INCREASE IN CYTOCHROME CONTENTS OF LIVER MITOCHONDRIA ON FEEDING RATS A LOW CASEIN DIET

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Summary WILLIAMS et al. (1) investigated the changes in amounts of total mitochondrial protein and cytochromes in the liver of rats fed a protein-free diet for a long period, and found that there was a marked similarity between changes in amount of total mitochondrial protein and that of cytochromes. Present experiments were performed to clarify whether under a more mild protein-deficient state the relationships found by Williams are applicable or not by investigating the changes in contents of cytochromes per unit amount of mitochondrial protein when rats were fed a low casein diet. A 4% casein diet was used as a low casein diet, and a 25% casein diet was used as a control diet. Rats were fed the test diets for 70 to 90 days. The results show that the contents of all cytochromes a, b, and c₁+c assayed and expressed as nmoles per mg of mitochondrial protein were significantly higher in rats fed a low casein diet than those in rats fed a control diet. These results suggest that relationships found by Williams in a severe protein-deficient state would not be applicable in a more mild protein-deficient state.

WILLIAMS et al. (1) employing rats fed on a protein-free diet for a long period, investigated the changes in amounts of total mitochondrial protein and cytochromes in the liver, and found that there was a marked similarity between changes in amounts of total mitochondrial protein and those of cytochromes in the responses to protein deficiency. Furthermore, they also investigated changes in cytochrome oxidase activity (2), succinic dehydrogenase activity (3) and amounts of mitochondrial phospholipid (2), and all the measured enzyme activities and amounts of phospholipid corresponded to protein deficiency in a similar way. From these facts, they suggested that there was a “common regulatory mechanism” controlling the levels of these components under the stress of protein deficiency.
In their experiments, rats were kept in a very severe protein-deficient state, being fed a protein-free diet for a 100-day period. The deficient state was so severe that at the end of this period some of the rats became moribund due to protein deficiency. Under a more mild protein-deficient state, however, it is questionable whether these relationships found by them might be applicable.

The author has investigated many changes in properties of rat liver mitochondria induced by feeding a low casein diet containing only 4% casein, and found that oxygen consumption per unit amount of mitochondrial protein was higher in rats fed a low casein diet than in rats fed a control diet (4). Simultaneously, it was shown also by the author (5) that the composition of rat liver mitochondria was changed by a low casein diet. That is, in rats fed a low casein diet, the ratio of lipid to that of protein increased in the rat liver mitochondria. From these facts, it could be considered that the ratio of oxidative enzyme proteins in mitochondria to the total mitochondrial protein might be increased by feeding rats a low casein diet. Cytochromes are fundamental respiratory components in mitochondria, and contents of cytochromes compared with the total mitochondrial protein therefore might be increased by feeding rats a low casein diet. This hypothesis is contradictory to Williams's opinion, assuming that his "common regulatory mechanism" is applicable to a more mild protein-deficient state. Therefore, the present experiments were undertaken to clarify whether under a more mild protein-deficient state as feeding a 4% casein diet Williams's opinion is applicable or not.

In the present experiments, because of the difficulty of quantitatively recovering in mitochondrial fraction total liver mitochondria, amounts of cytochromes and total mitochondrial protein are not expressed per unit weight of liver DNA as in Williams's experiments. Instead, the contents of cytochromes in purified mitochondrial fraction were measured, and their contents per unit amount of mitochondrial protein were calculated and compared.

**EXPERIMENTAL**

*Animals and diets.* Male rats of the Wistar strain, weighing about 170 g, were used. They were housed in individual cages in a temperature-controlled room at about 22°C.

The composition of diets is shown in Table 1. As in previous reports (4, 5), the low casein diet contained 4% casein, and the control diet contained 25% casein. The salt and vitamin mixtures were made according to HARPER (6).

*Plan of experiments.* For the determination of contents of cytochromes in mitochondrial fraction, two experiments, shown as experiments 1 and 2, were carried out. In both experiments, the rats were divided into 2 groups, one group was fed a low casein diet, and the other a control diet. Both groups were fed the test diets *ad libitum* for 70 to 90 days.

*Preparation of mitochondrial fraction.* Two kinds of media were used for
Table 1. Composition of diet. Diets were supplemented further with 6,000 IU of vitamin A, 600 IU of vitamin D and 100 mg of vitamin E per kilogram diet. Mineral mixture B and vitamin mixture were made according to Harper (6).

|                | Low casein diet (%) | High casein diet (%) |
|----------------|---------------------|----------------------|
| Casein         | 4                   | 25                   |
| α-Starch       | 85.6                | 64.6                 |
| Corn-oil       | 5                   | 5                    |
| Mineral mixture B | 5                 | 5                    |
| Vitamin mixture| 0.25                | 0.25                 |
| Choline chloride| 0.15               | 0.15                 |

the preparation of the mitochondrial fraction. Preparation medium 1 contained 0.21 M mannitol, 0.07 M sucrose, 0.1 mM EDTA, 0.01 M Tris-HCl buffer (pH 7.4) and 0.2% bovine serum albumin. Preparation medium 2 was the same as in preparation medium 1 except that bovine serum albumin was omitted.

The rats were killed and the livers were removed and homogenized in preparation medium 1 with a Teflon homogenizer. After removal of nuclei and cell debris by centrifugation at 600×g for 5 min, the mitochondria was sedimented by centrifugation at 10,000×g for 8 min. The sedimanted mitochondrial fraction was washed once with preparation medium 1, and then, twice with preparation medium 2. For the accurate determination of contents of cytochromes in mitochondrial fraction, it is necessary to accurately determine the contents of protein in the mitochondrial fraction. For this purpose, the final two washes with preparation medium 2 were necessary to eliminate contaminated bovine serum albumin together with other impurities. After each washing, the mitochondria was sedimented by centrifugation at 10,000×g for 8 min.

In the present experiments, care was taken to obtain mitochondrial fraction undamaged and pure as possible, while it was difficult to recover the mitochondria quantitatively in the mitochondrial fraction for the following reasons. First, to minimize damage to mitochondria in homogenization of liver cell by the Teflon homogenizer, the homogenizer was not rotated by a motor, but merely by hand. Therefore, cell homogenization was not complete, and cell debris was precipitated considerably in the nuclear fraction. Second, to minimize the contamination by other cell organelles, especially microsomes and lysosomes, as much of the “fluffy layer” as possible was discarded completely in each washing procedure, and hence, in each wash, some loss of mitochondria occurred.

**Determination of contents of cytochromes in mitochondrial fraction.** The determination of cytochrome content in a mitochondrial fraction was performed essentially based on the method described by Williams (7).

The mitochondrial fraction prepared from 5 g of the liver was suspended in 5 ml of 0.2 M phosphate buffer (pH 7.2), and lysed by adding deoxycholate and cholate to a final concentration of 0.5%. One-half of the lysate was oxidized with
a small amount of potassium ferricyanide. The other half was reduced at first with 30 μl of 1 M sodium ascorbate in the presence of 10 μl of 0.1 M potassium cyanide. A few grains of sodium hydrosulfite was further added to this reduced sample to complete the reduction. Difference spectra (oxidized-reduced) were recorded at 500 to 650 nm using a Hitachi 356 split-beam spectrophotometer. Calculation of contents of cytochromes a, b, and c1+c from such difference spectra was made according to Williams (7).

Protein contents in mitochondrial fraction were determined by a semimicro-Kjeldahl method after digesting with sulfuric acid (8).

RESULTS

Table 2 shows the means of initial body weights, final body weights, final liver weights and number of rats per group used for the determination of the contents of cytochromes in the mitochondrial fraction. It can be seen from Table 2 that body weights of rats fed a 4% casein diet increased a little during feeding on the test diets in both experiments.

Table 2. Initial body weight, final body weight, final liver weight and number of rats per group used for the determination of mitochondrial cytochromes. Each value is shown as the mean ± standard deviation.

| Group      | Number of rats per group | Initial body weight (g) | Final body weight (g) | Final liver weight (g) |
|------------|--------------------------|-------------------------|-----------------------|------------------------|
|            |                          | Experiment 1            |                       |                        |
| 4% Casein  | 5                        | 174±10                  | 195±8                 | 5.6±0.3                |
| 25% Casein | 5                        | 174±10                  | 331±10                | 11.0±0.8               |
|            |                          | Experiment 2            |                       |                        |
| 4% Casein  | 6                        | 174±10                  | 207±12                | 6.0±0.4                |
| 25% Casein | 6                        | 173±11                  | 330±22                | 10.3±1.1               |

Figure 1 shows typical difference spectra of liver mitochondrial cytochromes from rats fed low and high casein diets. As can be seen in Fig. 1, there was no marked difference in the pattern of cytochromes between two groups. For calculation of contents of cytochromes, difference in absorbancy was always corrected for the base line.

In the present experiments, as described before, changes in contents of cytochromes in mitochondrial fraction were measured and compared instead of expressing amounts of cytochromes and total mitochondrial protein per unit amount of liver DNA because of the difficulty of recovering in the mitochondrial fraction the total liver mitochondria quantitatively. Therefore, Table 3 shows the contents of cytochromes a, b and c1+c in mitochondrial fraction expressed as nmoles per mg of mitochondrial protein. It can be seen in Table 3 that in both experiments, contents of all cytochromes measured were higher in rats fed a low casein
Fig. 1. Typical difference spectra of the liver mitochondria from rats fed low and high casein diet. Trace 1, base line, trace 2, 4% casein, trace 3, 25% casein.

Table 3. Contents of cytochromes a, b and c₁+c in the liver mitochondria from rats fed low and high casein diets. Each value is shown as the mean ± standard deviation.

| Group     | Cytochrome a (n mole/mg protein) | Cytochrome b (n mole/mg protein) | Cytochrome c₁+c (n mole/mg protein) |
|-----------|----------------------------------|----------------------------------|-------------------------------------|
|           | Experiment 1                     |                                  |                                     |
| 4% Casein | 0.376±0.013*                     | 0.387±0.022*                     | 0.570±0.060*                       |
| 25% Casein| 0.246±0.034**                    | 0.219±0.054**                    | 0.412±0.064**                      |
|           | Experiment 2                     |                                  |                                     |
| 4% Casein | 0.307±0.050*                     | 0.361±0.026*                     | 0.483±0.031*                       |
| 25% Casein| 0.219±0.030**                    | 0.224±0.032**                    | 0.367±0.020**                      |

The values of * are significantly different from those of corresponding ** (p<0.01).

diet than those in control rats, and the differences between two groups were always statistically highly significant.

DISCUSSION

In the experiments by WILLIAMS et al. (1), changes in amounts of cytochromes and mitochondrial protein were expressed per unit amount of liver DNA. Since DNA per cell is unchanged, these values would represent the changes of these components per liver cell. Their experiments show that amounts of cytochromes and
mitochondrial protein per unit amount of liver DNA decreased almost by the same proportion in severe protein deficient state (I). And, therefore, although not calculated, from the figures represented, it is clear that the ratio of cytochromes to mitochondrial protein was not changed by a severe protein deficient state. It would mean that cytochromes and total mitochondrial protein are lost a liver cell by the same proportion in severe protein deficient state.

In the present experiments, as described before, total amounts of mitochondria could not be recovered quantitatively in the mitochondrial fraction, therefore, changes in amounts of cytochromes and mitochondria could not be expressed per unit amount of liver DNA, and hence changes in the amount of cytochrome and mitochondria per liver cell cannot be discussed on the basis of the present experiments. However, it can be considered that by investigating the changes in contents of cytochromes in a purified mitochondrial fraction, it may be possible to clarify whether all the proteins constituting mitochondria change by the same proportion or not. That is, if contents of cytochromes in mitochondrial fraction did not change, it would mean that cytochromes and mitochondrial protein change by the same proportion as in WILLIAMS's experiments. If not, it would mean that, contrary to Williams's experiments, cytochromes and mitochondrial protein change differently in response to protein deficiency. With these considerations, in the present experiments, changes in cytochrome contents in mitochondrial fraction in a mild protein deficient state were investigated.

As the results of such investigations, it can be seen in Table 3 that the contents of all cytochromes in mitochondrial fraction increased in rats fed a low casein diet, and hence, the results of the present experiments are different from those of WILLIAMS's experiments, i.e., in regard to a severe protein deficient state, indicating that cytochromes and mitochondrial protein change differently in response to more mild protein deficiency.

As reasons for the difference between the two phenomena, the following can be considered. First, the difference in degree of protein deficiency is one reason. In WILLIAMS's experiments, rats were fed a protein-free diet for a long period. Under such a severe condition, much of the body proteins would decrease. But, in the present experiments, the body weights of rats fed a 4% casein diet were not only maintained, but even showed a slight increase, as can be seen in Table 2. This means that the nitrogen balance of rats fed a 4% casein diet is maintained, and body proteins do not decrease. Under such conditions, the animals would adapt themselves as much as possible for their existence by choosing the more important proteins rather than less the important ones. However, as in case of WILLIAMS's experiments, i.e., using a protein-free diet for a long period, animals could not maintain even important proteins for their existence. Cytochromes are fundamental respiratory components in mitochondria and, unlike other non-catalytic mitochondrial structural proteins, are very essential proteins for the existence of animals. Therefore, in the present experiments, rats fed a 4% casein
diet would adapt themselves to maintain as much as possible such essential enzyme proteins as cytochromes, but with a protein-free diet, such as that used by Williams, it would be impossible and even essential enzyme proteins would be lost.

Secondly, some individual amino acids in casein might have a protective action for the loss of cytochromes, though not for all the mitochondrial proteins. Williams (3) found that methionine and cystine protect against the loss of certain enzymes in the electron transport system of liver mitochondria.

In any case, contrary to Williams's "common regulatory mechanism," the results of the present experiments suggest that in a mild protein-deficient state the degrees of decreased amounts were not the same among various proteins constituting mitochondria and essential enzyme proteins for energy metabolism as cytochromes would be maintained as much as possible.

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