0014-4754/96/020149-06$1.50 + 0.20

Morphological and biochemical effects of excessive amounts of biotin on embryonic development in mice

T. Watanabe

Department of Hygiene and Preventive Medicine, Yamagata University School of Medicine, 2-2-2 Iida Nishi, Yamagata 99023 (Japan), Fax +81 236 24 4680
Received 18 April 1995; accepted 19 June 1995

Abstract. Pregnant mice received excessive amounts of biotin either subcutaneously (sc) or orally during gestation. There were no differences in the successful pregnancy rates and number of dead or resorbed fetuses between the control and biotin-treated groups. In biotin-treated groups no increased incidence of fetuses with external malformations was clearly demonstrable. However, biotin accumulated in maternal and embryonic organs; especially, the serum biotin level in the biotin-treated dam was 200-fold higher than that in the control dam. There was a difference in biotinidase activity in maternal serum and placenta between the control and biotin-treated groups. It was concluded that excessive amounts of biotin affected the specific activity of biotinidase in pregnant mice, but did not disturb normal reproductive functions and embryonic development.
Key words. Biotin excess; embryonic development; reproductive function; biotinidase; estradiol.

The importance of biotin for the general metabolism and normal development of mammals and birds has long been recognized[1–3]. Biotin is an essential cofactor for the normal function of the carboxylases, transcarboxylases and decarboxylases involved in the fixation of carbon dioxide. These enzymes occupy an important position in such metabolic pathways as gluconeogenesis, fatty acid synthesis, the catabolism of amino acids and the regulation of carbohydrate metabolism. Biotin is likewise considered to be an important component in the maintenance of normal reproduction and embryonic development. Biotin deficiency can lead to a reduction in the activity of biotin-dependent enzymes, and may cause widespread metabolic effects on dams, resulting in abnormal morphology and development in the embryos. Early studies demonstrated that when domestic fowls were maintained on a biotin-deficient diet abnormal developments, such as micromelia and syndactyly, occurred in chick embryos[4–6]. Recently the author has found that maternal biotin deficiency was strongly teratogenic in mice and hamsters[7–9]. Maternal biotin deficiency induced a high percentage of embryos with morphological abnormalities and skeletal defects, such as cleft palate, micrognathia, micromelia and deformities of the cervical vertebral arch. This may be called 'congenital biotin deficiency syndrome'[10,11]. Therefore, biotin is important for maintaining reproductive functions and normal embryonic development in birds and rodents.

However, experimental studies of the effects of excessive amounts of biotin during gestation are limited to a few investigations in insects and rats. In insects, Benschoter and Paniagua[12,13] first demonstrated sterilizing effects of biotin in both the Mexican fruit fly (*Anastrepha ludens*) and the house fly when given excessive amounts of biotin in the diet (0.1–2.0%). The inhibitory effects on reproduction were limited to females. No morphological changes were observed in the testes in male flies. Pillai et al.[14] found that reduced fertility resulted from biotin excess in yellow fever mosquitoes, and Cohen and Levinson[15] reported that feeding superfluous biotin to hidebeetles (*Dermestes maculatus*) resulted in detrimental effects on both oogenesis and embryogenesis, but appeared to be harmless to the larvae and adults. However, the mechanism responsible for causing this decline in fertility or complete sterility in female insects is not clearly understood.

In rats, an acute dose of biotin (50 and 100 mg/kg body weight (b. wt.) subcutaneously (sc)) caused irregularities in the estrus cycle, atrophic changes in the ovary, and resorption of fetuses and placenta[16,17]. The effects of a dietary intake of excess biotin on the reproduction and embryos of mammals have not yet been determined. The present study was undertaken to investigate the reproductive and developmental effects of excessive amounts of biotin in mice.

Materials and methods

Experimental animals and diets. Nulliparous mice of the ICR strain were purchased from CLEA Japan, Co. (Tokyo), at 8 weeks of age. They were maintained on a commercial pellet diet (CRF-1; Oriental Yeast Co., Tokyo) for at least 2 weeks before the experiment to allow acclimatization to our animal facility. The females were exposed to males of the same strain during 2 h from 0900 to 1100. The day on which vaginal plugs were found at the end of the mating period was desig-

nated as day 0 of gestation. All mice were housed in polycarbonate cages with hardwood chip bedding in rooms with a temperature of 23 ± 2 °C and relative humidity ($50 \pm 10\%$). The light:dark cycle (12 h:12 h, light on at 0900) was held constant. All experiments were performed in accordance with the Recommendations for Laboratory Animal Care of the Yamagata University School of Medicine.

Pregnant animals were randomly divided into 4 groups. Untreated control animals were fed the commercial pellet CRF-1 diet. Animals in the 'oil' group were given 3 doses each of 0 mg or 50 mg biotin/kg b.wt. suspended in olive oil, by subcutaneous injection. Those in the 'NaOH' group were injected with 0.1 N NaOH at pH 11.0 (adjusted by HCl), with or without biotin (50 mg/kg b.wt). The volume injected was 0.5 ml in three abdominal regions on days 0, 6 and 12 of gestation. In the dietary group, animals were given the powdered CRF-1 diet, with or without supplementary biotin (1,000 mg biotin/kg diet; 0.1% biotin) throughout the gestation period. According to the manufacturer's report, the nutrient composition of the CRF-1 diet (gross energy, 15 MJ/kg) was as follows (g/kg): crude protein, 231; nitrogen-free extract, 528; crude ash, 66; crude fiber, 33; crude fat, 60; water, 82. Throughout the experimental period, pregnant animals were allowed access to food and tap water ad libitum. In our preliminary study, since there was no difference in daily food intake between the dietary control and biotin groups, no pair-fed control group was set up.

Morphological and histological evaluation. Pregnant dams in each group were examined daily for general signs and mortality. The females were killed by cervical dislocation on day 17 of gestation, and the numbers of implantation sites and dead or resorbed embryos were recorded following laparotomy. All litters were removed from the uterus, and dissected free of the surrounding membranes. Living fetuses were weighed individually and examined for morphological development under a dissecting microscope. Any gross external malformations were recorded. For histological examination, some placentae were washed with PBS solution and immediately fixed in Bouin's solution for 10 days. After dehydration and embedding in paraffin, specimens were sectioned (3 μm thick) and placed on glass slides. These sections were later deparaffinized in xylene and finally stained with hematoxylin and eosin.

Biochemical evaluation. In the dietary group, maternal blood was drawn directly from the heart shortly after killing by cervical dislocation on day 17 of gestation, and serum was prepared by centrifuging the clotted blood. Maternal and fetal livers and placentae were also excised and stored at −40 °C until biochemical analysis. Biotin concentrations were microbiologically measured with *Lactobacillus plantarum* ATCC 8014 according to the method of Fukui et al.[18].

Biotinidase is considered as a key enzyme for regulating the metabolism of biotin, and is found in placenta and various tissues[19]. It has been demonstrated that this enzyme cleaves biocytin, resulting in the regeneration of free biotin, and that this is necessary for the normal recycling of biotin, namely for biotin transport[20]. Therefore, the determination of biotinidase activity may be a reliable index of biotin status. On day 17 of gestation, biotinidase activity in the liver of the pregnant mice in the dietary group was determined by the method of Wolf et al.[21]. The concentrations of estradiol-E_2 in the maternal serum were measured by a time-resolved fluoroimmunoassay (TR-FIA) using a commercial kit (no. 1244-056) from Kabi Pharmacia, Co., Ltd., Tokyo[22].

For the kinetic study of serum biotin levels, some nonpregnant females received a single subcutaneous dose of 50 mg biotin/kg b.wt. in oil or NaOH solution, or were fed the CRF-1 diet containing 1,000 mg biotin/kg diet (the biotin diet), and serum was collected after 0, 0.5, 1, 3, 6 and 12 days. Each group consisted of 3 female mice. The sampling procedure and the biotin determination were essentially the same as those detailed above.

Statistical analyses. To detect statistically significant differences between the experimental groups, Fisher's exact test and the non-parametric Mann-Whitney U test were used as appropriate[23]. Statistical analysis of these data was performed on a personal computer using a commercial program, StatView statistical software (Abacus Concepts, Inc., Berkeley, CA, USA).

Results

Biotin kinetics. The relationship between serum biotin level and the time after administration of biotin in nonpregnant mice was as follows. The basal level of serum biotin was 6.1 ng/ml before the experiment. In the group treated with biotin in oil, the serum biotin level increased rapidly, reaching a maximum of 358.0 ng/ml after 12 h, and after 24 h decreased to 23.3 ng/ml. The rate of change of the serum biotin level in the NaOH/biotin-treated group was different from that in the oil/biotin-treated group. In the 'NaOH group', the serum biotin level increased gradually, reaching a maximum of 28.7 ng/ml after 3 days, and decreasing to 17.0 ng/ml after 6 days. In the dietary biotin-treated group (daily intake of biotin; about 5 mg), serum biotin level increased to 726.6 ng/ml after 24 h and reached a plateau of 1370.0 ng/ml after 4 days of feeding. This level remained constant during the entire period of feeding the biotin diet.

Reproductive functions. The effects of excessive amounts of biotin on reproduction and pregnancy in mice are shown in table 1. Dams exhibited no clinical signs (dermatitis and hair loss) or behavioral changes (ataxia) that might have been caused by excessive amounts of

Table 1. Effects of excessive amounts of biotin on reproduction and pregnancy of mice on day 17 of gestation.

	Untreated control	Oil		NaOH		Dietary[a]	
		control	biotin-treated[b]	control	biotin-treated[b]	control	biotin-treated[c]
Number of females							
mated	10	11	10	13	12	10	16
with implantation sites	9(0.90)[d]	9(0.82)	8(0.80)	12(0.92)	12(1.00)	8(0.80)	15(0.94)
Number of corpora lutea	15.8 ± 1.23[e]	16.7 ± 1.15	16.3 ± 1.98	15.9 ± 1.61	15.3 ± 1.30	15.3 ± 1.20	15.9 ± 1.59
Number of implantation sites	14.9 ± 1.85	16.4 ± 1.26	15.6 ± 2.23	15.3 ± 1.93	14.0 ± 1.93	14.1 ± 2.15	15.0 ± 2.28
Number of dead or resorbed fetuses	1.1 ± 0.99	2.7 ± 2.21	2.4 ± 1.80	1.8 ± 1.79	1.3 ± 0.94	0.8 ± 0.97	1.0 ± 1.26
Number of live fetuses	13.8 ± 2.20	13.8 ± 3.05	13.1 ± 2.03	13.6 ± 2.66	12.8 ± 2.45	13.4 ± 2.29	14.0 ± 1.90
Maternal body weight gain during gestation (g)	32.7 ± 2.34	28.6 ± 5.37* (87.5)[f]	27.8 ± 2.17** (85.0)	29.0 ± 3.06** (88.7)	28.5 ± 3.79** (87.2)	29.6 ± 3.02* (90.5)	30.0 ± 2.89* (91.7)
Fetal body weight (g)	1.10 ± 0.04	1.00 ± 0.07	1.03 ± 0.12	1.05 ± 0.05	1.07 ± 0.06	1.05 ± 0.05	1.05 ± 0.09
Placenta weight (mg)	109 ± 11	106 ± 13	105 ± 12	105 ± 9	112 ± 12	106 ± 9	105 ± 10
Food intake (g/day)	-[g]	-	-	-	-	6.2 ± 0.44	6.5 ± 0.12

[a]Powdered type of CRF-1 (Oriental Yeast Co., Tokyo).
[b]Total biotin dose 150 mg/kg body weight.
[c]Biotin content in the diet, 1,000 mg/kg diet.
[d]Successful pregnancy rate (the ratio of the number of dams with implantation to those mated).
[e]Each value represents mean ± SD.
[f]Relative values of untreated control values (percentage).
[g]Blank values were not measured.
*,**Significantly different from the untreated control value ($p < 0.05$ and $p < 0.01$, respectively), by Mann-Whitney U test.

Table 2. Effects of excessive amounts of biotin on embryonic development of mice on day 17 of gestation.

	Untreated control	Oil		NaOH		Dietary[a]	
		control	biotin-treated[b]	control	biotin-treated[b]	control	biotin-treated[c]
Number of females with live fetuses	9	9	8	12	12	8	15
Number of live fetuses examined	124	124	105	163	153	107	210
Number of malformed fetuses (%)[d]	2(1.6)	7(5.6)	1(1.0)	2(1.2)	3(2.8)	7(3.3)	
Number of malformations (%)[d]							
exencephaly	0	0	1(1.0)	0	0	0	0
cleft palate	0	1(0.8)	0	0	1(0.7)	0	1(4.8)
open eyelid	2(1.6)	3(2.4)	0	1(0.6)	1(0.7)	3(2.8)	3(1.4)
syndactyly	0	2(1.6)	0	1(0.6)	1(0.7)	0	3(1.4)
stubby tail	0	1(0/8)	0	0	0	0	0

[a]Powdered type of CRF-1 (Oriental Yeast Co., Tokyo).
[b]Total biotin dose 150 mg/kg body weight.
[c]Biotin content in the diet, 1,000 mg/kg diet.
[d]The incidence of fetuses with malformations among live fetuses examined.

biotin during gestation. Also, no mortality was detectable in dams during gestation in any experimental group. The oil and NaOH used as vehicles (controls) did not affect the successful pregnancy rate or the number of implantation sites. However, the number of resorbed or dead fetuses was slightly higher in the oil groups, whereas no increases were found in the NaOH groups. There were no differences in these maternal parameters between the untreated control and dietary control groups. At the end of gestation, maternal weight gain in the three experimental control groups was about 90% of that in the untreated control group. However, maternal weight gain was not affected by excessive amounts of biotin. Fetal parameters such as litter size, fetal body weight and placental weight were likewise similar in all experimental groups. The amounts of daily food intake did not differ between the dietary control and biotin-treated groups.

Embryonic development. Table 2 shows the teratogenic effects of excessive amounts of biotin in mice. External malformations detected in the present study were confined to a slight increase in the incidence of cleft palate and digital anomalies. External malformations were observed spontaneously at the incidences of 1.6%

Table 3. Biochemical analysis of maternal and fetal organs in dietary biotin-treated mice on day 17 of gestation.

Biochemical parameters		Control group	Biotin-treated group
Maternal serum	total biotin[1]	4.4(3.3–5.4)[a](4)[b]	815(780–850)*(4)
	biotinidase[2]	2.6(2.0–3.2)(6)	3.1(2.8–3.3)*(5)
	estradiol-E_2[3]	24.6(13.6–28.5)(5)	28.3(13.6–51.5)(5)
Maternal liver	total biotin[4]	0.64(0.62–0.65)(3)	1.36(1.16–1.62)*(4)
	biotinidase[5]	5.6(3.8–8.6)(6)	6.2(4.7–8.0)(5)
Fetal liver	total biotin[4]	0.65(0.53–0.98)(14)	50.3(44.0–58.3)**(10)
	biotinidase[5]	3.4(2.7–4.3)(14)	3.6(3.2–4.4)(10)
Placenta	total biotin[4]	0.22(0.18–0.30)(14)	37.0(25.0–43.8)**(10)
	biotinidase[5]	2.0(1.8–2.3)(14)	2.5(2.1–2.9)**(10)

[a]Each value represents mean (min-max).
[b]Values in parentheses refer to number of samples determined.
Total biotin; [1]ng/mg, [4]ng/mg wet weight.
Biotinidase activity; [2]n mol/ml/min, [5]p mol/mg/min.
Estradiol-E_2; [3]pg/ml.
*,**Significantly different from control values ($p < 0.05$ and $p < 0.01$, respectively), by Mann-Whitney U test.

and 2.8% of the fetuses in the untreated control and dietary control groups, respectively. These incidences were within the control ranges in our previous studies[8,24]. The incidence of malformations in the oil control group was 5.6%, which was slightly higher than that in the untreated control group. However, the excessive amount of biotin induced only one fetus (1.0%) with exencephaly in the oil group. No teratogenic effects were seen in the fetuses of dams treated with an excessive amount of biotin in the NaOH group. In the dietary biotin-treated group, 3.3% of the fetuses showed orofacial malformations and syndactyly, the incidence of which did not differ significantly from the 2.8% of the dietary control group.

Histological and biochemical examination. No histological changes in placentae, ovaries or maternal liver were detected in animals treated with dietary biotin. As shown in table 3, the concentration of serum biotin in the group given dietary biotin was 200-fold higher than that in the dietary control group. There was a difference in biotinidase activity of maternal serum and placenta between the dietary control and biotin-treated groups. However, no changes in estradiol-E_2 content were observed in the dietary biotin-treated group.

Discussion

In the present study using pregnant mice, biotin accumulated in serum, liver and placenta in the dietary biotin-treated group. Likewise, the serum biotin level in nonpregnant mice increased markedly after injection or ingestion of excessive amounts of biotin. However, high concentrations of serum biotin did not cause any histological changes in maternal organs, though slightly affected biotinidase activity. In addition, no specific effects of biotin excess on reproductive function were observed in mice given excessive amounts of biotin during gestation. These mouse fetuses showed no external malformations or histological changes in organs, in contrast to the findings of our previous studies using biotin-deficient mice[7–9].

In previous studies of the effects of biotin excess, long-term administration to rabbits (1.6 μmol/day) was found to prevent the formation of cholesterol-induced arteriosclerotic plaques in the aorta, although the cholesterol level in the plasma was not affected[3,25]. Several oral doses appeared to lower the metabolic rate, although no influence on metabolic turnover or renal function was observed with a single dose. Intravenous administration of high doses of biotin in biotin-deficient rats has no effect on the heart rate, blood pressure, circulation, respiration, or gastric secretion[26]. Therefore, excessive biotin intake may have no large direct influence on physiological function or on the circulatory system in experimental animals. In humans, it was demonstrated that the specific activities of three carboxylases in the mitochondria were raised by administration of large doses of biotin, but the activity of acetyl-CoA carboxylase located in the cytosol was not affected[27,28]. However, no adverse effects of oral or intramuscular treatment of biotin excess in infants with seborrheic dermatitis were detected[29,30]. Some recent studies showed a beneficial influence of biotin in the treatment of patients with inborn errors of metabolism (multiple carboxylase deficiency), sternocostoclavicular hyperostosis, and diabetes mellitus[31,32]. Although in most cases patients were treated with excessive amounts of biotin (5–20 mg daily), administered either orally or parenterally over a prolonged period, no adverse effects of biotin treatment have been reported.

As to reproductive effects of biotin excess, Paul et al.[16,17,33–35] found abnormal effects on the reproductive performance of female rats injected subcutaneously with 20.5 or 41 μmol biotin in 0.1N NaOH per 100 g b.wt. (5

and 10 mg/100 g b.wt., respectively)[16]. Treatment with excessive biotin doses led to irregularity in the estrus cycle, atrophic changes in the ovary, and fetal resorption. An acute dose (10 mg/100 g b.wt.) of biotin treatment before implantation, prevented development of fetuses and placenta, and an excess of biotin, even at post-implantation, exhibited a growth-inhibitory effect on fetuses and placentae. In addition, the biotin-treated rats showed changes in vaginal leukocyte numbers, especially an increase in leukocyte infiltration into the vaginal lumen during the dioestrus stage[17]. However, Mittelholzer[36] observed no disturbance of the reproductive performance of female rats with excessive administration of biotin (5 mg and 50 mg/kg b.wt. sc). To our knowledge, there is little information on effects of biotin on human reproductive function.

The underlying mechanism of reproductive and embryonic effects of biotin excess in experimental animals remains to be elucidated. Estrogen and progesterone are essential hormones for the regulation of the reproductive cycle and the growth and development of embryos. Paul et al.[16] showed that estrogen secretion was inhibited by excessive amounts of biotin, resulting in reduced liver and uterine glycogen content in female rats. Administration of estrogen (estradiol-17β) to biotin-treated rats ameliorated the detrimental effects on reproduction. It is therefore suggested that the reproductive changes following biotin treatment reflect the inhibition of estrogen production in the ovary. However, in the present study using female mice there was no difference in the estradiol-E_2 concentration of sera near term between dietary control and excess biotin-treated groups. Therefore, an excess amount of biotin may have no growth-inhibitory effects on the placenta in pregnant mice.

Biotinidase is present in various tissues, and is important for the recycling of endogenous biotin[19]. In this experiment, biotinidase activity was enriched in maternal serum and placenta in biotin-treated mice with an excessive intake of biotin from the diet. The biotin concentration in the fetal liver was higher than that in the maternal liver in the dietary biotin-treated group. These observations indicate that pregnant dams can actively supply the developing embryos with biotin through the placenta by means of this enzyme.

According to a previous histological investigation of material and fetal organs in rats, biotin treatment enhances the formation of the corpora lutea[16]. However, both the corpora lutea and stroma frequently showed atrophic changes, which is one piece of evidence that there is some biochemical alteration in biotin-treated rats. However, in pregnant mice fed excessive amounts of biotin in the present study, no histological changes in maternal liver or ovary were observed near term. There may be interspecies differences between rats and mice in the reproductive effects of excessive amounts of biotin as well as of biotin deficiency.

In conclusion, excessive amounts of biotin affected the specific activity of biotinidase in pregnant mice, but apparently did not disturb normal reproductive function or embryonic development. To confirm this, further biochemical and histological studies will be needed.

Acknowledgments. The author would like to thank Mr. T. Fukui, Byotai-Seiri Laboratory, Tokyo, for his skillful technical assistance, and Japan Roche, Inc., Tokyo, for supplying biotin.

1 Dakshinamurti, K., and Chauhan, J., Vitam. Horm. *45* (1989) 337.
2 Hommes, F. A., World Rev. Nutr. Diet. *48* (1986) 34.
3 Bonjour, J-.P., in: Handbook of Vitamins: Nutritional, Biochemical, and Clinical Aspects, p. 403. Ed. L. J. Machlin. Marcel Dekker, New York 1984.
4 Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., Anat. Rec. *100* (1948) 29.
5 Couch, J. R., Cravens, W. W., Elvehjem, C. A., and Halpin, J. G., J. Nutr. *35* (1948) 57.
6 Cravens, W. W., McGibbon, W. H., and Sebesta, E. E., Anat. Rec. *90* (1944) 55.
7 Watanabe, T., J. Nutr. *113* (1983) 574.
8 Watanabe, T., J. Nutr. *123* (1993) 2101.
9 Watanabe, T., and Endo, A., J. Nutr. *119* (1989) 255.
10 Watanabe, T., Congenital Anom. *30* (1990) 79.
11 Watanabe, T., Dakshinamurti, K., and Persaud, T. V. N., J. Nutr. *125* (1995) 2114.
12 Benschoter, C. A., and R. Paniagua, G., Ann. entomol. Soc. Am. *59* (1966) 298.
13 Benschoter, C. A., J. econ. Entomol. *60* (1967) 1326.
14 Pillai, M. K. K., and Madhukar, B. V. R., Naturwissenschaften *56* (1969) 218.
15 Cohen, E., and Levinson, H. Z., Experientia *24* (1968) 367.
16 Paul, P. K., Duttagupta, P. N., and Agarwal, H. C., Curr. Sci. *42* (1973) 613.
17 Paul, P. K., Duttagupta, P. N., and Agarwal, H.C., Curr. Sci. *42* (1973) 206.
18 Fukui, T., Iinuma, K., Oizumi, J., and Izumi, Y., J. nutr. Sci. Vitaminol. *40* (1994) 491.
19 Wolf, B., Heard, G. S., McVoy, J. R. S., and Raetz, H. M., J. inherit. metab. Dis. *7* (Suppl. 2) (1984) 121.
20 Koivusalo, M., and Pispa, J., Acta physiol. scand. *58* (1963) 13.
21 Wolf, B., Grier, R. E., Allen, R. J., Goodman, S. I., and Kien, C. L., Clinica chim. Acta *131* (1983) 272.
22 Hemmilä, I., Dakubu, S., Mukkala, V-.M., Siitari, H., and Lövgren, T., Analyt. Biochem. *137* (1984) 335.
23 Siegel, S., in: Nonparametric Statistics: for the Behavioral Sciences, p. 68. McGraw-Hill Kogakusha, Tokyo 1956.
24 Watanabe, T., and Endo, A., Teratology *30* (1984) 91.
25 Körner, W. F., and Völlm, J., in: Klinische Pharmakologie und Pharmakotherapie, p. 381. Ed. H. P. Kuemerle, E. R. Kuemerle, E. R. Garrett, and K. H. Spitzy. Urban and Schwarzenberg, München 1976.
26 György, P., and Langer, B. W., Jr., in: The Vitamins. Chemistry, Physiology, Pathology, Methods, Vol. II, 2nd edn., p. 261. Ed. W. H. Sebrell, Jr., and R. S. Harris. Academic Press, New York 1968.
27 Stirk, J-.H., Alberti, K. G. M. M., and Bartlett, K., Biochem. Soc. Trans. *11* (1983) 185.
28 Bartlett, K., Horsburgh, T., and Gompertz, D., Clin. Sci. *58* (1980) 111.
29 Dupré, A., Lassère, J., Christol, B., Bonafé, J. L., Rumeau, H., Arbarel, N., Coberand, S., Périole, N., and Sorbara, A. M., Rev. Med. Toulouse *13* (1977) 675.
30 Bonjour, J. P., Int. J. Vitam. Nutr. Res. *47* (1977) 107.
31 Wolf, B., Hsia, Y. E., Sweetman, L., Feldman, G., Boychuk,

R. B., Bart, R. D., Crowell, D. H., Di Mauro, R. M. and Nyhan, W. L., Pediatrics *68* (1981) 113.
32 Maebashi, M., Makino, Y., Furukawa, Y., Ohinata, K., Kimura, S., and Sato, T., J. clin. Biochem. Nutr. *15* (1993) 65.
33 Paul, P. K., and Duttagupta, P. N., J. nutr. Sci. Vitaminol. *21* (1975) 89.
34 Paul, P. K., and Duttagupta, P. N., J. nutr. Sci. Vitaminol. *22* (1976) 181.
35 Paul, P. K., in: CRC Handbook Series in Nutrition and Food, Section E; Nutritional Disorders, p. 47. Ed. M. Rechcigl, Jr. CRC Press, West Palm Beach 1978.
36 Mittelholzer, E., Int. J. Vitam. Nutr. Res. *46* (1976) 33.