 1. *Mitochondrial content of succinyl-CoA, acetyl-CoA and CoASH during the oxidation of proline*

(a) Mitochondrial protein (9mg) was added to 15 ml of O₂-saturated medium comprising 0.12M-KCl-20mM-Tes (potassium salt)-10mM-potassium phosphate-10mM-L-proline. Samples (2ml) were withdrawn and expelled into 1.3 ml portions of 16% (v/v) HClO₄ in 40% (v/v) ethanol at the times shown. Extracts were neutralized and assayed for CoASH and thioester as described in Hansford (1974), except that the activities of citrate synthase, malate dehydrogenase and phosphotransacetylase were doubled. ADP was added to 4mM at the time indicated. (b) The trace of oxygen consumption was obtained from an electrode in the same suspension. Numbers on the trace refer to extracts in (a). ○, CoASH; □, acetyl-CoA; ●, succinyl-CoA.

Table 4. *ATP, ADP and AMP content of beetle mitochondria during controlled proline oxidation*

Mitochondria (0.7 ml of 10 mg/ml suspension) were added to a medium comprising 0.12 M-KCl-20 mM-Tes (potassium salt)-10 mM-potassium phosphate-10 mM-L-proline, at zero time. The pH of the solution was 7.2 and the final volume 5.8 ml. At the times indicated 2 ml samples, withdrawn by syringe, were expelled into HClO₄. Extraction and neutralization were as described by Hansford (1975), and analysis was by the enzymic methods described by Williamson & Corkey (1969) and a Farrand filter fluorimeter (Hansford, 1975) was used.

Time of sampling (min)	ATP (nmol/mg of protein)	ADP (nmol/mg of protein)	AMP (nmol/mg of protein)	Sum of ATP, ADP, AMP
0.98	11.6	1.03	1.64	14.3
1.48	11.6	1.08	1.20	13.9

Fig. 2. *Effect of added ATP on the mitochondrial content of succinyl-CoA, acetyl-CoA and CoASH during proline oxidation*

Mitochondrial suspension (1.1 ml:12.9 mg of protein) was added to a medium comprising 0.12 M-KCl-20 mM-Tes (potassium salt)-10 mM-potassium phosphate-10 mM-L-proline, pH 7.2, final volume 11.2 ml. At the times indicated samples (2 ml) were expelled into HClO₄. ATP was added to give a concentration of 5 mM. ○, CoASH; □, acetyl-CoA; ●, succinyl-CoA.

the initial controlled state before the addition of ADP was a true state 4 (in the nomenclature of Chance & Williams, 1956) with respiration limited by availability of ADP. To attempt to answer this question, mitochondria oxidizing proline in the absence of added ADP were extracted and analysed for ATP, ADP and AMP (Table 4). The large majority of the nucleotide was ATP, but ADP constituted a significant fraction (about 8%). If this ADP is indeed available to the ATP synthetase then this respiratory state may not be a true state 4. Fig. 2 presents the results of an experiment in which a high concentration of ATP was added to mitochondria oxidizing proline and in the ambiguous metabolic state discussed above. This addition should serve to remove the small amount of matrix ADP by translocation. The succinyl-CoA content falls on adding ATP. Thus the difference in mitochondrial succinyl-CoA content at the beginning and end of the experiment shown in Fig. 1 probably reflects the formation of ATP during the period of

state-3 respiration, and the attainment of true state-4 respiration in the latter phase of the experiment. In a discussion of the mechanism of the response of succinyl-CoA content to matrix ATP content, three different phases of the experiment shown in Fig. 1 have to be discriminated. In the first phase the adenylate energy charge is high (0.86 calculated from the data of Table 4). The elevated succinyl-CoA content suggests a high activity of glutamate dehydrogenase and 2-oxoglutarate dehydrogenase relative to that of succinyl-CoA synthetase (operating in the direction of succinyl-CoA cleavage) and subsequent reactions. Data presented below show that glutamate dehydrogenase is indeed active at this energy charge. In the second phase the addition of ADP leads to a presumably low (but undetermined) matrix energy charge, activating glutamate dehydrogenase slightly but succinyl-CoA synthetase (and possibly 'malic' enzyme, as discussed below) to a greater extent. This results in the fall in succinyl-CoA content which is observed. The final phase is one in which the energy charge of the matrix adenylates is probably essentially 1, as there is a large pool of extramitochondrial ATP. Under these conditions glutamate dehydrogenase is essentially inactive, and the succinyl-CoA content fails to rise significantly (Fig. 1).

Mitochondrial isocitrate, 2-oxoglutarate and malate were also assayed during proline oxidation, in experiments involving transitions from controlled to active (state 3) to controlled (state 4) respiration. Fig. 3 presents the mean results obtained from two experiments closely matched in protein concentration, concentrations of reagents and times of addition. It also presents values for the content of pyruvate, citrate and glutamate, which were obtained from one of the pair of experiments only. On adding ADP there is a fall in mitochondrial isocitrate content, but a slight rise in citrate content. Similar changes are seen when ADP is added to fly mitochondria oxidizing pyruvate plus proline (Johnson & Hansford, 1975) and have been attributed to an activation of NAD-isocitrate dehydrogenase and a failure of aconitase to maintain equilibrium in this state of high flux. There is no appreciable change in glutamate but a

Fig. 3. Mitochondrial content of pyruvate, glutamate and some tricarboxylate-cycle intermediates during proline oxidation

Mitochondria (14mg of protein) were incubated in 19ml of the medium described in the legend to Fig. 1. Where indicated on the oxygen-electrode trace 50 μ mol of ADP was added. Sampling was carried out as described previously (Johnson & Hansford, 1975) and at the times indicated. Sample contents shown in the table are nmol/mg of mitochondrial protein and are means from duplicate experiments for isocitrate, 2-oxoglutarate and malate. For the remainder the values are from one of the pair of duplicates.

Sample ...	Content			
	1	2	3	4
Glutamate				
Total	16.1	16.6	17.3	22.3
Mitochondrial	4.4	5.8	5.1	8.1
2-Oxoglutarate				
Total	3.67	3.25	2.96	2.89
Mitochondrial	0.78	0.44	0.15	0.24
Malate				
Total	4.50	4.63	5.11	7.59
Mitochondrial	1.76	1.44	1.60	3.08
Pyruvate				
Total	1.44	6.40	8.39	3.25
Mitochondrial	0.61	0.64	0.96	0.40
Citrate				
Total	6.37	7.98	10.52	14.50
Mitochondrial	1.92	2.30	5.25	8.17
Isocitrate				
Total	0.59	0.44	0.76	0.99
Mitochondrial	0.21	-0.03	0.29	0.41

fall in 2-oxoglutarate content. Finally, there is a slight decrease in mitochondrial malate content, which is in striking contrast with results with the blowfly, where ADP addition during pyruvate/proline or pyruvate/ATP/HCO₃⁻ oxidation invariably leads to a large increase (Johnson & Hansford, 1975). The changes

Fig. 4. Fluorescence of mitochondrial NAD(P)H during proline oxidation

Mitochondria (approx. 0.6mg of protein) were added to 2ml of a medium comprising 0.12M-KCl-20mM-Tes (potassium salt)-10mM-potassium phosphate, pH7.2. Where indicated, 20 μ mol of proline and 2 μ mol of ADP were added. The point corresponding to the depletion of the ADP (state 3→4) was determined by comparison with an oxygen-electrode experiment under identical conditions. Fluorescence was monitored as described in Hansford (1975).

of metabolite concentration as the mitochondria enter the final controlled state (state 4) are probably more informative, in view of the questionable nature of the initial controlled state. Fig. 3 shows that there is a clear rise in citrate and isocitrate content, suggesting a control at isocitrate dehydrogenase. In addition there is a rise in glutamate, though no clear change in 2-oxoglutarate content, which is consistent with a control at glutamate dehydrogenase, although it does not prove it. Finally, there is a rise in malate and a fall in pyruvate content, suggesting a control at 'malic' enzyme.

A fluorescence study of mitochondrial NAD(P)H content during proline oxidation (Fig. 4) shows that ADP leads to a slight oxidation, and that the completion of ADP phosphorylation is associated with a fairly large (34%) reduction. There is a subsequent phase of reoxidation, however, in which the nucleotide may become as little reduced (10%) as it is during ADP phosphorylation. This reoxidation of NADH during state 4 may reflect P_i uptake, and tends to corroborate the finding that the initial state is less than a state 4 in terms of adenylate energy charge (Table 4). This trace would tend to suggest that nicotinamide nucleotide redox state is not an important factor in adjusting the activities of tricarboxylate-cycle enzymes to the different demands of state-3 and state-4 respiration.

Fig. 5. Mitochondrial content of CoASH, acetyl-CoA and succinyl-CoA during the oxidation of pyruvate at low concentration

(a) Mitochondrial suspension (1 ml) containing 8.8 mg of mitochondrial protein was added to a medium comprising 0.12 M-KCl-20 mM-Tes (potassium salt)-10 mM-potassium phosphate-2.5 mM-KHCO₃-0.5 mM-ATP-0.46 mM-pyruvate (pH 7.2), total vol. 16.3 ml. Samples (2 ml) were subsequently taken at the times indicated, extracted and assayed as described in the Materials and Methods section and the legend to Fig. 1. Where indicated, ADP was added to the suspension to a concentration of approx. 4.2 mM. ○, CoASH; □, acetyl-CoA; ●, succinyl-CoA. (b) The oxygen-electrode trace is from the same experiment, and the medium was O₂-saturated.

Control of pyruvate oxidation

It was clear from the formation of citrate during proline oxidation (Fig. 3) and from the non-stoichiometry of NH₃ and pyruvate accumulation (Table 3) that these mitochondria possess the enzymes necessary for carrying out the portion of the tricarboxylate cycle from oxaloacetate to 2-oxoglutarate, and this indeed was confirmed by the rapid oxygen consumption obtained in the presence of pyruvate, ATP and KHCO₃ (Table 1). When CoASH and CoA thioesters were determined during pyruvate oxidation (Fig. 5) a pattern of change was obtained on the addition of ADP identical with that previously described in the blowfly (Hansford, 1974). Thus there was a large decrease in acetyl-CoA on adding ADP and an increase in CoASH and succinyl-CoA content. The changes reversed on depletion of the ADP, leaving only acetyl-CoA detectable in state 4. This was interesting, as the experiment was carried out at a low pyruvate concentration, such that the pyruvate remaining during the final state-4 phase of the experiment was calculated from the oxygen consumption to be not more than 40 μM. The implication is that the steady-state intramitochondrial concentration of pyruvate is less than 40 μM during state-4 proline oxidation (Fig. 1), as under those conditions the acetyl-CoA content is found to be much lower. This is consistent with the small amount of pyruvate found

(Fig. 3), which was mainly in the incubation medium, as expected from the presence of a pyruvate carrier found in other mitochondria (Papa *et al.*, 1971) and the ratio of medium:mitochondrial matrix volume of 10³ used in these experiments. The matrix pyruvate content determined by centrifugation and subtraction (Fig. 3) is much higher, but may well be in error, as it represents the difference between two relatively large numbers.

Measurement of the other tricarboxylate-cycle intermediates during controlled and active pyruvate oxidation (Fig. 6) gave results very similar to those obtained with the blowfly (Johnson & Hansford, 1975). Thus there is a fall in isocitrate and citrate content on activating respiration, identifying control at isocitrate dehydrogenase, and there is a marked rise in malate content. The latter change contrasts with the change obtained on activating proline oxidation (Fig. 3). These data again represent mean values obtained from two closely matched experiments, using different mitochondrial suspensions.

A fluorescence experiment (not shown) revealed that mitochondrial NAD(P) is essentially totally oxidized during controlled pyruvate oxidation, and becomes more reduced (24%) on stimulating respiration with ADP. This finding is also true of the blowfly (Hansford, 1975) and has been interpreted as an indication of the importance of adenine nucleotides in

Fig. 6. Mitochondrial content of tricarboxylate-cycle intermediates during pyruvate oxidation

Mitochondria (12mg of protein) were incubated in 19ml of the medium described in the legend to Fig. 5 with the single exception that the pyruvate concentration was 2.5mM. ADP (50μmol) was added where indicated on the oxygen-electrode trace. Sampling was carried out as described previously (Johnson & Hansford, 1975) and at the times indicated. Sample contents shown in the table are nmol/mg of mitochondrial protein and are means from duplicate experiments. Statistical significance of state 4 (samples 1 and 4)—state 3 (samples 2 and 3): * $P < 0.001$, † $P < 0.1$.

		Content			
	Sample ...	1	2	3	4
Citrate					
	Total	12.11	8.63	9.82	14.48
	Mitochondrial	9.11	4.00	5.18	8.24*
Isocitrate					
	Total	0.93	0.64	0.64	1.16
	Mitochondrial	0.56	0.16	0.15	0.50*
2-Oxoglutarate					
	Total	4.92	4.63	4.23	4.18
	Mitochondrial	0.36	0.43	0.40	-0.03†
Malate					
	Total	1.79	3.17	3.06	2.17
	Mitochondrial	0.11	1.33	1.20	0.15*

controlling dehydrogenase activity in insect flight muscle.

Effect of adenylate energy charge on glutamate dehydrogenase

Fig. 7 presents the results of an experiment in which glutamate dehydrogenase activity was measured in the direction of glutamate oxidation, by using a soluble fraction derived from the beetle mitochondria by sonication. Adenine nucleotides of total concentration 5mM were added to the cuvettes to give

Fig. 7. Effect of adenine nucleotide energy charge on the activity of glutamate dehydrogenase, operating in the direction of glutamate oxidation

Beetle mitochondria were subjected to ultrasonic irradiation (see the Materials and Methods section) and then to ultracentrifugation at 100000g for 30min. Portions (50μl) of the supernatant fluid were used for the measurement of glutamate dehydrogenase activity. The reaction mixture was as described in the Materials and Methods section, except that Triton X-100 was omitted, and the volume was 2.5ml. ATP, ADP and AMP were present in different proportions to give the energy-charge values indicated. The total concentration of adenine nucleotide was 5mM, and energy charge was calculated as described by Atkinson (1968), assuming an equilibrium constant of 1 for the adenylate kinase reaction. The reaction was initiated by the addition of L-glutamate to a final concentration of 40mM, immediately after the addition of the enzymically active fraction.

the energy-charge values shown. The enzyme is maximally active in the range 0.5–0.7. There is a slight decrease in activity at charge values less than 0.5, reflecting the lesser activation by AMP than by ADP. This range, however, is probably not of physiological interest. There is a sharp decline in activity at charge values of more than 0.9, and no detectable activity at values above 0.98. This behaviour accords well with the general findings and predictions concerning catabolic enzymes advanced by Atkinson (1968). Godinot & Gautheron (1972) have described the activation of glutamate dehydrogenase from heart by phosphate and by ADP. The effect of ADP appears to be more pronounced in the beetle. The work of Godinot & Gautheron (1972) raises the possibility that the dependence of activity on energy charge might be different at lower glutamate concentrations. The concentration used in the present work (40mM) was probably saturating. The effect of P_i on the enzyme activity was not investigated.

Fig. 8. Effect of ATP, succinate and ATP plus succinate on NAD-linked 'malic' enzyme activity

Beetle mitochondria were suspended in 1.5 ml of 50 mM-Hepes (potassium salt), pH 7.4, 3 mM-dithiothreitol and 0.5 mM-MnCl₂, giving a protein concentration of 2.0 mg/ml. The suspension was subjected to ultrasonic disintegration (see the Materials and Methods section) and then ultracentrifugation at 100000g for 30 min. Portions (20 μl) of the supernatant fluid were used for the assay of 'malic' enzyme activity. The reaction was initiated by adding the enzyme to 1 ml of reaction medium comprising 50 mM-Hepes (potassium salt) (pH 7.4), 5 mM-MnCl₂, 1 mM-NAD⁺, 2.5 mM-dithiothreitol, 2 mM-KCN, rotenone (0.5 μg/ml), oligomycin (0.5 μg/ml), with malate, succinate and ATP as shown. Increase in E_{340} was recorded. The reaction catalysed by malate dehydrogenase (EC 1.1.1.37) was at equilibrium by the time recording was begun, as confirmed by a lack of increase in E_{340} on adding 25 μg of purified malate dehydrogenase. ●, No additions; ○, +2 mM-succinate; Δ, +2 mM-succinate+2 mM-ATP; □, +2 mM-ATP.

Properties of a crude 'malic' enzyme derived from beetle mitochondria

Sauer (1973) has demonstrated inhibition by ATP of a 'malic' enzyme from adrenal-cortex mitochondria, and the data of Fig. 3 made it seem likely that some such mechanism was involved in the state 3 to 4 transition. Assays of mitochondrial fractions prepared either by solubilization with Triton X-100 or by sonication showed that the enzyme is indeed inhibited by ATP, and this is shown in Fig. 8. Succinate acts as an activator, as described by Sauer (1973) and for an NADP-linked mitochondrial enzyme by Frenkel (1972). The apparent K_m for malate at a saturating NAD⁺ concentration is 1.1 mM and the K_i for ATP is 0.86 mM, the latter value being derived from the linear Lineweaver-Burk plots obtained in the presence of succinate, and the presence or absence of ATP (Fig. 8). Comparison of these values for K_m and K_i with the malate and ATP contents actually determined (Fig. 3 and Table 4 respectively) suggests that 'malic' enzyme may indeed be rather inactive during controlled proline oxidation. The experimental

Fig. 9. Effect of pyruvate on NAD-linked 'malic' enzyme activity

The 100000g supernatant fraction of a mitochondrial sonicate was used as the source of enzyme, and conditions were as described in the legend to Fig. 8. Pyruvate present at (□) 0.5 mM and (○) 2.5 mM; (●) no added pyruvate.

finding that these mitochondria will oxidize pyruvate in the presence of ATP and HCO₃⁻ (Table 1) or, suboptimally, pyruvate alone (not shown) suggested that under these conditions there must be a conservation of C₄ intermediate, and thus a limitation of 'malic' enzyme activity. Accordingly, experiments were carried out to see whether pyruvate inhibits 'malic' enzyme activity, and this was found to be so (Fig. 9). This is thought to be a novel property of a 'malic' enzyme, and seems to be more than product inhibition, in that the inhibition by pyruvate is non-competitive with respect to malate.

Discussion

The striking rate of proline oxidation demonstrated in beetle flight-muscle mitochondria by de Kort *et al.* (1973) and confirmed in the present study made it possible to explore the way in which insect flight muscle achieves the active oxidation of glutamate. Table 3 shows that a considerable proportion of the glutamate oxidation is associated with NH₃ production, and therefore involves glutamate dehydrogenase. By contrast, it has been found that glutamate oxidation in mammalian mitochondria occurs mainly via transamination. Even in liver, which contains an active glutamate dehydrogenase, the transaminase

Table 5. *Computed fluxes during proline oxidation*

(1) Oxygen consumption is from the data of Table 3(a), corresponding to state-3 respiration. (2) Pyruvate production is approximate, based on comparison of Table 3(a) and 3(b), and assuming that the pyruvate/O₂ ratio is the same at a given O₂ consumption in the two experiments. (3) Pyruvate production $\times 6$. (4) [NH₃ production (from Table 3(a) – pyruvate production) $\times 11$. (5) Sum of columns (3) and (4). (6) Column (1) – column (5). (7) Column (6)/5.

(1)	(2)	(3)	(4)	(5)	(6)	(7)
Oxygen consumption (ng-atoms/mg of protein)	Pyruvate production (nmol/mg of protein)	Oxygen equivalence of pyruvate (ng-atoms/mg)	Oxygen equivalence of CO ₂ (ng-atoms/mg)	Oxygen consumption dependent on glutamate dehydrogenase activity (ng-atoms/mg)	Oxygen consumption dependent on transaminase (ng-atoms/mg)	Expected alanine production (nmol/mg)
917	14	84	759	843	74	15
2230	26	156	1830	1986	244	49
3000	35	210	2080	2290	710	142

pathway predominates, with the exact proportions depending on the mitochondrial energy status (Borst, 1962; Chappell, 1964; de Haan *et al.*, 1967; Papa *et al.*, 1967). Table 5 uses the results from Table 3 for oxygen consumption, pyruvate production and NH₃ production, which are parameters measurable with some accuracy, and computes the alanine production expected, based on an assumption of 11 g-atoms of oxygen/mol of proline oxidized via glutamate dehydrogenase and 5 g-atoms of oxygen/mol of proline oxidized via alanine aminotransferase. These assumptions seem to be justified, since it is known from Fig. 3 that the accumulation of other intermediates is small. It is found that the computed alanine production is of the same order of magnitude as that measured, the measurement being admittedly rather imprecise. The metabolism of glutamate in the Japanese beetle thus differs from that described by Bursell (1963, 1965, 1966, 1967) in the Tsetse fly, where the transamination pathway operates to the exclusion of glutamate dehydrogenase. The operation of the transaminase pathway would lead to the production of ten times the alanine actually found, and to no NH₃. The assay of glutamate dehydrogenase in beetle mitochondrial extracts shows that it has the activity necessary for a major role in proline catabolism, and moreover that it possesses the extreme sensitivity to adenylate energy charge often seen in enzymes catalysing rate-limiting steps in catabolic pathways (Atkinson, 1968). This sensitivity to energy charge would be expected to favour an oxidation of glutamate by transamination during state-4 proline oxidation. In fact, some NH₃ is still produced in the resting state (Table 3), though no claim is made about stoichiometry. It should be remembered that this initial state of proline oxidation may not be truly state 4 and that the energy charge of 0.86 found experimentally in this initial state (Table 4) would permit a fairly high dehydrogenase activity (Fig. 7). NH₃

production was not investigated in a true state 4, i.e. after a cycle of ADP-stimulated respiration.

The active proline oxidation seen in these mitochondria provides intramitochondrial tricarboxylate-cycle intermediates which are not free to leave the mitochondria, as supported by the data in Table 1 and shown in Figs. 3 and 6. This would be osmotically disastrous if an exceptionally active 'malic' enzyme were not present in these mitochondria (Table 2), allowing the formation of pyruvate from malate. The pyruvate may then leave the mitochondria or be further oxidized. The activity of this 'malic' enzyme is in the range 300–400 nmol/min per mg of mitochondrial protein, which may be compared with the activity of 17.6 nmol/min per mg of mitochondrial protein reported by Sauer (1973) in adrenal-cortex mitochondria and described as the highest specific activity reported. There has been some question of the role that mitochondrial 'malic' enzymes play (Davis *et al.*, 1972; Brdiczka & Pette, 1971). The peculiar metabolism of the beetle mitochondria makes this activity comprehensible and indeed mandatory. It is considered that 'malic' enzyme is more likely to be involved physiologically than oxaloacetate decarboxylase, which has been suggested to participate in proline oxidation in the Tsetse fly (Bursell, 1965), as the tissue concentration of oxaloacetate is probably extremely low. In this context Dean & Bartley (1973) suggest that in liver mitochondria the K_m of oxaloacetate decarboxylase is approximately two orders of magnitude higher than the oxaloacetate concentration. It may be that the Tsetse fly possesses high 'malic' enzyme activity, and that the decarboxylation of oxaloacetate represents a portion of the enzyme mechanism. This was shown to be true for the NADP-linked 'malic' enzyme (see Kun, 1963), but not for the NAD-linked enzyme from *Ascaris* muscle (Fodge *et al.*, 1972). The finding that the 'malic' enzyme is inhibited by

pyruvate (Fig. 9) may explain why these mitochondria can oxidize pyruvate alone, without depleting the tricarboxylate cycle of intermediate required in catalytic quantities. It is not known to what extent the oxidation of pyruvate derived from glycolysis occurs in the living insect.

There have been recent studies on the steady-state contents of tricarboxylate-cycle intermediates in flight-muscle mitochondria from one insect, the blowfly, under defined metabolic conditions (Hansford, 1974; Johnson & Hansford, 1975). The present work extends the former study to an insect in which the operation of the cycle is significantly different. When exogenous pyruvate is the substrate (Figs. 5 and 6) the patterns of change are very similar to those described in the blowfly (Johnson & Hansford, 1975) and clearly implicate a primary control at isocitrate dehydrogenase. With proline as substrate this control is not so obvious, but it is suggested by the rise in citrate and isocitrate content on the depletion of ADP (Fig. 3). The same change in metabolic state gives rise to an increased glutamate content and a somewhat equivocal fall in 2-oxoglutarate, consistent with a control at glutamate dehydrogenase. This might be translated into a control at proline dehydrogenase, if proline dehydrogenase from the beetle shows the sensitivity to inhibition by glutamate which is seen in some other insects (Norden & Venturas, 1972). Finally, the fall in malate content on eliciting state-3 respiration, markedly different from what occurs in the fly and from what occurs in the beetle when pyruvate is the substrate, suggests an adenine nucleotide control at 'malic' enzyme. Although the equilibrium constant for this reaction ($K_{eq} = 19.6M^{-1}$) would at first sight make effective control seem unlikely, it remains a real possibility, as the CO_2 content of the flight muscle may be low. Thus the beetle mitochondria may provide the first evidence for the functioning in the mitochondrion of the ATP control shown to apply to mitochondrial 'malic' enzymes isolated from adrenal cortex (Sauer, 1973) and from heart (Lin & Davis, 1974). It is noteworthy that during controlled pyruvate oxidation (Figs. 5 and 6) acetyl-CoA content is very high relative to that of succinyl-CoA, and citrate synthase activity is apparently limited by the oxaloacetate concentration, as the malate content is very low. By contrast, malate content (and presumably oxaloacetate) is high during controlled proline oxidation, and citrate synthase activity is probably limited by the high succinyl-CoA/acetyl-CoA ratio, the latter being a mechanism proposed to apply in mammalian heart (La Noue *et al.*, 1972) under conditions giving rise to oxidized nicotinamide nucleotide.

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