Dietary protein level alters oxidative phosphorylation in heart and liver mitochondria of chicks

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To determine the effects of dietary protein level on cardiac and hepatic mitochondrial oxidative phosphorylation, chicks were fed on semi-purified diets of different protein levels (7, 25, 43 and 61 % of metabolizable energy content) for 7, 14 and 21 d. All diets were formulated to contain equivalent fat, mineral and vitamin contents on a gross energy basis. Cardiac and hepatic mitochondrial oxidative phosphorylation rates were assessed polarographically with pyruvate and malate as substrates. Cardiac mitochondria isolated from chicks fed on a 43 or 61 % protein-energy diet for 7 d exhibited significantly reduced ADP:oxygen (ADP:O) ratios when compared with mitochondria isolated from chicks fed on a lower-protein-energy diet. Feeding low- (7 %) protein-energy diets for 14 d resulted in a relatively increased ADP:O ratio in the heart. Responses of ADP:O ratios to protein level in hepatic mitochondria showed more dependency on protein level than in heart muscle; at all feeding periods the ADP:O ratio decreased with an increase in protein level. As a result, ATP synthesized in the liver, expressed as nmol/mg mitochondrial protein per min, significantly decreased with increased dietary protein level. A parallel correlation was observed, in chicks fed on diets with different levels of protein, between ADP:O ratio for liver mitochondria and body fat. These results suggest that the reduction in oxidative phosphorylation in the heart and liver of animals fed on a higher protein-energy diet may partly contribute to the depression of body fat.

Dietary protein: Oxidative phosphorylation: Liver and heart mitochondria: Body fat: Chicken

Several studies have characterized metabolic responses to dietary protein level. These responses involved lipogenesis or fatty acid metabolism (Masoro *et al.* 1950; Yeh & Leveille, 1969) and protein metabolism (Smith *et al.* 1982; Sampson *et al.* 1986; Muramatsu *et al.* 1987) in rats and chicks. As far as the effect of dietary protein is concerned, all the experiments to date have been restricted to studies on substrate and energy metabolism in the whole body (Hartsook & Hershberger, 1963; Leveille & Cloutier, 1987), with little attention given to bioenergetic function in the mitochondria of specific organs.

It is well known that dietary fat affects mitochondrial function. Rats chronically fed on a diet containing long-chain fatty acids showed a decline in the oxidation of substrates at coupling sites I and II as well as a decrease in ATP synthesis in heart mitochondria (Clandinin, 1978). In chicks the low energetic efficiency of a diet high in rapeseed oil was clearly shown to be due to the uncoupling of oxidative phosphorylation (Renner *et al.* 1979). Liver mitochondria isolated from rats fed on diets supplemented with 20 and 50 g/kg diet of ethyl ester concentrate of *trans*-fatty acids containing 52 % (w/w) *t,t*-18:2

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showed depressed ADP:oxygen (ADP:O) ratio, rates of O_2 consumption and lower ATP synthesis rates than control rats (Schrijver & Privett, 1984). Thus, there is an apparent association of dietary composition with mitochondrial energetic efficiency.

Recently we reported that cardiac mitochondria isolated from rats fed on a 70% protein-energy diet for 23 d exhibited significantly reduced ADP:O ratios when compared with mitochondria isolated from rats fed on a lower-protein-energy diet, and that the impaired ADP:O ratio of rats fed on very high-protein-energy diets was not due to a deficiency of dietary fat (Toyomizu & Clandinin, 1992). Therefore, it is of interest to examine in growing chicks: (1) whether oxidative phosphorylation in the cardiac and hepatic mitochondria is affected by the protein level of the diet fed and (2) whether changes in oxidative phosphorylation are associated with corresponding changes in body composition with the dietary protein level. Here, dietary protein-induced changes in the oxidative phosphorylation capacity of isolated hepatic and cardiac mitochondria were associated with differences in the amount of body fat retained. Findings of the present study constitute the first-reported study of mitochondrial respiration and oxidative phosphorylation in the heart and liver of chicks fed on diets with different protein levels.

MATERIALS AND METHODS

Animals and diets

Ninety-six male chicks (Arbor Acres, Ijichi, Shukeijyo Co. Ltd., Japan) at 11 d of age were divided into four groups of twenty-four chicks each. Each replicate consisted of two birds housed in a wire-bottomed aluminum cage under controlled light (13 h light and 11 h dark) and temperature ($24 \pm 1°$). After a 3 d adaptation period chicks weighing 419 (SD 16) g were fed on an experimental diet and water *ad lib.* Body-weight and food intake were recorded weekly and daily respectively. The carbohydrate source was a 2:3:5 (by wt) mixture of yellow maize, α-maize starch and glucose. The fat source was soya-bean oil. The protein source was a 660:328:12 (by wt) mixture of isolated soya-bean protein, soya-bean meal and DL-methionine. All the diets contained the same amounts of cellulose, fat, minerals and vitamins per 100 kJ metabolizable energy. Four experimental diets of 7, 25 (control diet), 43 and 61% protein-energy level (PME) were formulated on a metabolizable energy (ME) basis by substituting the carbohydrate level (CME) at a constant fat level (FME). The nutrient composition of the diets fed in these experiments is illustrated in Table 1.

Measurement of mitochondrial respiration

Eight chicks from each group at 7, 14 and 21 d of the feeding regimen were killed by cervical dislocation. Hearts at 7 and 14 d and livers at 7, 14 and 21 d were immediately excised, and surplus fat or atrial tissue was removed. For isolation of mitochondria, four replicates of two pooled hearts or livers in each group were made. Hearts were placed in cold 210 mM-mannitol containing 70 mM-sucrose and 0·1 mM-EDTA and cut into pieces for mitochondrial isolation as described in detail previously (Clandinin, 1978; Renner *et al.* 1979). Rates of O_2 utilization were measured with 10 mM-pyruvate, 2 mM-malate and 10 mM-malonate as substrates for oxidative phosphorylation in the following reaction mixture (pH 7·4): 15 mM-potassium chloride, 30 mM-potassium dihydrogen phosphate, 25 mM-Tris-hydrochloride, 45 mM-sucrose, 10 mM-mannitol, 5 mM-magnesium chloride, 7 mM-EDTA, 20 mM-glucose, 2 g albumin/l, 0·015 mM-cytochrome C, 0·5 mM-NAD and mitochondrial protein (approximately 300 μg) (Clandinin, 1978). Liver mitochondrial isolation was performed according to the modified method of Johnson & Lardy (1967). Briefly, a homogenate (100 g/l) was prepared in cold buffer containing 250 mM-sucrose and 10 mM-Tris-hydrochloride (pH 7·4), and nuclei and cell debris were removed by centrifugation at

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

Protein levels as metabolizable energy...	7	25	43	61
Isolated soya-bean protein†	45·91	198·57	350·19	499·94
Soya-bean meal†	22·82	98·69	174·04	248·46
DL-methionine†	0·84	3·61	6·37	9·09
Soyabean oil	45·79	46·00	46·22	46·35
Yellow maize‡	158·58	113·86	69·44	25·29
α-Maize starch‡	237·87	170·78	104·16	37·93
Glucose‡	396·45	284·64	173·60	63·21
Cellulose	13·16	13·18	13·20	13·34
Calcium phosphate, dibasic	52·84	45·96	39·12	32·28
Calcium carbonate	4·24	6·28	8·30	10·30
Potassium chloride	6·81	4·02	1·24	0·00
Sodium chloride	5·14	4·89	4·63	4·37
Trace mineral mixture§	5·31	5·29	5·27	5·24
Vitamin mixture§	4·24	4·23	4·22	4·20
Total	1000·00	1000·00	1000·00	1000·00

* All the diets contained the same amount of cellulose, fat, minerals, and vitamins per unit metabolizable energy.

† Protein consisted of a 660:328:12 (by wt) mixture of isolated soya-bean protein, soya-bean meal and DL-methionine.

‡ Carbohydrate consisted of a 2:3:5 (by wt) mixture of yellow maize, α-maize starch and glucose. Yellow maize contains 50 g lucene (*Medicago sativa*)/kg.

§ See Akiba & Matsumoto (1978).

600 ***g*** for 10 min; mitochondria were pelleted by centrifugation at 5000 ***g*** for 20 min then washed twice in buffer followed by centrifugation at 5500 ***g*** for 15 min. Rates of O_2 utilization were measured with 10 mM-pyruvate and 2·5 mM-malate as substrates, 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-EDTA 1 g albumin/l and mitochondrial protein (approximately 1 mg) (Brady & Hoppel, 1983).

To avoid bias we measured the O_2 consumption of each mitochondrial preparation from the four dietary groups according to a systematically randomized order that was different on each day. Oxidation rates, expressed in ng O_2/mg mitochondrial protein per min, were measured polarographically in a total reaction volume of 2 ml at 37° using an O_2 monitor equipped with two O_2 sensors. The state 3 respiratory rate in the presence of ADP (235 nmol portions), the state 4 respiratory rate after exhaustion of ADP, respiratory control indices and ADP:O ratios were determined on the third and subsequent cycles as described by Chance & Williams (1956) and Chappell (1964). The ADP and AMP concentrations were determined by enzymic analysis (Jaworek *et al.* 1974). One equivalent of AMP was considered to be equal to two equivalents of ADP as shown by Hoppel *et al.* (1979). The solubility of O_2 at 37° was assumed to be 0·39 μg atoms O_2/ml (Clandinin, 1978). Protein was measured by a colorimetric method (Lowry *et al.* 1951).

Chemical analysis of carcasses

After removing the liver from chicks fed on an experimental diet for 21 d, gastrointestinal contents were discarded and the carcasses stored at −20°. Each frozen carcass was individually homogenized and analysed as described in our previous paper (Toyomizu *et al.* 1982). Protein content (nitrogen × 6·25) of carcasses was determined by the Kjeldahl method. Carcass fat was extracted with diethyl ether and determined gravimetrically.

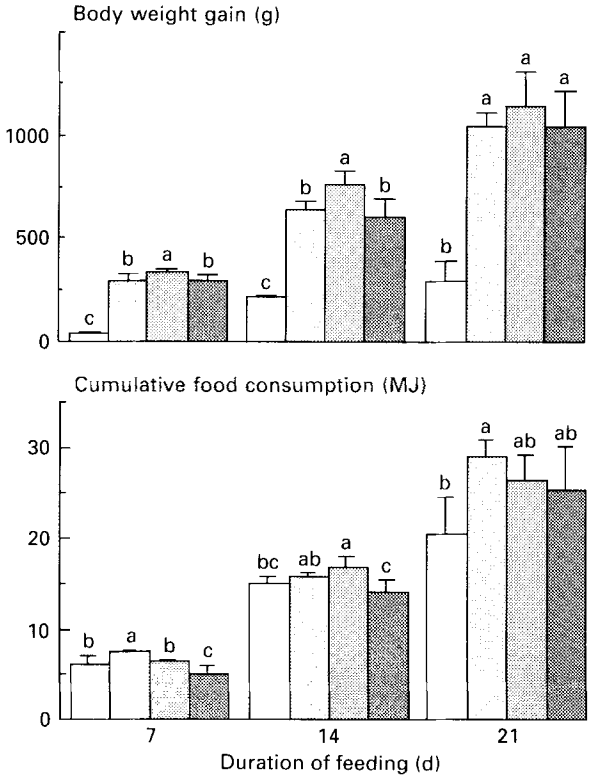

Fig. 1. Effect of dietary protein level on body-weight gain and cumulative food consumption at different feeding periods: □, 7% PME; □, 25% PME; □, 43% PME; ▨, 61% PME. Differences in means were tested by Duncan's multiple-comparison test. Values are means with their standard deviations represented by vertical bars. Means with different superscript letters within the duration of feeding were significantly different ($P < 0.05$).

Statistical procedure

With two-way analysis the effect of treatment on mitochondrial functions was first examined to separate the effect of dietary protein level from the effect due to the duration of feeding. The significance level for individual group comparisons was $P < 0.05$ using Duncan's least significant difference multiple-range test (Statistical Analysis System Institute, 1985).

RESULTS

Results of body-weight gain and cumulative food consumption for chicks fed on diets of different protein levels for 7, 14 and 21 d are shown in Fig. 1. Regardless of the feeding period, the body-weight gain increased to a maximal level at 43% dietary protein-energy and decreased thereafter. Food intake, expressed as metabolizable energy, for chicks fed on a 7 or 61% protein-energy diet was lower than for chicks fed on a 25 or 43% protein-energy diet for 7, 14 and 21 d. The intake for chicks fed on a 61% protein-energy diet was not significantly different from that for chicks fed on a 7% protein-energy diet for 14 and 21 d.

Determination of cardiac and hepatic mitochondrial respiration with pyruvate and malate as substrates indicated no significant differences in state 3 or state 4 among chicks fed on diets of different protein levels for every 7, 14 or 21 d (Table 2). The rates of O_2 uptake and ADP:O ratios observed were similar in magnitude to those previously reported

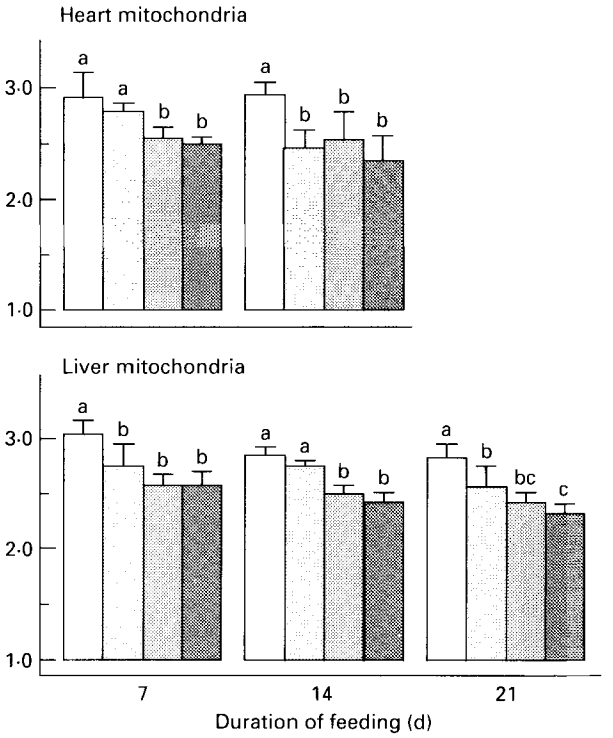

Fig. 2. Effect of dietary protein level on ADP:oxygen (ADP:O) ratio in chick heart mitochondria and liver mitochondria at different feeding periods: □, 7% PME; □, 25% PME; ▨, 43% PME; ▩, 61% PME. Differences in means were tested by Duncan's multiple-comparison test. Values are means with their standard deviations represented by vertical bars. Means with different superscript letters within the duration of feeding were significantly different ($P < 0·05$).

for rat cardiac and hepatic isolated mitochondria (Clandinin, 1978; Schrijver & Privett, 1984). The ADP:O ratio observed significantly decreased with an increase in protein level for both cardiac and hepatic mitochondria isolated from rats after 7, 14 and 21 d of feeding (Fig. 2).

The rate of ATP synthesis also significantly decreased with increased protein level in chick liver mitochondria at 14 d. The respiratory control ratio of heart and liver mitochondria had a tendency to decrease with an increase in dietary protein level (Table 2). The lower ADP:O ratios, the decreased ATP synthesis and, to some extent, the lower respiratory control ratio in the higher-protein groups reflected the malfunction of the respiratory enzyme systems or defective coupling of oxidation to phosphorylation, or both.

The body composition of chicks fed on experimental diets is shown in Table 3. The weight of carcass water, carcass protein and carcass ash varied between animal groups because of differences in final body-weight. However, when values were corrected for carcass weight some differences in the body composition were still noted except for ash. Both weight and percentage of carcass fat decreased with increasing dietary protein level, in agreement with our previous observation with chicks force-fed 4084 kJ (on the basis of calculated metabolizable energy) purified diet for 10 d (Toyomizu *et al.* 1982). On the assumption that body fat and body protein contains 39·3 and 23·4 kJ/g respectively, the estimated energy efficiency was calculated as the body energy gain:metabolizable energy intake ratio. As a result, the mean values for chicks fed on diets of 7, 25, 43 and 61% PME

Table 2. *Oxidative activity of isolated heart and liver mitochondria, with pyruvate and malate as substrate, from chicks fed on diets of different levels of dietary protein-energy (PME) during the 7, 14 and 21 d experimental periods**

(Mean values for four replicates of two pooled hearts or livers in each group)

	Period of feeding (d)	Dietary PME (%)				Pooled SE	Statistical significance of variance ratio (F)† on:		
		7	25	43	61		Duration of feeding	Diet	Duration of feeding × diet
Heart mitochondria (24 df)									
State 3	7	347	377	369	378	45·5	NS	NS	NS
(ng atom/min per mg protein)	14	404	472	359	413				
State 4	7	83	79	105	114	15·3	$P < 0·01$	NS	NS
(ng atom/min per mg protein)	14	107	124	131	145				
Respiratory	7	4·26	4·98	3·68	3·55	0·335	$P < 0·05$	$P < 0·01$	NS
control index‡	14	4·00	3·97	2·96	2·92				
ATP synthesized	7	1012	1055	935	944	114	NS	NS	NS
(nmol/min per mg protein)	14	1188	1145	898	985				
Liver mitochondria (36 df)									
State 3	7	159	142	151	156	12·2	$P < 0·001$	NS	NS
(ng atom/min per mg protein)	14	144	123	134	135				
	21	99	85	100	67				
State 4	7	25	27	31	29	2·69	NS	NS	NS
(ng atom/min per mg protein)	14	28	27	27	31				
	21	28	24	27	24				
Respiratory	7	6·49	5·38	5·14	5·52	0·381	$P < 0·001$	NS	NS
control index‡	14	5·25	4·64	5·05	4·41				
	21	3·61	3·40	3·65	2·87				
ATP synthesized	7	483	389	385	395	30·7	$P < 0·01$	$P < 0·001$	NS
(nmol/min per mg protein)	14	404	335	333	324				
	21	276	214	239	153				

NS, not significant

* For details of dietary treatments, see Table 1.

† Two-way factorial analysis of treatment effects of diet and duration of feeding.

‡ Respiratory control index was calculated as the state 3: state 4 oxidation rates ratio.

Table 3. *Body composition of chicks fed on diets containing different levels of dietary protein-energy (PME) for 21 d**

(Mean values for four replicates of two pooled chicks in each group; 12 df)

Dietary PME (%)...	7	25	43	61	SED	Statistical significance: $P <$
Water						
g	359·1[b]	873·5[a]	986·5[a]	922·4[a]	62·4	0·001
% of carcass	54·9[c]	65·2[b]	69·0[a]	68·7[a]	0·829	0·001
Fat						
g	173·4[a]	155·5[a,b]	123·1[b,c]	89·6[c]	17·0	0·01
% of carcass	26·2[a]	11·6[b]	8·6[c]	6·7[c]	1·07	0·001
Protein						
g	100·3[b]	259·0[a]	280·0[a]	276·5[a]	16·4	0·001
% of carcass	15·4[c]	19·4[b]	19·6[a,b]	20·7[a]	0·528	0·001
Ash						
g	20·2[b]	40·7[a]	40·3[a]	39·9[a]	3·27	0·001
% of carcass	3·1	3·0	2·8	3·0	0·176	NS

[a,b,c] Means with different superscript letters were significantly different ($P < 0·05$).
NS, not significant; SED, standard error of difference.
* For details of dietary treatments, see Table 1.

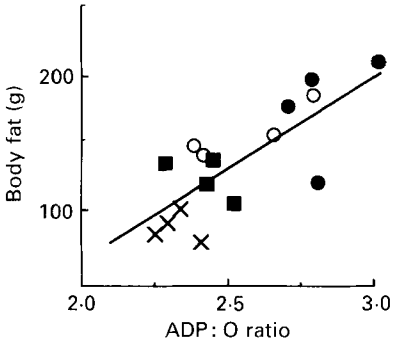

Fig. 3. Relationship between body fat and ADP:oxygen (ADP:O) ratio in chick liver mitochondria: (●), 7% PME; (○), 25% PME; (■), 43% PME; (×), 61% PME. Each symbol represents two animals, with ADP:O ratio observed from two pooled livers *v.* the mean of the corresponding body fat. The regression line is described by $Y = 128·4\ X - 187·4$ where Y is body fat (g) and X is ADP:O ratio (r 0·745, n 16, residual SD 27·3, $P < 0·01$).

were calculated to be 0·280, 0·305, 0·304 and 0·263 respectively, and there were no significant differences. Although the energy efficiency was independent of variables for mitochondrial function, consistent change in oxidative phosphorylation capacity and body fat associated with changes in dietary protein was observed; the overall correlation between the ADP:O ratio measured with mitochondria from two pooled livers and mean values for the corresponding carcass fat weight is significant (Fig. 3; r 0·745, n 16, residual SD (RSD) 27·3).

DISCUSSION

The ADP:O ratio indicates how efficiently mitochondria use the molecular substrate O_2 for flavoprotein-mediated ATP synthesis. Recent studies with domestic animals indicate that variations exist in the efficiency of mitochondrial function among sheep, swine and chicken

breeds varying in performance (Wolanis *et al.* 1980; Dzapo & Wassmuth, 1983; Brown *et al.* 1986), but nutritional studies on mitochondrial function were mainly restricted to modulations of dietary fat (Divakaran & Venkataraman, 1977; Renner *et al.* 1979; Schrijver & Privett, 1984). The present study indicates that the level of dietary protein is an important determinant of oxidative phosphorylation in chick heart and liver mitochondria. To the authors' knowledge, virtually no findings are available to confirm the direct effect of the amount of carbohydrate on mitochondrial functions. Earlier studies demonstrated that although fatty acid compositional differences were observed in hepatic mitochondria of rats fed on a 650 g sucrose/kg or a 650 g maize-starch/kg diet, these differences had little effect on the membrane-associated, succinate-supported respiration in the livers (Wander & Berdanier, 1985). Also, neither changes in dietary fat level in compensation for carbohydrate level nor starvation influenced mitochondrial oxidation rate or ADP:O ratio in livers (Brady & Hoppel, 1983), implying that carbohydrate sources or levels largely do not have an effect on mitochondrial respiration. Our recent work with rats showed that high-protein diets reduced the ADP:O ratio in heart mitochondria compared with a medium-protein diet, and that the impaired ADP:O ratio of rats fed on high-protein diets was not due to a deficiency of dietary fat (Toyomizu & Clandinin, 1992). In the present study we have extended the former observation by assessing whether or not dietary protein could alter both heart and liver mitochondrial function in chicks.

State 3 respiration rate is a measure of how rapidly mitochondria are able to utilize the molecular substrate O_2 during phosphorylation of exogenous ADP to synthesize ATP. State 4 respiration rate reflects a basal level of internal ADP recycling when external ADP concentration is low, and a dielectric breakdown of the inner membrane allowing proton recycling and possibly Ca^{2+} recycling. The present results of measurement of state 3 or state 4 showed there are apparently no differences among the respiration rates of liver or heart mitochondria in chicks fed on experimental diets, indicating that oxidative rate in heart or liver mitochondria is not much affected by dietary protein level whether ADP is present or not. The ATP synthesis rate, dependent on both the rate and efficiency of substrate utilization, was an overall measure of the ability of mitochondria to conserve energy needed by cells for diverse metabolic and synthetic activities. Dietary protein-induced changes in the ADP:O ratio (i.e. ATP synthesis: state 3) of liver and heart mitochondria were reflected in changes in the ATP synthesis rate; however, the changes in heart mitochondria were not great enough to be significant.

Results obtained with chickens demonstrated that specific metabolic changes reflected in differences in energy efficiency or body fat, or both, can be induced by feeding rapeseed oil (Renner *et al.* 1979) and *trans*-fatty acid (*t,t*-18:2) (Schrijver & Privett, 1984) and can be attributed to the uncoupling of oxidative phosphorylation. As a consequence of affecting phosphorylation capacity of mitochondria, modifying dietary protein level may possibly affect energy efficiency or body composition.

The mechanism involved in the protein effect on energy efficiency, however, appears more complex inasmuch as the level of dietary protein fed has effects on protein or amino acid metabolism and carbohydrate metabolism as well as lipid metabolism. In fact, the present results show some discrepancy between estimated energy efficiency and oxidative phosphorylation, in particular in the 7% protein-energy group; even increased oxidative phosphorylation in the mitochondria isolated from the liver and heart of chicks fed on a low-protein diet for 7, 14 and 21 d did not induce an elevation in energy efficiency at 21 d. It is unlikely that alteration in energy efficiency for the whole body of animals fed on diets with various protein levels would only depend on the oxidative phosphorylation. Krebs (1964) concluded that the ATP yield from metabolizable energy equivalents of protein is less than that from the comparable metabolizable energy equivalents of carbohydrate and

fat. In the low-protein group in the present study, elevations of futile metabolic cycles or activation of the thermogenesis system, the increase in α-glycerophosphate shuttle activity as shown in rats fed on a low-protein diet (Tyzbir *et al.* 1981) may also have occurred.

Chemical energy in food is converted to work, heat or stored energy (Baldwin & Bywater, 1984; Leveille & Cloutier, 1987). It is assumed that the thermic effect of exercise was similar between groups in the present experiment. In light of the findings of Kleiber (1975) that percentages of body fat and body water are the most variable, and that protein and mineral change very little, body fat appears to be most changeable in response to the differences in oxidative phosphorylation capacity. Thus, in the present study the decreased body fat of chicks fed on higher-protein diets may reflect the impaired ADP:O ratios of the liver and heart. As referred to previously, the partial oxidation of protein and amino acid results in reduced availability of ATP compared with carbohydrate and fat. Apparently coupled with this are the different efficiencies for fat deposition with which dietary amino acids and dietary carbohydrate are converted to body fat. Besides these, other factors, i.e. substrate availability and the rate of generation of reducing equivalents to support fatty acid biosynthesis, conceivably limit lipogenesis or fatty acid synthesis when animals are fed on higher-protein diets. It was suggested that a limitation in the availability of cytoplasmic reducing equivalents may initiate the reduction in hepatic fatty acid synthesis in chicks fed on a high-protein diet (Yeh & Leveille, 1969). However, it seems unlikely that the production of glycerophosphate or acetyl-CoA for fatty acids would be limiting, since even the diets with high levels of protein still allow for a substantial amount of carbohydrate in the diet. In any event, both factors would also be coupled with dietary protein-induced changes in oxidative phosphorylation of the liver and heart in chicks. The parallelism, as shown in Fig. 3, between body fat and ADP:O ratio should be interpreted including all these contributing reactions.

The molecular control mechanism through which dietary protein levels affected oxidative phosphorylation capacity in the liver and heart is unknown. In an early study, change in the thickness of the lipid bilayer was demonstrated to alter the permeability of mitochondria membrane to protons (Zsigmond & Clandinin, 1986). It is possible that change in dietary protein intake might alter the composition of membrane phospholipid-linked fatty acid. Impaired oxidative phosphorylation could be induced by such a modification of the lipid bilayer if production of ATP from ADP in the mitochondria is according to the chemiosmotic hypothesis. It would also be conceivable that any one of three proton pumps in the respiratory chain which act in parallel with respect to the proton circuit (Nicholls, 1982) might somehow be modified by dietary protein.

Christensen *et al.* (1983) reported that hepatic ATP synthesis capacity reflects the oxidative metabolism of the whole body in rats from 5 weeks to 5 months of age. However, it has also been observed in several studies that liver mitochondria do not respond as well as heart mitochondria to different dietary fat compositions as far as their oxidative activity is concerned (Houtsmuller *et al.* 1970; Christophersen & Bremer, 1972). Therefore, further studies utilizing additional substrates and other organs, such as the kidney, skeletal muscle and intestines, would be necessary to clarify the effect of dietary protein-induced mitochondrial function on the whole body.

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