The substrate-specific impairment of oxidative phosphorylation in liver mitochondria from high-protein-fed chickens

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Chickens fed on semi-purified low (7%) or high (61%) protein-energy diets for 14 or 17 d were used for determinations of oxidative phosphorylation and specific amounts of mitochondrial protein in liver. The ADP:oxygen (ADP:O) values obtained when pyruvate + malate were used as substrates were significantly reduced in the high-protein-fed group after the 4th day compared with those for the group fed the low-protein diet, while the differences in ADP:O values between the two treatments when L-glutamate was used as substrate were found to be significant on the 14th day. At any feeding period no significant differences in ADP:O values were observed between the two groups when α-ketoglutarate, malate, or octanoate + malate were used as substrates, nor in specific amounts of mitochondrial protein in liver. The dependency of the pyruvate + malate-supported respiration rate on the temperature in the reaction medium was also determined. The results of an Arrhenius plot showed that transition temperatures, and the lower and upper energies of activation, were similar for the groups fed on low- and high-protein diets. Furthermore, no morphological changes in mitochondria were observed among chickens fed on diets with various protein levels for 14 d. From these results we concluded that the reduction of ADP:O value with pyruvate + malate or L-glutamate substrates in chickens fed on a high-protein diet was substrate-specific, and was not due to functional damage to the respiratory chain for electron flow from NAD-linked substrates to the ubiquinone pool, nor to modulation of properties of the inner mitochondrial membrane.

Mitochondria: Oxidative phosphorylation: Chicken liver

We proposed that the impaired oxidative phosphorylation capacities seen with increasing dietary protein level may be associated with functional damage to the respiratory chain for electron flow from NAD-linked substrate to the ubiquinone pool, since the responses of hepatic mitochondria to dietary protein were observed only when pyruvate + malate and L-glutamate were used as substrates but not with succinate or ascorbate + N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD; Tanaka *et al.* 1993). However, the change in oxidative phosphorylation capacity would not necessarily be specified by the only entrance site for electron flow. For example, Hoppel *et al.* (1979) demonstrated that the state 3 oxidation rate for β-hydroxybutyrate and succinate declined in rats fed on a riboflavin-deficient diet, but that the oxidations of pyruvate, glutamate, and α-ketoglutarate were not changed. Therefore, whether the mechanism through which the ADP:oxygen (ADP:O) value for pyruvate + malate is impaired in chickens fed on a high-protein diet can be identified with that for L-glutamate has to be investigated and the responses of the ADP:O value to dietary protein level for NAD-linked substrates other than pyruvate + malate or L-glutamate are not yet known.

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Alternatively, the impaired ADP:O value could be due to dysfunction of F_0F_1-ATPase, that is, oligomycin-sensitive ATPase (*EC* 3.6.1.3), since membrane fluidity could be one of the important determinants of activity of F_0F_1-ATPase (Zsigmond & Clandinin, 1986; Echegoyen *et al.* 1993). To check this possibility the effects of dietary protein level on the dependency of liver mitochondrial respiration on the temperature of the reaction medium were determined using an Arrhenius plot.

We also reported that the ATP synthesis per mg mitochondrial protein decreased with increasing dietary protein level in chicken liver mitochondria (Toyomizu *et al.* 1992; Tanaka *et al.* 1993). As mitochondrial proliferation could occur by compensatory mechanisms to protect hepatic energy metabolism (Krahenbuhl *et al.* 1991), the question of whether the amount of mitochondrial protein in liver would be affected by dietary protein level was posed.

In order to demonstrate the time courses of mitochondrial functions with various NAD-linked substrates and of specific amounts of mitochondrial protein over a period of 14 or 17 d after feeding of experimental diets, we chose only two levels of dietary protein, low (7% energy) and high (61% energy); our previous study had indicated that the ADP:O value significantly decreased with increasing protein level (7, 25, 43, and 61% of metabolizable energy content; Toyomizu *et al.* 1992). We used pyruvate+malate as substrates for respiration in order to assess the temperature-dependency with an Arrhenius plot.

MATERIALS AND METHODS

Animals and diets

Male chickens (Cobb) were obtained from a commercial hatchery (Ishida Poultry and Eggs Co. Ltd., Nagaoka 940, Japan) at 1 d of age, and were housed in electrically heated batteries. They were provided *ad lib.* with water and a commercial starter diet for 9 d in Expts 1 and 2, and for 12 d in Expt 3. In these experiments, chickens were housed in a wire-bottomed aluminium cage under conditions of controlled light (14 h light, 10 h dark) and temperature ($25 \pm 2°$). For these three experiments, four kinds of experimental diets were prepared, providing protein at 7, 25 (control diet), 43, and 61% of total energy on a metabolizable energy (ME) basis by replacing carbohydrate at a constant fat level (Table 1). The carbohydrate source was a yellow maize–α-maize starch–glucose (2:3:5, by wt) mixture. The fat source was soyabean oil. The protein source was an isolated soyabean protein–soyabean meal–L-methionine–L-lysine monohydrochloride (657:326:12:5, by wt) mixture. All the diets contained the same amount of crude fibre, crude fat, minerals and vitamins on a ME basis.

In Expt 1, after a 5 d adaptation period to the control diet, eighty-eight chickens were used for measurement of oxidative phosphorylation with diverse NAD-linked substrates. In one series, forty chickens were fed on diets containing either 7 or 61% protein-energy for 2, 4, 6, 9 and 14 d, and in another series, forty-eight chickens were fed on the two diets for 2, 4, 6, 9, 13 and 17 d (Expt 1.1). Two further groups of eight chickens corresponding to these two trials were used to determine the zero time baseline. In the latter series we also attempted to determine mitochondrial protein contents in liver (Expt 1.2).

In Expt 2, liver mitochondria isolated from four chickens in each of two groups fed on diets containing 7 and 61% protein-energy for 16 d after a 5 d adaptation period were used to evaluate the dependency of pyruvate+malate-supported respiration on the temperature of the reaction medium.

In Expt 3, for the study of the ultrastructural morphology of isolated liver mitochondria we used five chickens in each of four groups fed on diets containing either 7, 25, 43 or 61% protein-energy for 14 d after a 2 d adaptation period.

Table 1. *Composition of experimental diets* (g/kg)*

Protein levels as metabolizable energy (% total energy)...	7	25	43	61
Isolated soyabean protein†	47·12	201·23	354·62	507·33
Soyabean meal†	23·42	100·02	176·26	252·16
L-Methionine†	0·86	3·67	6·48	9·27
L-Lysine monohydrochloride†	0·37	1·59	2·80	4·01
Soyabean oil	46·41	46·74	47·06	47·40
Yellow maize‡	160·33	115·22	70·35	25·69
α-Maize starch‡	240·49	172·84	105·52	38·53
Glucose‡	400·82	288·06	175·87	64·21
Cellulose	16·95	13·79	10·53	7·29
Calcium phosphate, dibasic	27·89	25·20	22·53	19·87
Calcium carbonate	13·11	12·59	12·06	11·55
Potassium chloride	8·61	5·76	2·92	0·00
Sodium chloride	3·88	3·60	3·32	3·05
Trace mineral mixture§	5·40	5·39	5·37	5·36
Vitamin mixture§	4·32	4·31	4·30	4·29
Total	1000·00	1000·00	1000·00	1000·00
Metabolizable energy (MJ/kg), calculated	14·01	13·97	13·93	13·90

* All the diets contained the same amount of crude fibre, crude fat, minerals and vitamins on a metabolizable energy basis.

† Protein consisted of an isolated soyabean protein–soyabean meal–L-methionine–L-lysine monohydrochloride (657:326:12:5, by wt) mixture.

‡ Carbohydrate consisted of a yellow maize–α maize starch–glucose (2:3:5, by wt) mixture.

§ See Akiba & Matsumoto (1978).

All chickens were killed by cervical dislocation.

Expt 1.1. Mitochondrial isolation from liver, and oxidative metabolism

Liver mitochondria were prepared according to the methods of Hoppel *et al.* (1979). The oxidation rates of isolated liver mitochondria were measured in the following reaction mixture (pH 7·0): 80 mM-KCl, 50 mM-3(*N*-morpholino)propanesulphonic acid (Mops), 5 mM-KH_2PO_4, 1 mM-ethylene glycol bis (β-aminoethylether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA), and 1 g albumin/l. Substrate concentrations were 10 mM-pyruvate + 2·5 mM-malate plus 10 mM-malonate, 10 mM-L-glutamate, 10 mM-α-ketoglutarate, 2·5 mM-malate, 0·8 mM-octanoate + 2·5 mM-malate, and 0·16 mM-palmitate + 2·5 mM-malate. When palmitate + malate were used as substrates, 2 mM-L-carnitine, 2 mM-ATP, and 1 mM-coenzyme A were added to the reaction mixture. To avoid bias we measured O consumption of each mitochondrial preparation from the two dietary groups according to a systematically randomized order that was different on each day. Mitochondrial protein used was 50 μg/ml and oxidation rate expressed in ng O_2/mg mitochondrial protein per min was measured polarographically in a total reaction volume of 2 ml at 37° using an O_2 monitor (Toyomizu & Clandinin, 1993). The state 3 respiration rate in the presence of ADP and the ADP:O value were determined on the third and subsequent cycles as described by Chance & Williams (1956) and Chappell (1964). The exact ADP concentration was determined spectrophotometrically (Jaworek *et al.* 1974). Protein was measured by a colorimetric method (Lowry *et al.* 1951).

Expt 1.2. Enzyme activities and mitochondrial protein content in liver

Liver was dissected and immediately worked up for the determination of enzyme activities for both liver homogenates and the isolated mitochondria. Liver homogenates were prepared according to the method of Rafael *et al.* (1984); the liver was carefully minced with a pair of scissors, homogenized in ice-cold 50 mM-KH_2PO_4 buffer (pH 7·2), 1 mM-EDTA, and 50 g/l of the minced liver by means of a tightly fitting 20 ml glass–glass homogenizer (20 strokes at 200 rev./min). Liver mitochondria were isolated as described above. Cytochrome oxidase (*EC* 1.9.3.1) activity was determined polarographically in freshly prepared unfrozen samples of the liver homogenates and the mitochondrial suspension, as described by Sottocasa *et al.* (1967). Succinate-cytochrome c reductase (*EC* 1.3.99.1) was assayed in frozen-and-thawed samples of liver homogenates and mitochondrial stock suspension at 25° according to the method of Sottocasa *et al.* (1967). The enzyme activities are expressed in nanokat/mg mitochondrial protein per min or nanokat/mg liver per min.

Expt 2. Temperature dependency of oxidation rate in liver mitochondria

The dependence on temperature of mitochondrial O consumption was determined essentially according to the method of Wander & Berdanier (1985). Briefly, this involved incubating with a 300 mOsm medium (pH 7·2–7·4) containing 200 mM-sucrose, 10 mM-Tris (hydroxymethyl)aminomethane-hydrochloride buffer, 5 mM-$MgCl_2$, 5 mM-KH_2PO_4, 5 mM-EGTA, 10 mM-pyruvate, 2·5 mM-malate, and 10 mM-malonate in a 2·0 ml chamber. O consumption was measured at 3° intervals from 4° to 40° in the presence of 0·22 mM-ADP. To ensure that the system was saturated with O, air was bubbled through the buffer before the addition of the substrate, the mitochondria, and the ADP at the temperature being investigated; thereafter a stable 100% line was recorded. Osmolarity was adjusted by addition of sucrose using an osmometer (Semi-micro osmometer, Knauer, Germany).

The Arrhenius plot consisted of measuring pyruvate + malate-supported O consumption rate in state 3 at 3° intervals and calculating the activation energies and the transition temperatures using the equation derived by Arrhenius:

$$\frac{d\ln k}{dt} = \frac{Ea}{RT^2}, \tag{1}$$

where k is the rate constant, R the gas constant (8·312 J/(mol.k)), Ea the activation energy, and T the transition temperature in degrees Kelvin. Integration of equation 1 and conversion to base 10 logarithms gives

$$\log\left(\frac{k_2}{k_1}\right) = \frac{Ea}{2{\cdot}303R}\left(\frac{1}{T_1} - \frac{1}{T_2}\right), \tag{2}$$

from which it can be seen that the value of Ea can be obtained from the slope of the straight line obtained when the logarithm of k is plotted against the reciprocal of the absolute temperature.

Expt 3. Electron microscopy

Isolated liver mitochondria were fixed according to the methods of Yamauchi *et al.* (1990). Specimens were embedded in Spurr's plastic mixture (Spurr, 1969). Thin sections were cut with a Reichert Om4 ultramicrotome equipped with a glass knife. Sections were picked up on 400 mesh grids. Specimens were examined with a Jeol 100B electron microscope at 80 kV.

Statistics

Expts 1.1 and 1.2. The data were analysed using the Statistical Analysis System (1985). As oxidative measurements were done using mitochondria individually isolated from liver for comparing between times of measurements among treatment groups, the unpaired *t* test was used to determine whether significant differences existed within a group compared with values for zero time baseline and whether significant differences existed between the low (7% energy)- and high (61% energy)-protein-fed groups.

Expt 2. Wolfe & Bagnall (1979) commented that breaks calculated from Arrhenius plots involve mathematical or biochemical artefacts and can be varied by experimental design. Minimum Akaike's information criterion estimate (MAICE) provides a versatile method used in wide fields of statistical model building (Akaike, 1974). Therefore, the variable two-line fits were done with the MAICE to calculate the slopes for the Arrhenius plot and the inflections were determined (Iwaya-Inoue *et al.* 1989). The unpaired *t* test was used to determine whether significant differences existed between dietary treatments. All data are expressed as mean values and standard deviations (SD).

RESULTS

The time courses of ADP:O value and state 3 oxidation rate in liver mitochondria of chicks fed on low- and high-protein diets are shown in Fig. 1 when pyruvate + malate (a), L-glutamate (b), α-ketoglutarate (c), malate (d), octanoate + malate (e), or palmitate + malate (f) were used as substrates (Expt 1.1). When pyruvate + malate were used as substrates the ADP:O values for the first few days gradually decreased in the high-protein-fed group and increased slightly in the low-protein-fed group. On the 4th day of feeding their differences were found to be significant and tended gradually to become more so ($P < 0{\cdot}05$). State 3 oxidation rates in both groups increased slightly but definitely, except for the high-protein-fed group on the 4th and 9th days when the rates were significantly lower in the high-protein-fed group than the low-protein-fed group. When L-glutamate was used as a substrate, differences in the ADP:O values between the two groups were found to be significant on the 14th day of feeding ($P < 0{\cdot}05$) whereas no significant differences were observed in the state 3 oxidation rates at any feeding period. No significant differences were observed in the ADP:O values nor in state 3 rates for α-ketoglutarate, malate, octanoate + malate, or palmitate + malate at any feeding period between the two groups.

In order to estimate mitochondrial protein content the specific activities of cytochrome c oxidase and succinate-cytochrome c reductase were determined as mitochondrial marker enzymes in the liver homogenate and in the mitochondria (Expt 1.2). The activities of these enzymes in both liver homogenates and mitochondria (Fig. 2) were similar in magnitude to those previously reported for rat liver (Rafael *et al.* 1984). The cytochrome c oxidase activities in both liver and mitochondria decreased gradually after feeding the two kinds of diets, and they tended to be higher in chickens fed on the 61% protein-energy diet than in those fed on the 7% protein-energy diet (Fig. 2(a)). In the high-protein-fed group succinate-cytochrome c reductase activities in both liver and mitochondria showed a transient increase on the 2nd day, returned to their original levels by the 13th day, and then increased immediately. In the low-protein-fed group they showed a transient decrease on the 4th day and increased gradually, and they tended to be significantly higher in chickens fed on the 61% protein-energy diet than in those fed on the 7% protein-energy diet (Fig. 2(b)). On the basis of enzyme activities in the mitochondrial preparations and in the liver homogenates the mitochondrial protein content/g liver was calculated. No significant differences were observed between the two groups in the estimate of mitochondrial protein

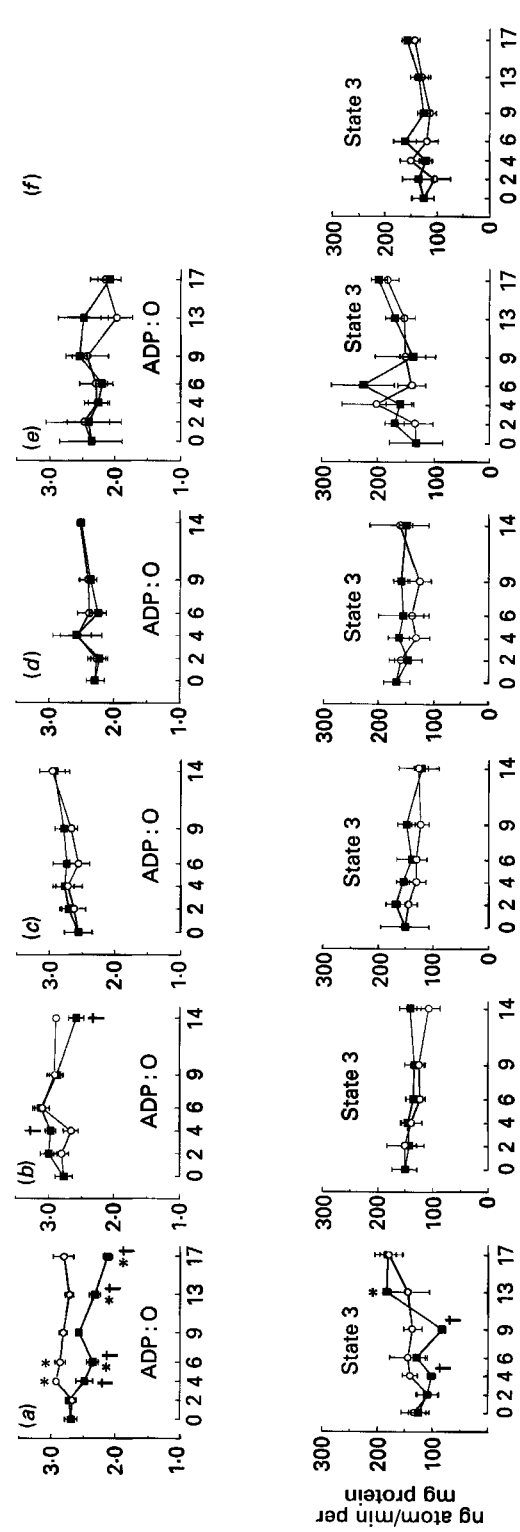

Fig. 1. ADP:oxygen (ADP:O) value and state 3 oxidation rate in liver mitochondria from chicks fed on diets containing either 7 (○) or 61 (■)% energy as protein, followed for 14 or 17 d. The substrate groups shown are (a) pyruvate + malate, (b) L-glutamate, (c) α-ketoglutarate, (d) malate, (e) octanoate + malate and (f) palmitate + malate. Values are means for four chickens per treatment and eight chickens at baseline, with standard deviations indicated by vertical bars. * Mean values were significantly different from baseline, $P < 0{\cdot}05$. † Mean values were significantly different from those for chickens on the low-protein diet, $P < 0{\cdot}05$. For details of diets and procedures, see Table 1 and pp. 798–801.

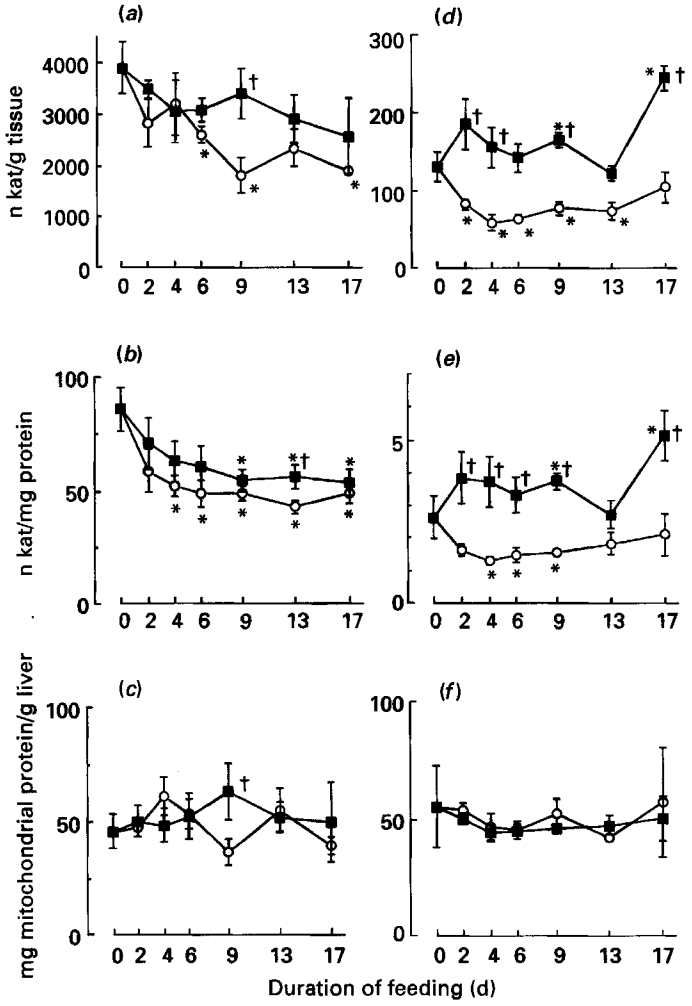

Fig. 2. Activities of cytochrome oxidase (*EC* 1.9.3.1) and succinate-cytochrome c reductase (*EC* 1.3.99.1) in liver and mitochondria from chicks fed on diets containing 7 (○) or 61 (■) % energy as protein, followed for 17 d. (a) Liver and (b) mitochondrial cytochrome oxidase activities; (c) specific amounts of mitochondrial protein calculated from the activities of cytochrome oxidase in liver and mitochondria; (d) liver and (e) mitochondrial succinate-cytochrome c reductase activities; (f) specific amounts of mitochondrial protein calculated from activities of succinate-cytochrome c reductase in liver and mitochondria. Values are means for four chicks per treatment and eight chicks at baseline, with standard deviations indicated by vertical bars. * Mean values were significantly different from baseline, $P < 0.05$. † Mean values were significantly different from those for chicks fed on the low-protein diet, $P < 0.05$. For details of diets and procedures, see Table 1 and pp. 798–801.

content/g liver calculated using the two enzyme activities at any feeding period except for those from cytochrome c oxidase on the 9th day of feeding.

Fig. 3 shows two Arrhenius plots of the rates of pyruvate+malate-supported O consumption for liver mitochondria from the high- and low-protein-fed groups, against the reciprocal of the absolute temperature (Expt 2). The Arrhenius plots for both groups were biphasic, that is, two linear portions with different slopes intersecting at 21·9 (SD 1·7)° for the low-protein-fed group and at 23·0 (SD 0·9)° for the high-protein-fed group. No differences in the transition temperature between the two groups were observed ($P = 0.35$).

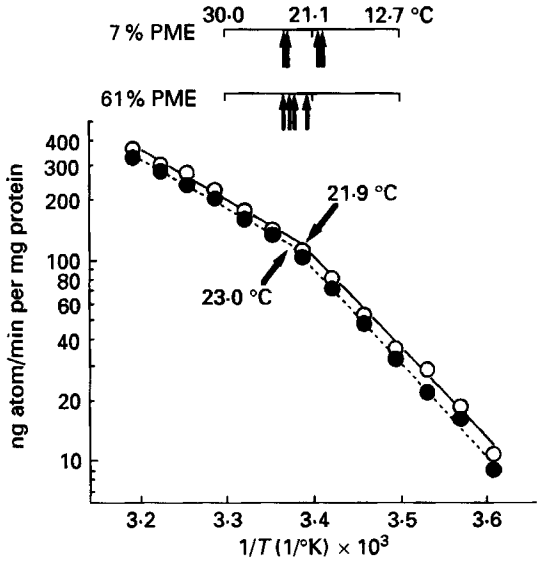

Fig. 3. Arrhenius plots of the rate of state 3 oxidation with pyruvate+malate as substrates against the reciprocal of absolute temperature (°K) in liver mitochondria from chicks fed on diets containing 7 (○) or 61 (●)% energy as protein (PME) for 16 d. Oxygen consumption is expressed in ng O_2/mg mitochondrial protein per min.

The values for activation energies (kJ/mol) of pyruvate+malate oxidation process below and above the transition temperature were 87·2 (SD 6·0) and 50·0 (SD 6·5) respectively in mitochondria from chickens fed on the low-protein-energy diet, and 90·0 (SD 1·7) and 47·6 (SD 3·3) respectively in those from the high-protein-fed chickens. There were no significant differences in activation energies below ($P = 0{\cdot}47$) and above ($P = 0{\cdot}60$) the transition temperature between the two groups.

Irrespective of dietary protein level, mitochondrial pellets obtained from chickens were typical in appearance (Expt 3, results not shown), showing little evidence of contamination by other cellular constituents and no differences among dietary treatments.

DISCUSSION

In comparison with the low-protein-fed group the ADP:O values for pyruvate+malate were significantly reduced in the high-protein-fed group throughout the course of the experiment after the 4th day, while state 3 oxidation rate tended to decrease in the high-protein-fed group from the 4th to the 9th day (Fig. 1(a)). The details of response pattern of the ADP:O value to dietary protein level and the degree of the reduction in the value for chickens fed on the diet containing 61% protein-energy were similar to those observed previously (Tanaka *et al.* 1995), whereas the effect of dietary protein on the state 3 oxidation rate was not entirely in agreement with the previous results, which showed reduction in the rate of state 3 oxidation in the high-protein-fed group after the 2nd day. These findings suggest that decreased ADP:O values with pyruvate+malate in liver mitochondria from chickens fed on a high-protein-energy diet might not be a direct consequence of changes in the state 3 respiration. When L-glutamate was used as a substrate the differences in ADP:O value between treatments were found to be significant on the 14th day (Fig. 1(b)), in agreement with our previous results which showed that chickens fed on a high-protein diet for 21 d exhibited a decreased ADP:O value with L-glutamate (Tanaka *et al.* 1993). The present results clearly show that there was a time-lag

between the appearance of impairment of ADP:O value in the high-protein-fed group for pyruvate + malate and that for L-glutamate. At any feeding period, no significant differences were observed in the ADP:O values for α-ketoglutarate, malate, octanoate + malate, or palmitate + malate between the two groups (Fig. 1(c), (d), (e) and (f)). From these results we could conclude that the mechanism through which the ADP:O value for pyruvate + malate in chickens fed on a high-protein-energy diet was impaired is not the same as that for L-glutamate, and that the impairment of ADP:O value for pyruvate + malate or L-glutamate is substrate-specific rather than due to functional damage to the respiratory chain for electron flow from NAD-linked substrates to the ubiquinone pool.

Changes in thickness of the lipid bilayer and lipid composition alter mitochondrial ATPase function (Zsigmond & Clandinin, 1986), thereby possibly reducing the ADP:O value. The present results of the Arrhenius plot showed that the transition temperature and lower and upper energies of activation were similar for the 7 and 61% protein-fed groups (Fig. 3), implying that in both groups the physical properties of the lipids of the membrane were maintained. We have already demonstrated that no changes in sensitivity of mitochondrial ATPase activity to oligomycin, expressed as a percentage of total ATPase activity, were observed among chickens fed for 21 d on diets with various protein levels. The present results, and this demonstration, suggest that the attenuated ADP:O values in the high-protein-fed group may not be associated with modulation of properties of the inner mitochondrial membrane, such as degrees of fluidity and integrity.

We have reported that the reduction in oxidative phosphorylation in the heart and liver of animals fed on a high-protein-energy diet may partly contribute to the depression of body fat (Toyomizu *et al.* 1992). However, to protect such a decrease in efficiency of oxidative phosphorylation in chickens fed on a high-protein-energy diet, compensatory mechanisms involving increasing mitochondrial protein content could be proposed. In fact, it was shown that rats treated with a cobalamin analogue had decreased state 3 oxidation rates for diverse substrates but increased mitochondrial content per hepatocyte (Krahenbuhl *et al.* 1991). Further, Zaragozá *et al.* (1987) observed a significant increment in the volumetric density, numerical density and size of hepatocyte mitochondria in rats treated with a high-protein diet. In the present experiment no differences were observed, during any feeding period, in the specific amounts of mitochondria in the liver, calculated from the activities of mitochondrial marker enzymes, cytochrome oxidase or succinate-cytochrome c reductase, in the liver and in the mitochondria (Fig. 2(a) and (b)). Furthermore, neither megamitochondria nor gross structural damage to mitochondria was detected in any dietary treatment. Therefore, we conclude that compensatory proliferation, enlargement of mitochondria, and morphological alterations in mitochondria were not induced by feeding a high-protein diet.

Other possible mechanisms through which impairments of ADP:O value occur in high-protein-fed chickens may exist associated with specific changes in the ATP-consuming reaction. In chickens fed on a high-protein-energy diet the ATP generated during oxidative phosphorylation would be consumed in the reactions from pyruvate or glutamate to the other product, resulting in a lower value of ADP to O_2 consumption in the electron transport chain. On the high-protein-energy diet the time-lag between the appearance of impaired ADP:O value for pyruvate + malate and that for L-glutamate (Fig. 1(a) and (b)) might be associated with a difference in the induction period after feeding a high-protein-energy diet between both enzymes requiring ATP. Further studies are necessary to elucidate the mechanism of the substrate-specific reduction in ADP:O value in chickens fed on a high-protein-energy diet, and, ultimately the metabolic and bioenergetic responses to dietary protein by the whole body.

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REFERENCES

Akaike, H. (1974). A new look at the statistical model identification. *Institute of Electrical and Electronics Engineers Transactions on Automatic Control* **AC-19**, 716–723.

Akiba, Y. & Matsumoto, T. (1978). Effects of force-feeding and dietary cellulose on liver lipid accumulation and lipid composition of liver and plasma in growing chicks. *Journal of Nutrition* **108**, 739–748.

Chance, B. & Williams, G. R. (1956). The respiratory chain and oxidative phosphorylation. *Advances in Enzymology* **17**, 65–134.

Chappell, J. B. (1964). The oxidation of citrate, isocitrate and *cis*-aconitate by isolated mitochondria. *Biochemical Journal* **90**, 225–237.

Echegoyen, S., Oliva, E. B., Sepulveda, J., Díaz-Zagoya, J. C., Espinosa-García, M. T., Pardo, J. P. & Martinez, F. (1993). Cholesterol increase in mitochondria: its effect on inner-membrane functions, submitochondrial localization and ultrastructural morphology. *Biochemical Journal* **289**, 703–708.

Hoppel, C., DiMarco, J. P. & Tandler, B. (1979). Riboflavin and rat hepatic cell structure and function: mitochondrial oxidative metabolism in deficiency states. *Journal of Biological Chemistry* **254**, 4164–4170.

Iwaya-Inoue, M., Sakaguchi, K. & Kaku, S. (1989). Statistical studies using AIC method to decide the question of "Break" or "Straight" in Arrhenius plots of water proton NMR relaxation times in chilling-sensitive *Vigna* and insensitive *Pisum* seedlings. *Plant Cell Physiology* **30**, 309–316.

Jaworek, D., Gruber, W. & Bergmeyer, H. U. (1974). Adenosine-5′-diphosphate and adenosine-5′-monophosphate. In *Methods of Enzymatic Analysis*, vol. 4, pp. 2127–2131 [H. U. Bergmeyer, editor]. New York: Academic Press.

Krahenbuhl, S., Chang, M., Brass, E. P. & Hoppel, C. L. (1991). Decreased activities of ubiquinol: ferricytochrome c oxidoreductase (complex III) and ferrocytochrome c: oxygen oxidoreductase (complex IV) in liver mitochondria from rats with hydroxycobalamin[c-lactam]-induced methylmalonic aciduria. *Journal of Biological Chemistry* **266**, 20998–21003.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193**, 265–275.

Rafael, J., Patzelt, J., Schäfer, H. & Elmadfa, I. (1984). The effect of essential fatty acid deficiency on basal respiration and function of liver mitochondria in rats. *Journal of Nutrition* **114**, 255–262.

Sottocasa, G. L., Kuylenstierna, B., Ernster, L. & Bergstrand, A. (1967). An electron-transport system associated with the outer membrane of liver mitochondria: a biochemical and morphological study. *Journal of Cell Biology* **32**, 415–438.

Statistical Analysis Systems (1985). *SAS User's Guide, Statistics*, 5th edn. Cary, NC: SAS Institute Inc.

Spurr, A. R. (1969). A low-viscosity epoxy resin embedding medium for electron microscopy. *Journal of Ultrastructure Research* **26**, 31–43.

Tanaka, M., Ishibashi, T., Okamoto, K. & Toyomizu, M. (1993). Tissue- and substrate-dependent responses of oxidative phosphorylation to dietary protein level in chicks. *British Journal of Nutrition* **70**, 459–469.

Tanaka, M., Ishibashi, T. & Toyomizu, M. (1995). Time course of oxidative phosphorylation in liver mitochondria of chickens fed on high-protein diet. *British Poultry Science* **36**, 143–154.

Toyomizu, M. & Clandinin, M. T. (1993). Effects of dietary protein and fat level on oxidative phosphorylation in rat heart mitochondria. *British Journal of Nutrition* **69**, 97–102.

Toyomizu, M., Kirihara, D., Tanaka, M., Hayashi, K. & Tomita, Y. (1992). Dietary protein level alters oxidative phosphorylation in heart and liver mitochondria of chicks. *British Journal of Nutrition* **68**, 89–99.

Wander, R. C. & Berdanier, C. D. (1985). Effects of dietary carbohydrate on mitochondrial composition and function in two strains of rats. *Journal of Nutrition* **115**, 190–199.

Wolfe, J. & Bagnall, C. (1979). Statistical tests to decide between straight line segments and curves as suitable fits to Arrhenius plots or other data. In *Low Temperature Stress in Crop Plants*, pp. 527–533 [J. M. Lyons, D. Graham and J. K. Raison, editors]. New York: Academic Press.

Yamauchi, K., Isshiki, Y., Zhou, Z.-X. & Nakahiro, Y. (1990). Scanning and transmission of microscopic observations of bacteria adhering to ileal epithelial cells in growing broiler and white leghorn chickens. *British Poultry Science* **31**, 129–137.

Zaragozá, R., Renau-Piqueras, J., Portolés, M., Hernández-Yago, J., Jordá, A. & Grisoía, S. (1987). Rats fed prolonged high protein diets show an increase in nitrogen metabolism and liver megamitochondria. *Archives of Biochemistry and Biophysics* **258**, 426–435.

Zsigmond, E. & Clandinin, M. T. (1986). Modulation of mitochondrial ATPase sensitivity to inhibitors and stimulators by diet-induced changes in membrane lipid. *International Journal of Biochemistry* **18**, 505–511.

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