Biochemical Pharmacology, 1966, Vol. 15, pp. 971–976. Pergamon Press Ltd., Printed in Great Britain.

IMPAIRMENT OF HEPATIC DRUG METABOLISM IN CALCIUM DEFICIENCY*

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(*Received* 7 *October* 1965; *accepted* 22 *December* 1965)

Abstract—The duration of action of hexobarbital but not that of barbital, a non-metabolized barbiturate, is prolonged in calcium-deficient rats. This finding led to the investigation of the effects of calcium deprivation on drug metabolism *in vitro*. Calcium deficiency lowers the rate of metabolism of drugs by both oxidative and reductive pathways in liver microsomes. This impairment of hepatic drug metabolism appears to result from a decrease in the specific activity of the enzymes in the liver microsomes.

IT IS well known that the diet and nutritional status of an animal can markedly affect the rate of metabolism of drugs by liver microsomes. For example, drug metabolism in liver preparations is impaired by starvation,[1, 2] alloxan administration,[3] pretreatment with thyroxine,[4] and feeding with diets deficient in protein[5] or ascorbic acid.[6] The present studies were undertaken to determine the effects of calcium depletion on the activity of the microsomal drug enzymes. The data show that an appreciable decrease in the activity of these enzymes in rats follows the appearance of the early signs of calcium depletion.

METHODS

Immature male Sprague-Dawley rats (50 g) were used in these studies. Animals were maintained on distilled water and the calcium-deficient test diet obtained from Nutritional Biochemicals Corp. Control animals were pair fed either Purina rat chow or the calcium-deficient diet fortified with 1% calcium in the form of the carbonate. As previously reported,[7] as a means of maintaining approximately equal body weights, it was necessary to restrict the food intake of the control rats to a level slightly below that of the calcium-deficient animals.

Preparation of microsomes. The rats were killed by cervical dislocation. Their livers were removed and immediately chilled in crushed ice. All subsequent operations were carried out at 4°. The livers were homogenized in a motor-driven Teflon-glass homogenizer with 3 volumes of isotonic KCl (1·15%) containing 1×10^{-3} M ethylenediamine tetracetic acid (EDTA). The homogenate was centrifuged at 10,000 *g* (av.) for 30 min in an International model HR-1 centrifuge (rotor no. 856). The supernatant fraction was then centrifuged at 105,000 *g* (av.) for 1 hr in a Spinco model L preparative ultracentrifuge (rotor no. 40). The supernatant phase (soluble fraction) was

* A preliminary report of this work has appeared: P. Joiner and L. Hurwitz, *J. Tenn. Acad. Sci.* 39, 76 (1965). This work was supported in part by United States Public Health Service Grants AM-02235 and R01 AM 08758.

removed, and the microsomal pellet was homogenized with sufficient isotonic KCl (containing EDTA) to give a volume of 12 ml. Aliquots of this preparation were used for enzyme assays and protein estimations.

Enzyme assays. A typical incubation mixture consisted of 1·7 ml of 0·2 M phosphate buffer (pH 7·4) containing glucose-6-phosphate (50 μmoles) and NADP (0·5 μmole), 1·0–1·5 ml of microsomes (obtained from 250–375 mg liver), 1·0–1·5 ml of soluble fraction (obtained from the livers of control rats), nicotinamide (100 μmoles), $MgCl_2$ (150 μmoles), and semicarbazide (60 μmoles). To this mixture one of the following drug substrates was added: aminopyrine, 5 μmoles; hexobarbital, 1·9 μmoles; or *p*-nitrobenzoic acid, 10 μmoles. The final volume was brought up to 6 ml with water. Reaction mixtures containing aminopyrine or hexobarbital were incubated in a Dubnoff metabolic shaker for 30 min at 37° under oxygen; those containing *p*-nitrobenzoic acid were incubated under nitrogen.

The rate of metabolism of hexobarbital was measured by estimating the disappearance of substrate.[8] The reduction of *p*-nitrobenzoic acid was determined by measuring the *p*-aminobenzoic acid formed.[9] The rate of demethylation of aminopyrine was measured by estimating the amount of formaldehyde formed. Formaldehyde was estimated by the Nash procedure after the precipitation of proteins with $ZnSO_4$ and $Ba(OH)_2$.[10] In these studies the dye was extracted into isoamyl alcohol and assayed in a spectrophotometer.

Protein estimations. Microsomal protein was measured by the biuret procedure.[11] Interfering materials were removed from the reaction mixture by extraction with a mixture of ether and ethanol, as follows. Biuret reagent (4 ml) was added to tubes containing microsomes (1 ml) obtained from 250 mg liver. The contents were thoroughly mixed and allowed to stand for 30 min at room temperature. Absolute ethanol (0·5 ml) and ether (4·5 ml) were added, and the tubes were agitated on a vortex mixer for 15 sec. The mixture was then centrifuged, and the organic phase and the debris at the interface were removed by careful aspiration. The optical density of the aqueous phase was measured at 540 mμ. Bovine serum albumin obtained from Sigma Chemical Co. was used as a standard.

Duration of action of barbiturates. Rats received hexobarbital (100 mg/kg) or barbital (150 mg/kg) i.p., and the time between injection and the return of the righting reflex was measured.

RESULTS

Rats maintained on the experimental diet for about 30 days manifested the first early signs of calcium deficiency, which included depressed growth rate, alopecia, loss of appetite, and black fluid feces. The duration of action of hexobarbital was measured in rats fed the calcium-deficient diet for 30 and 43 days. The results in Table 1 show that calcium deprivation for 30 days prolonged the duration of action of hexobarbital only about 30 per cent. The sleeping times of rats fed the diet for 43 days, however, were increased almost 80 per cent over those of control animals. Calcium depletion for 36 days did not increase the sleeping times of rats receiving barbital, a non-metabolized barbiturate. It thus appeared likely that prolongation of the duration of action of hexobarbital was due to a decrease in its rate of oxidation by liver microsomes.

Liver microsomes obtained from rats that were maintained on a control or on a calcium-deficient diet for a period of 33 or 40 days were incubated with hexobarbital, aminopyrine, or *p*-nitrobenzoic acid, as described in Methods. The rates of metabolism of these substrates per milligram microsomal protein in preparations from control and calcium-deficient animals were compared. After 33 days there appeared to be little or no difference in the rates of metabolism of the substrates between the two

TABLE 1. EFFECT OF CALCIUM DEPLETION ON THE DURATION OF ACTION OF HEXOBARBITAL

Diet	Days on diet	Sleeping time (min)
Calcium-deficient + calcium	30	34 ± 7 (8)
Calcium-deficient	30	44 ± 5* (6)
Calcium-deficient + calcium	43	34 ± 7 (8)
Calcium-deficient	43	60 ± 17* (17)

Results are expressed as the mean values ± standard deviation. The number of animals is in parentheses.

* Significantly different from control values ($P < 0.05$).

groups (Table 2). By contrast, feeding with the calcium-deficient diet for 40 days resulted in a pronounced impairment in the metabolism of hexobarbital, aminopyrine, and *p*-nitrobenzoic acid. The results in Table 2 show that after 40 days the specific activity of liver microsomes from calcium-deficient rats was 30–50 per cent below that of control preparations. It is noteworthy that the effects of calcium depletion on the duration of action of hexobarbital became evident earlier than did a measurable decrease in the specific activity of the microsomal enzymes.

The body weights of rats fed the calcium-deficient diet for 33 or 40 days did not differ appreciably from those of controls fed either Purina laboratory chow or the experimental diet fortified with calcium. The liver weights of the experimental animals, however, were substantially higher than those of the controls after both 33 and 40 days (Table 3). Moreover, a significant decrease in the concentration of microsomal protein in the livers was measurable at 40 days. Since the percentage decrease in the concentration of microsomal protein was less than would be expected if it resulted simply from the increase in liver weight, it is obvious that total protein synthesis was not impaired in the livers of the calcium-deficient rats. These findings indicate that the impairment of hepatic drug metabolism evoked by calcium depletion results from an inactivation (loss of specific activity) of microsomal enzymes rather than from a reduction in total protein synthesis.

From the data that provide the basis for Table 3, it is possible to calculate the μmoles hexobarbital metabolized/100 g rat. When this was done with the data from the animals kept on the diet for 40 days, it was found that the calcium-deficient rats metabolized significantly less hexobarbital than the controls. Controls metabolized 13·03 μmoles/100 g rat ± 2·50 (S.D.); calcium-deficient rats metabolized 7·90 μmoles/100 g rat ± 2·66 ($P < 0.05$).

TABLE 2. EFFECT OF CALCIUM DEPLETION ON THE ACTIVITY OF ENZYMES IN RAT LIVER MICROSOMES

Expt.	Diet	Duration of feeding	Enzyme activity*					
			Hexobarbital metabolism		Aminopyrine demethylation		*p*-Nitrobenzoic acid reduction	
		(days)	(mμmoles ± S.D.)	Relative activity	Formaldehyde (mμmoles ± S.D.)	Relative activity	(mμmoles ± S.D.)	Relative activity
1	Purina	33	90 ± 13		26 ± 4		43 ± 5	
	Calcium-deficient + calcium		108 ± 25	100	25 ± 6	100	40 ± 6	100
	Calcium-deficient		89 ± 29	82	25 ± 6	100	32 ± 5†	80
2	Calcium-deficient + calcium	33	152 ± 22	100	43 ± 7	100	49 ± 5	100
	Calcium-deficient		141 ± 42	93	41 ± 9	95	48 ± 7	98
3	Purina	40	124 ± 17		37 ± 7		58 ± 7	
	Calcium-deficient + calcium		118 ± 12	100	39 ± 4	100	48 ± 5	100
	Calcium-deficient		77 ± 40‡	65	23 ± 3†	59	33 ± 4†	69
4	Calcium-deficient + calcium	40	139 ± 12	100	33 ± 5	100	38 ± 5	100
	Calcium-deficient		67 ± 17†	48	21 ± 4†	64	27 ± 4†	71

* Enzyme activities are expressed as mμmoles substrate metabolized by 1·0 mg microsomal protein in 30 min. Results are reported as the mean values obtained with 6 rats ± standard deviation.

† Significantly different from control values ($P < 0.05$).

‡ P value approaches significance.

TABLE 3. EFFECT OF CALCIUM DEPLETION ON LIVER WEIGHT AND MICROSOMAL PROTEIN

Diet	Duration of feeding (days)	Liver weight	Liver weight	Increase	Microsomal protein	Decrease
		(g ± S.D.)	Body weight (mg/g ± S.D.)	(%)	Liver weight (mg/g ± S.D.)	(%)
Purina	33	2·8 ± 0·1	29·0 ± 2·1		35·0 ± 1·6	
Calcium-deficient + calcium		3·6 ± 0·1	30·7 ± 1·6		35·2 ± 3·9	
Calcium-deficient		6·5 ± 1·4*	46·3 ± 6·1*	51	32·5 ± 3·5	8
Calcium-deficient + calcium	40	4·6 ± 0·2	33·7 ± 2·7		27·5 ± 1·9	
Calcium-deficient		6·6 ± 0·9*	54·9 ± 8·8*	63	21·5 ± 2·2*	22

Results are expressed as the mean value obtained from 6 rats ± S.D.
* Significantly different from control values ($P < 0.05$).

DISCUSSION

Starvation is known to lower the rate of metabolism of a number of drugs by liver preparations. Dixon *et al.*[1] demonstrated that liver microsomes from starved animals oxidize hexobarbital, chlorpromazine, aminopyrine, and acetanilide more slowly than do those from control animals. Other studies have shown that fasting also reduces the oxidation of meperidine,[2] *p*-methoxyacetanilide, and monomethyl-4-amino antipyrine* by liver preparations.

The results presented in this paper show that the feeding of rats with a diet deficient in calcium impairs the oxidation of aminopyrine and hexobarbital as well as the reduction of *p*-nitrobenzoic acid by liver microsomes. Since this impairment can be prevented by feeding the test diet fortified with calcium, the reduction of drug metabolism cannot be attributed to an additional deficiency in the synthetic diet. This reduction of drug metabolism follows the development of the early signs of calcium deficiency which include loss of appetite, depressed growth rate, and alopecia. All these signs are rapidly reversed if the animal is returned to a diet containing a normal amount of calcium.

It is possible that an impaired utilization of the dietary intake may be responsible for the effects of calcium deficiency on drug metabolism. This, however, appears unlikely, since the effects of calcium deprivation on the drug-metabolizing enzymes differ in several important aspects from those of starvation. Fasting reduces drug metabolism in liver microsomes within 36–72 hr, but the impairment due to calcium deficiency can be measured only after about 5 weeks. Only the oxidative pathways of drug metabolism are depressed by starvation;[1] calcium deficiency, however, impairs both oxidative and reductive pathways. Dixon *et al.*[1] have suggested that the reduced hepatic drug metabolism in starved animals is the result of a loss of enzymic protein from the microsomes. The findings presented in this paper show that calcium depletion evokes a decrease in the specific activity of the microsomes (mμmoles drug

* J. V. Dingell and J. R. Gillette, unpublished results.

metabolized/mg protein). This decrease in activity is measurable in incubation mixtures containing the soluble fraction obtained from normal livers. It is thus reasonable to suggest that the observed inactivation arises from either a structural change in the enzyme or a specific inhibition of the synthesis of enzymic protein.

REFERENCES

1. R. L. DIXON, R. W. SHULTICE and J. R. FOUTS, *Proc. Soc. exp. Biol. Med* **103**, 333 (1960).
2. J. S. ROTH and J. BUKOVSKY, *J. Pharmac. exp. Ther.* **131**, 275 (1961).
3. R. L. DIXON, L. G. HART and J. R. FOUTS, *J. Pharmac. exp. Ther.* **133**, 7 (1961).
4. A. H. CONNEY and L. GARREN, *Biochem. Pharmac.* **6**, 257 (1961).
5. R. KATO, E. CHIESARA and P. VASSANELLI, *Biochem. Pharmac.* **11**, 211 (1962).
6. A. H. CONNEY, G. A. BRAY, C. EVANS and J. J. BURNS, *Ann. N.Y. Acad. Sci.* **92**, 115 (1961).
7. M. KLEIBER, M. D. D. BOELTER and D. M. GREENBERG, *J. Nutr.* **19**, 517 (1940).
8. J. R. COOPER and B. B. BRODIE, *J. Pharmac. exp. Ther.* **114**, 409 (1955).
9. J. R. FOUTS and B. B. BRODIE, *J. Pharmac. exp. Ther.* **119**, 197 (1957).
10. J. COCHIN and J. AXELROD, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
11. A. G. GORNALL, C. J. BARDAWILL and M. M. DAVID, *J. biol. Chem.* **177**, 751 (1949).