EFFECT OF DIETARY PROTEIN DEFICIENCY ON RAT HEPATIC DRUG-METABOLIZING ENZYME SYSTEM

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Abstract—We have examined the effect of dietary protein deficiency on rat hepatic drug-metabolizing enzyme system for a period of two months. Cytochrome P-450 and b5 contents in liver microsomes, which were plotted on semilogarithmic paper as a function of the time of deficiency, showed biphasical reductions during protein deficiency: rapid decreases in the first 3 weeks were followed by more gradual decreases. However, the three enzymatic activities examined, i.e. aminopyrine demethylase, aniline hydroxylase and p-nitroanisole demethylase, were not reduced at a uniform rate. In the earlier phase, activities of the former two enzymes were reduced more rapidly than that of the last phase. This biphasical and non-uniform reduction of enzymatic activities suggests the existence of two or more cytochrome P-450 subspecies in non-depleted male rats. Intraperitoneal administration of well-known environmental pollutants, polychlorinated dibenzofurans and biphenyls (100 μg and 100 mg/kg, respectively) to the depleted rats resulted in a marked induction of drug-metabolizing enzymes. However, as the deficiency became more severe (2 months), the induction declined to a considerable degree, especially in the case of polychlorinated biphenyl administration.

In 1952, V.A. Drill presented a review concerning the interaction between hepatotoxins and dietary factors, in which he showed that protein deficiency affected the toxicity of the various hepatotoxins (1). Since then, many laboratories have shown that protein deficiency decreases the microsomal oxidation rates of a great variety of drugs and other foreign chemicals (2-4). Boyd and his colleagues (5, 6) observed that many pesticides indicated increased toxicity in protein-depleted animals, as illustrated by a 2,100-fold increase in the toxicity of captan (5). In contrast, some compounds such as carbon tetrachloride (7) and heptachlor (8) have shown lower toxicities. These increased or decreased toxicities observed in protein-depleted animals are generally interpreted to be caused by the depression of foreign chemical metabolism during protein deficiency.

In the last decade, several investigators suggested that different forms of cytochrome P-450 existed in liver microsomes, and their arguments were actually proved by the purification of several cytochrome P-450 and P-448 subspecies different in spectral properties, minimal molecular weight, substrate specificities or immunological properties (9). In the present report we have examined the time course of the decrease of rat hepatic hemoprotein contents and drug-oxidation activities during protein deficiency, and obtained results suggesting that different cytochrome P-450 subspecies were reduced at very different rates. We have also examined the induction of these enzymes caused by polychlorinated...
biphenyls and dibenzofurans, well-known environmental pollutants, during a long period of deficiency.

**MATERIALS AND METHODS**

The disodium salt of glucose-6-phosphate, glucose-6-phosphate dehydrogenase (from baker's yeast, Type V) and ethylisocyanide were purchased from the Sigma Chemical Company; the latter compound being distilled before use. NADP was obtained from the Oriental Yeast Co., p-nitroanisole and aniline from the Wako Pure Chemical Industries, and aminopyrine from the Sanko Seikagaku Kogyo Co. p-Nitroanisole was recrystallized twice from ethanol.

The polychlorinated dibenzofuran mixture (PCDFs) and the polychlorinated biphenyl mixture (PCBs, Kanechlor-500) were purchased from the Wako Pure Chemical Industries; the gas chromatogram profile of the former compound indicated only two peaks corresponding to a tetra- (12.0%) and a pentachlorodibenzofuran (88.0%).

**Animals and diets:** Four week old male Wistar rats, obtained from CLEA Japan Inc., were housed in groups of four in wire-bottomed, stainless steel cages in an airconditioned room with a relative humidity of 50±5%, a constant temperature of 25±1°C and a 10 hr-14 hr day-night lighting schedule. After maintenance on a stock diet for 2 weeks, the rats were prefed a purified complete diet (basal diet) for 5 days ad lib. and thereafter fed a protein-free diet ad lib. during the respective experimental period.

The basal diet consisted of the following: casein 24%, cornstarch 56%, sucrose 5%, soybean oil 5%, cellulose powder 4%, Harper's salt mixture (10) (the Oriental Yeast Co.) 4%, Harper's vitamin mixture (10) (the Oriental Yeast Co.) 0.85%, choline chloride 0.15%, vitamin E 10 mg%, vitamin A 400 U%, and vitamin D 200 U%. The protein-free diet was kept isocaloric by replacing the casein with an equivalent amount of starch. Both diets were prepared by the Oriental Yeast Co..

At the various times of protein deficiency (the 0, 4, 16, 32 and 67 th days) the animals were given a single intraperitoneal administration of olive oil (1 ml/kg), PCDFs in olive oil (100 μg/kg) or PCBs in the same vehicle (100 mg/kg), and thereafter they were kept on the same diets for 5 days.

**Microsomal preparations:** The animals were decapitated and the dissected liver was perfused with 1.15% KCl solution containing 0.1 mM EDTA and then homogenized with 3 vol. of the same solution. The homogenate was centrifuged at 10,000×g for 20 min, and the resultant supernatant was further centrifuged at 105,000×g for 60 min. The pellet was suspended in the KCl-EDTA solution and centrifuged again to remove any hemoglobin present.

**Enzyme assays and spectral studies:** p-Nitroanisole demethylation was assayed according to the method of Kato and Gillette (11). Aminopyrine demethylation was assayed by a slight modification of the method of Cochin and Axelrod (12), involving the use of trichloroacetic acid as the protein coagulant instead of ZnSO₄-Ba(OH)₂. Aniline hydroxylation was measured as previously described (13).
The incubation mixture (1.25 ml) containing 0.8 mM NADP, 4 mM glucose-6-phosphate, 5 mM nicotinamide, 5 mM MgCl₂, 0.2 U/ml of glucose-6-phosphate dehydrogenase, 56 mM sodium phosphate buffer (pH 7.4), 0.1 mM EDTA, the microsomal fraction derived from 80 mg of liver and a substrate (1.2 mM p-nitroanisole, 4 mM aminopyrine or 0.5 mM aniline). After the mixture was preincubated for 5 min in the absence of substrate, the reaction was performed for 10 min at 37°C in the open air.

The CO-binding difference spectrum and the ethylisocyanide difference spectrum of reduced microsomes were recorded, as previously described (13).

RESULTS

As shown in Table 1, in the earlier stage of protein deficiency (about 10 days) the animals lost weight very rapidly, but thereafter a more gradual and linear weight loss was observed. Figure 1 shows the changes in liver weight, hepatic protein content and hepatic microsomal protein content during the protein deficiency. In this figure, values are all expressed on a body weight basis. In the earlier stage, a marked decrease was observed in these three parameters, but the decreases of the latter two were more significant than that of the former. Therefore, when expressed on a liver weight basis, the total and microsomal protein contents in the liver still showed moderate decreases. As the deficiency progressed, all these parameters decreased in parallel with the decrease in body weight.

TABLE 1. Effect of polychlorinated dibenzofurans and biphenyls on body weight, liver weight and hepatic microsomal protein content in protein-depleted male rats.

| Days of protein depletion | Treatment | No. of animals | Final body weight (g) | Body weight gain (g) | Liver weight (mg/g body weight) | Microsomal protein (mg/g liver) |
|--------------------------|-----------|----------------|----------------------|---------------------|-------------------------------|-------------------------------|
| 0                        | olive oil | 4              | 305±16               | 21±5                | 3.99±0.13                     | 18.1±2.2                     | 72.2±10.2                    |
|                          | PCDFs     | 4              | 305±30               | 19±5                | 4.56±0.27                     | 20.8±1.8                     | 94.6±3.7*                    |
|                          | PCBs      | 4              | 310±21               | 27±6                | 4.62±0.25                     | 19.2±1.9                     | 88.2±5.6*                    |
| 9                        | olive oil | 4              | 248±26               | -37±7               | 3.59±0.33                     | 15.0±2.2                     | 50.7±6.5                     |
|                          | PCDFs     | 4              | 248±15               | -39±5               | 3.71±0.35                     | 16.8±1.9                     | 65.5±3.7**                   |
|                          | PCBs      | 4              | 253±22               | -36±3               | 3.93±0.35                     | 17.0±2.5                     | 63.3±14.0                    |
| 21                       | olive oil | 4              | 234±15               | -53±3               | 3.67±0.19                     | 12.9±0.7                     | 47.3±3.7                    |
|                          | PCDFs     | 4              | 225±26               | -63±12              | 4.18±0.49                     | 15.0±3.7                     | 61.3±8.9*                   |
|                          | PCBs      | 4              | 237±18               | -55±12              | 3.86±0.36                     | 14.0±2.0                     | 54.1±8.0                    |
| 37                       | olive oil | 4              | 208±5                | -79±3               | 3.39±0.28                     | 13.5±2.5                     | 46.1±11.0                   |
|                          | PCDFs     | 4              | 215±9                | -80±3               | 4.35±0.17                     | 18.2±2.7*                    | 79.0±10.2**                  |
| 72                       | olive oil | 7              | 167±15               | -112±7              | 3.47±0.43                     | 14.4±3.3                     | 49.6±11.5                   |
|                          | PCDFs     | 6              | 175±12               | -113±6              | 4.37±0.50                     | 14.8±2.1                     | 64.3±7.9*                   |
|                          | PCBs      | 6              | 174±17               | -110±16             | 4.00±0.57                     | 14.9±3.1                     | 58.3±6.6                    |

* Animals were given olive oil alone (1 ml/kg, i.p.) or polychlorinated dibenzofurans (PCDFs, 100 μg/kg) or polychlorinated biphenyls (PCBs, 100 mg/kg) dissolved in olive oil on the 0, 4, 16, 32 or 67th days of protein depletion, and maintained on a basal (non-depleted group) or a protein-free diet for a further 5 days.
** Initial body weight of these animals was 242 g–321 g (about 7 weeks-old).
Values are expressed as mean±SD. Significantly different from each olive oil-injected group, *P<0.05; **P<0.01.
Figure 2 shows the decrease of cytochrome P-450 and b5 contents in rat liver during protein deficiency as a function of time after protein deprivation. The values were expressed on a microsomal protein basis and plotted on semilogarithmic paper. In the protein-depleted animals, these hemoproteins decreased from the liver at a faster rate than the total microsomal protein. However, such decreases of hemoproteins seem to be biphasic: in the earlier phase of 3 to 5 weeks they decreased almost linearly from microsomes, but the deficiency progressed, no further decreases were observed.

We have depicted in Fig. 3 the activities (specific activities) of microsomal drug oxidation in protein-depleted rats in logarithms as a function of time of deficiency. In this experiment, three enzymatic activities, i.e. aminopyrine demethylase, aniline hydroxylase and p-nitroanisole demethylase activities, were examined. All these enzymes showed reduced activities biphasically during protein deficiency: in the earlier phase very rapidly and in the later more slowly. However, in the earlier phase no uniform decrease was observed among the three activities: the reduction of the former two was much more significant than that of the last phase. When the deficiency continued for more than 5 weeks, these activities decreased at a slow and uniform rate. Enzymatic activities expressed on a cytochrome P-450 basis (shown in Fig. 4) more clearly indicate the above result: protein deficiency reduced the activities of aminopyrine demethylase and aniline hydroxylase per nmoles of cytochrome P-450 to 1/3.5 and 1/2.0, respectively, in 5 weeks of deficiency, but p-nitroanisole demethylase showed little reduction in its activity during this period.

Figures 5 and 6 show the induction of microsomal hemoproteins and drug-oxidation activities produced by well-known, inducers, PCDFs (13, 14) (100 μg/kg) and PCBs (100 mg/kg), in protein-depleted rats. Values are expressed on a body weight basis, which should
be a most appropriate basis so far as the relation between microsomal metabolism and biological activity in vivo is concerned. These doses of PCDFs and PCBs resulted in about 30 and 15% increases, respectively, in microsomal protein content, almost irrespective of the degree of deficiency (Table 1). In depleted rats, the per cent increases of cytochrome P-450 content by the administration of PCDFs were over 150% throughout the period of deficiency and this value was greater than a 100% increase observed in nondepleted rats. However, the levels observed in PCDF-treated depleted rats were only 60 to 90% of those in PCDF-treated nondepleted rats. On the other hand, the administration of PCBs gave a similar per cent increase in cytochrome P-450 content in the depleted and nondepleted rats (about a 100% increase) when the deficiency did not continue for a long time. The effects of these treatments on cytochrome b5 content were similar to those observed for cytochrome P-450 content (Fig. 5).

The enhancement of p-nitroanisole demethylase and aniline hydroxylase activities produced by PCDF-treatment was much more significant in protein-depleted rats than in nondepleted rats, when the per cent increase was compared. Nevertheless, induced levels of these activities in the depleted animals were only 70 and 60% of the levels seen in the nondepleted induced animals, respectively (Fig. 6).

After 3 weeks of deficiency, the administration of PCBs resulted in a greater per cent increase in three activities examined when compared with those of nondepleted rats.
However, when the deficiency continued for 10 weeks, the induction was again reduced. Despite this distinct induction in the depleted animals, the levels attained by these animals were much lower than those in PCB-treated nondepleted rats.

After 10 weeks of deficiency, the induction of cytochrome P-450 content and drug-oxidation activities caused by PCDF-treatment was well preserved, while that caused by PCB-treatment was only trivial.

The 455 nm to 430 nm peak ratio in the ethylisocyanide difference spectrum was remarkably increased by the administration of PCDFs, which was proof of a 3-methylcholanthrene type induction. This increase was more significant in depleted rats than nondepleted rats, indicating a well preserved inducing ability in depleted rats. The PCB-treatment did not enhance this ratio significantly, indicating that the induction produced by a small amount of PCBs was of the phenobarbital type, as previously reported (13).

DISCUSSION

Dietary protein deficiency brought about a marked reduction in liver mass and microsomal protein content, while a more significant reduction was observed in hepatic cytochrome
P-450 content and its drug-oxidation activities. In this experiment, we measured microsomal drug-oxidation activities for three substrates and found that during protein deficiency these activities are reduced in a biphasic manner: more rapidly in the earlier phase and more slowly in the later phase. However, in the earlier phase no uniform reduction was observed in these three activities: aminopyrine demethylase and aniline hydroxylase reduced their activities rapidly but p-nitroanisole demethylase only gradually. From these results it is suggested that at least two forms of cytochrome P-450 subspecies exist in the rat liver: one rapidly decreases in quantity during protein deficiency and prefers aminopyrine and aniline as substrates, while the other decreases gradually and prefers a greater variety of substrates.

In Figs. 2 and 3, the amount of a component in the nondepleted control rats was calculated by extrapolating the slowly decreasing component to zero time. In this way, this component was calculated to consist of about 70% cytochrome P-450 and p-nitroanisole demethylation activity but only about 30 and 40% aminopyrine demethylation and aniline hydroxylation, respectively.

Levin et al. reported the existence of at least two forms of cytochrome P-450 which turned over at the half-life of 7-8 and 42-46 hr, and in adult rats the ratio of rapidly turning over cytochrome P-450 to slowly turning over hemoprotein was 1.9 (15). This value resembles the ratio we obtained in this experiment: the ratio of slowly reducing cytochrome P-450 component to rapidly reducing component resulting from protein deficiency was about 2.3. Therefore, these slowly and rapidly reducing cytochromes during protein deficiency may correspond to the rapid and slow turn over of cytochromes, respectively, shown by Levin et al. (15).

We applied microsomes from protein depleted or nondepleted rats on the SDS-10% polyacrylamide gels and electrophoresis was carried out according to the method of Laemmli (16). We found that dietary protein deprivation specifically reduced the band with the highest molecular weight among the five polypeptide bands ranging from 45,000 to 55,000 daltons (unpublished data). This polypeptide band may correspond to the cytochrome P-450 subspecies, the existence of which has been suggested in the present report.

In Figs. 5 and 6, we have shown that during protein deficiency, the ability of rats to induce drug-metabolizing enzymes in response to PCDF- and PCB-administration was well maintained, unless the duration of deficiency was long (2 months). In fact, expressed as per cent increase, a similar or slightly greater induction was observed in the depleted rats than in the nondepleted rats. Waterlow and Stephen (17) and Garlick et al. (18), using a constant infusion method to measure the synthesis rate of fixed liver protein, found that fractional synthesis rate was well maintained for at least 3 weeks of protein deprivation. In contrast, the synthesis rates of liver export protein such as albumin and transferrin (19, 20) and of muscle protein (18) were markedly reduced. Such a good maintenance of liver protein synthesizing machinery during dietary protein deprivation may be convenient for the induction of drug-metabolizing enzymes. Thus, the animals probably adapt well to such an undesirable physiological state and are protected from the invasion of toxic foreign
A moderate protein depletion did not reduce the ability of rats to induce drug-metabolizing enzymes in response to foreign chemicals, but as the deficiency became severe (extending over 2 months) the ability declined to a considerable degree, in particular in the case of PCB-treatment. Several explanations concerning this decline of induction are proposed:

(a) protein deficiency may reduce the genetic substances involved in phenobarbital-type induction, at a faster rate; (b) phenobarbital-type induction may be more relevant to the inhibition of degradation of enzyme protein and such a severe reduction of basal enzyme level may no longer bring about any induction; (c) the tissue distribution patterns of PCBs and PCDFs are different from each other (21): PCDFs are distributed to a similar degree in the liver and adipose tissue, while PCBs are preferentially in the latter tissue; severe protein deficiency may reduce the protein involved in the transfer of PCBs from adipose tissue to liver; (d) and others.

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