Effect of riboflavin deficiency on reproductive performance and on biochemical indices of riboflavin status in the rat

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1. Young female rats were made riboflavin-deficient by feeding a purified diet containing casein (210 g/kg). This basal diet provided 0·40 mg riboflavin/kg diet, to which was added additional riboflavin at 0, 0·12 or 0·25 mg/kg diet. Control animals received the same diet with 15 mg added riboflavin/kg. The diets were given for 4 weeks before mating, then throughout pregnancy and for 15 d of lactation.

2. With no added riboflavin in the diet, reproduction was severely impaired and fetal resorption was usually observed. With 0·12 mg added riboflavin/kg diet, however, reproduction was usually successful, and the growth of dams and pups was only marginally depressed in comparison with pair-fed controls optimally supplied with riboflavin.

3. The activation coefficient (stimulated:basal activity) of erythrocyte glutathione reductase (NAD(P)H) (*EC* 1.6.4.2) was high, and the concentration of riboflavin in the liver was correspondingly low in the dams receiving diets containing 0·12 or 0·25 mg added riboflavin/kg and in their sucking pups at 15 d post partum. Riboflavin levels in the milk from both groups of dams were about eightfold lower than in controls. There was little evidence that the sucking pups could maintain their riboflavin level at the expense of that in the maternal tissues.

Severe maternal riboflavin deficiency, like a severe deficiency of many other nutrients, is known to impair reproductive efficiency in animals, resulting in failure to produce viable offspring (Coward *et al.* 1942) or in congenital malformations (Warkany & Schraffenberger, 1944). However, there are no recent studies of the effect of moderate riboflavin deficiency on gestation and subsequent rearing of sucking animals, using purified diets containing adequate amounts of all other nutrients known to be necessary for reproduction, and with the use of pair-fed control animals to match the effects of inanition.

The aims of the present study were first, to establish the minimum concentration of riboflavin in the diet of young female rats which is required to support gestation and the rearing of sucking offspring; second, to measure in these animals the values of key biochemical indices of riboflavin status, namely liver flavin concentrations and erythrocyte glutathione reductase (NAD(P)H) (*EC* 1.6.4.2; GR) activation coefficients, which indicate the extent of tissue depletion of riboflavin; and third, to examine some functional effects of marginal riboflavin deficiency in sucking pups, particularly those related to fatty acid utilization for energy release. The latter aspect is described in the accompanying paper (Duerden & Bates, 1985).

A brief description of the present study has been published as an abstract (Duerden & Bates, 1983).

ANIMALS AND METHODS

Female Norwegian hooded rats reared on Purina-chow diets were housed individually in suspended wire cages, mean body-weights being 150 g at the beginning of the experiment. Coprophagy was prevented during the initial 4-week period of establishment of riboflavin deficiency by the use of a circular collar of Plastizote fitted around the animal's neck, as previously described (Olpin & Bates, 1982). The size of the central hole was adjusted so

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that the collar could be pushed over the animal's head. It was removed at approximately fortnightly intervals to permit the animals to clean themselves and replaced, with adjustment of overall size and of the central hole, as the animals grew.

The basic riboflavin-deficient diet was similar to that used previously (Olpin & Bates, 1982). It contained (g/kg): sucrose 706, casein 'low in vitamins' (BDH, Poole, Dorset) 210, salt mixture (Greenfield *et al.* 1969) 50, arachis oil 30, choline chloride 2 and cystine 1·5. The riboflavin content of the casein was measured by fluorimetry (Bessey *et al.* 1949) and by microbiological assay using *Lactobacillus casei* (Barton-Wright, 1961), the mean estimated content being 1·95 mg/kg casein, which thus gave 0·40 mg/kg diet. The following vitamins were added (mg/kg diet): calcium pantothenate 20, thiamine hydrochloride 4, pyridoxine hydrochloride 9, nicotinamide 25, biotin 1·0, pteroylmonoglutamic acid 1·0, cyanocobalamin 0·05, menadione 9, α-tocopherol 250, retinyl acetate 2·1 retinol equivalents and ergocalciferol 0·0075. In addition to the riboflavin contributed by the casein, riboflavin added separately as the free vitamin was supplied at 0, 0·12, 0·25 or 15 mg/kg. Both pair-fed and *ad-lib.*-fed control animals received diets containing 15 mg riboflavin/kg. Pair-fed animals were individually paired with the riboflavin-deficient animals where possible, although this was not always feasible after mating, and some readjustment between pairs was necessary.

At 4 weeks after transfer to the special diets, collars were removed and the animals were mated with males given a Purina-chow diet. Presence of a vaginal plug was taken as evidence of successful mating. The females then continued to receive the same purified diets until term: collars were not applied during gestation and lactation. Litter sizes were adjusted to eight pups for each dam by removal or fostering within feed groups; food pots were raised from the floor of the cage to prevent access by the pups, and the pups were reared to day 15 post partum, receiving only their mother's milk.

On day 15, the pups were removed and killed by diethyl ether anaesthesia; blood samples were collected and stored frozen for measurement of GR activation coefficient and liver samples were excised and stored frozen for flavin and lipid analysis, or were used immediately for the isolation of mitochondria for measurement of respiration. Likewise, samples of interscapular brown adipose tissue were removed for the isolation of mitochondria (Duerden & Bates, 1985).

At 2 h after removal of the litters, the dams were anaesthetized with pentobarbitone and milked by suction from a chamber connected to a water pump after the injection of 8 units oxytocin; 0·5–2·5 ml milk was collected within 30 min. The riboflavin-deficient dams usually produced less milk than the controls. They were then killed by exsanguination and blood and liver samples were treated as for the pups.

The activation coefficient (stimulated: basal activity; AC) of erythrocyte GR (EGRAC) was measured in washed erythrocyte preparations by a previously-described method (Prentice & Bates, 1981), modified for use with a Roche Cobas Bioanalyser (Powers *et al.* 1983). The reaction time was 5 min, following addition of NADPH as start reagent. Hepatic FAD and FMN, together with free riboflavin, were measured by the method of Bessey *et al.* (1949), as described previously (Prentice & Bates, 1981). Milk riboflavin was measured by the same method.

Statistical analysis

Student's *t* test was used for the comparison between groups, after ascertaining that the values approximated to a normal distribution.
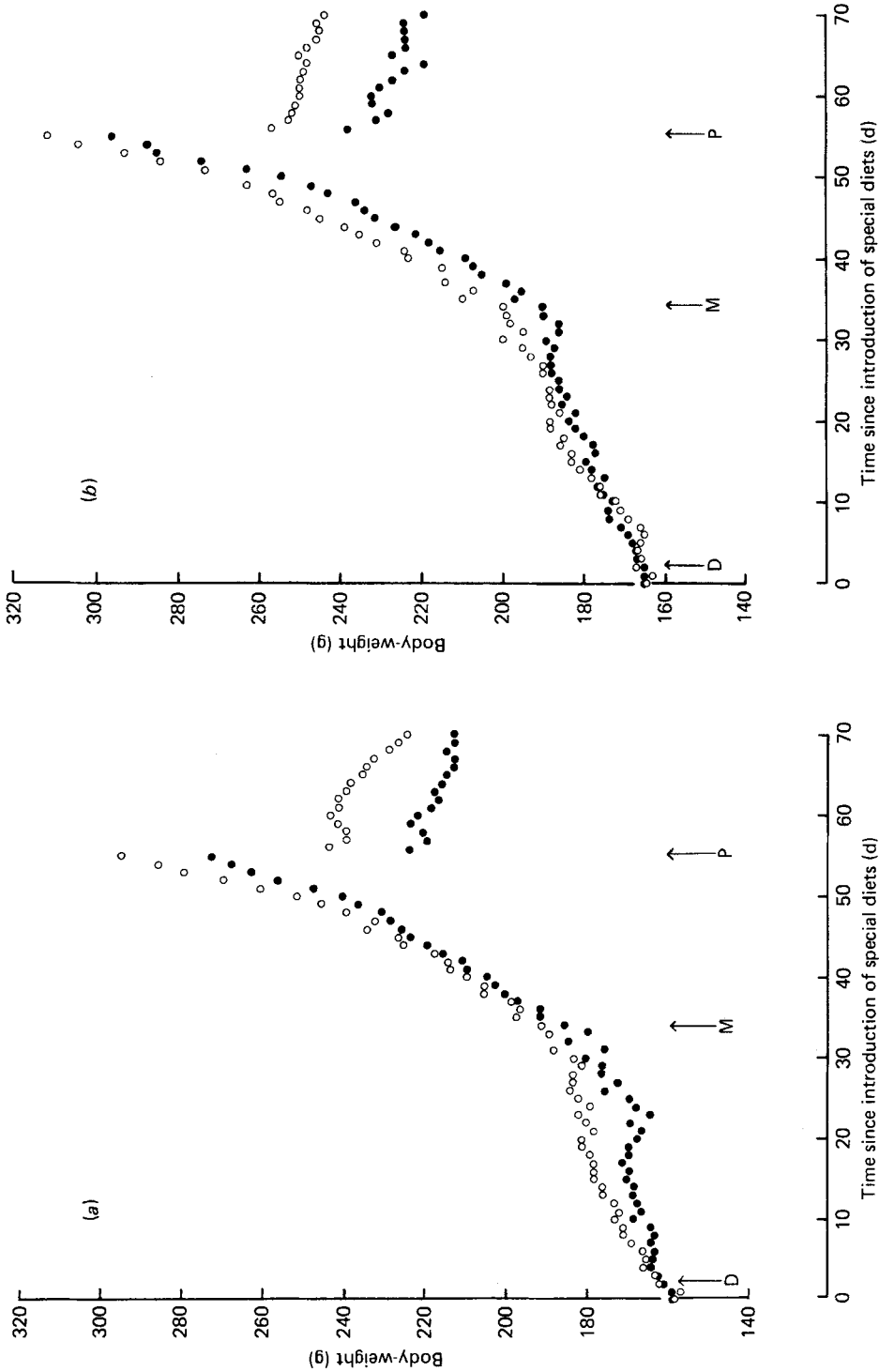
Fig. 1. Body-weights (g) of dams from time of introduction of special diets. Each point represents the mean daily value for the animals in each group; standard errors varied between 2 and 8 g. (a) Riboflavin-restricted (●——●) group receiving 0·12 mg added riboflavin/kg diet (n 6) and their pair-fed controls (○——○) (n 5). (b) Riboflavin-restricted (●——●) group receiving 0·25 mg added riboflavin/kg diet (n 5) and their pair-fed controls (○——○) (n 6). D, introduction of riboflavin-restricted diets; M, mating; P, parturition.
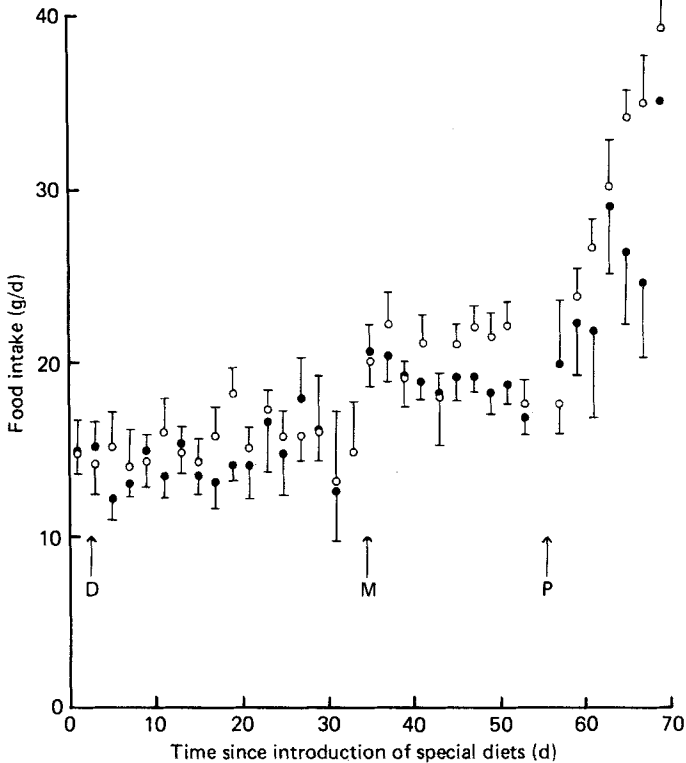
Fig. 2. Food intakes (g/d), at 2-d intervals, of dams from time of introduction of special diets. Values are means with their standard errors represented by vertical bars. Riboflavin-restricted (●—●) group receiving 0·12 mg added riboflavin/kg diet (*n* 6) and their pair-fed controls (○—○) (*n* 5).

RESULTS

Animals which received no added riboflavin, apart from that supplied by casein in their diets, failed to reproduce successfully. The frequent occurrence of vaginal bleeding, weight loss during the course of pregnancy and vaginal resorption sites indicated that although fertilization had occurred, it was followed by resorption during the course of gestation. However, animals which had been made riboflavin-deficient to this severe extent were found to recover sufficiently, on addition of only 0·12 mg riboflavin/kg diet, to achieve successful gestation after subsequent mating. There was thus no permanent damage to the reproductive system (cf. Coward *et al.* 1942). Congenital malformation was not frequently observed; it occurred in one fetus only, in one of the severely riboflavin-deficient groups.

In contrast to the failure of gestation when no riboflavin was added to the basal diet, reproduction was more frequently successful in animals which received 0·12 or 0·25 mg added riboflavin/kg diet. In the present experiment, five of eight animals in each riboflavin-restricted group achieved a complete cycle of gestation and lactation.

Fig. 1 shows the growth curves of these dams from the beginning of the experiment to the time of death at 15 d post partum. During the first 6 weeks of feeding on the special diets, growth was slow. This may have been due partly to the relatively low food intakes (Fig. 2), although the growth of the pair-fed animals was only 10% less than that of *ad lib.*-fed controls in a parallel experiment. Thus the effect of food restriction *per se* does not

Table 1. *Birth weights, litter numbers and body-weights at 15 d post partum in riboflavin-restricted and pair-fed control rats*

(Mean values with their standard errors; no. of pooled litters in parentheses)

Treatment	Riboflavin added to maternal diet (mg/kg)	Body-wt of litters at birth (g)		No. of pups in each litter		Body-wt of litters at 15 d post partum (g)	
		Mean	SE	Mean	SE	Mean	SE
Riboflavin-restricted (group 1)	0·12	45·4	4·63 (5)	8·75	1·03 (5)	168·3	9·12 (4)
Pair-fed to group 1	15	44·9	5·27 (5)	8·40	1·12 (5)	181·9	13·83 (5)
Riboflavin-restricted (group 2)	0·25	51·9	2·70 (5)	10·00	0·70 (5)	196·0	7·30 (5)
Pair-fed to group 2	15	51·0	2·94 (6)	9·50	0·50 (6)	202·8	10·98 (6)

None of the differences between riboflavin-restricted and corresponding pair-fed control groups were significant ($P > 0.05$).

appear to have been very marked, and it is likely that the application of collars also had some effect on growth rate.

After mating, the riboflavin-restricted groups increased their voluntary food intakes (Fig. 2) and showed accelerated weight gain due to growth of the fetuses. The group receiving 0·12 mg added riboflavin/kg diet gained weight almost as rapidly as its control group, although the mean body-weight at mating was somewhat lower (Fig. 1), and this was also reflected by the fact that no difference was observed between litter weights of control and deficient groups at birth (Table 1). Likewise, litter numbers were identical in deficient and control groups (Table 1).

After parturition, food intake accelerated as suckling commenced (Fig. 2), and again there was no major difference in body-weight gain between riboflavin-restricted and the respective control groups (Fig. 1), although from parturition onwards both of the riboflavin-deficient groups of dams maintained somewhat lower body-weights than their controls, and there was a trend towards lower body-weights of the litters at 15 d post partum (Table 1). Owing to large standard errors within the control groups, their litter weights were not significantly different from those of the riboflavin-restricted groups, but it is worth noting that a significant difference ($P < 0.05$) was detected between the two deficient groups (0·12 *v.* 0·25 mg added riboflavin/kg diet). This trend towards lower body-weights of the riboflavin-restricted pups as sucking continued was observed consistently in several experiments, but its magnitude was never very great.

In several experiments it was found that the minimum riboflavin intake which was compatible with successful lactation was lower than that needed for successful gestation.

The depletion of tissue levels of riboflavin, in both dams and pups at 15 d post partum, is indicated in Tables 2 and 3 by an increase in EGRAC and a reduction in hepatic flavin levels in the riboflavin-restricted groups.

In the present experiment, the magnitude of the difference in EGRAC between the riboflavin-restricted groups and pair-fed controls was somewhat greater for the dams than for the pups (Table 2). In other experiments, however, the riboflavin-restricted pups had a higher value of EGRAC than the riboflavin-restricted dams, and the overall picture from several experiments was that the dams and pups were affected to about the same extent. There was no indication that the lowest level of riboflavin intake affected the dams' EGRAC

Table 2. *Erythrocyte glutathione reductase* (NAD(P)H) (EC *1.6.4.2*) *activation coefficients* (*EGRAC*) *in riboflavin-restricted and pair-fed rats*

(Mean values with their standard errors; no. of animals (dams) or pooled litters (pups) in parentheses)

| | Riboflavin added to maternal diet (mg/kg) | EGRAC | | | |
| | | Dams | | Pups | |
Treatment		Mean	SE	Mean	SE
Riboflavin-restricted (group 1)	0·12	2·04***	0·03 (4)	1·78***	0·07 (4)
Pair-fed to group 1	15	1·28	0·03 (5)	1·21	0·03 (5)
Riboflavin-restricted (group 2)	0·25	2·00***	0·09 (5)	1·59***	0·03 (5)
Pair-fed to group 2	15	1·31	0·03 (6)	1·20	0·05 (6)

Mean values were significantly different from corresponding pair-fed controls: *** $P < 0·001$.

Table 3. *Hepatic flavin levels in riboflavin-restricted and pair-fed rats*

(Mean values with their standard errors; no. of animals (dams) or pooled litters (pups) in parentheses)

| | Riboflavin added to maternal diet (mg/kg) | Dams | | | | Pups | | | |
| | | FMN+ riboflavin† | | FAD† | | FMN+ riboflavin† | | FAD† | |
Treatment		Mean	SE	Mean	SE	Mean	SE	Mean	SE
Riboflavin-restricted (group 1)	0·12	1·48***	0·17 (4)	8·15***	0·61 (4)	1·26***	0·17 (4)	3·98***	0·21 (4)
Pair-fed to group 1	15	4·86	0·14 (5)	19·01	0·58 (5)	5·09	0·21 (5)	14·34	0·72 (5)
Riboflavin-restricted (group 2)	0·25	1·91***	0·16 (5)	8·82***	0·67 (5)	1·89***	0·06 (5)	5·55***	0·20 (5)
Pair-fed to group 2	15	4·58	0·16 (6)	16·81	0·48 (6)	5·19	0·16 (6)	15·65	0·34 (6)

Mean values were significantly different from corresponding pair-fed controls: *** $P < 0·001$.
† Flavin levels in μg/g wet liver.

more severely than the intermediate level, but there was some indication of a higher mean EGRAC in the more-severely-restricted group of pups (Table 2).

Hepatic flavin levels indicated a severe depletion for both dams and pups at both the low levels of riboflavin intake (Table 3), being slightly (but not significantly) more severe at the lower of the two intake levels used. An unexpected observation was that the extent of depletion of the pups was somewhat greater than was observed for the corresponding dams. Thus control:riboflavin-restricted values for FAD were 2·33 and 1·95 for the two groups of dams, compared with 3·60 and 2·87 for the two groups of pups, and for the riboflavin+ FMN fraction, the values for the ratio were 3·28 and 2·62 for the two groups of dams compared with 3·96 and 2·83 for the two groups of pups.

Milk riboflavin levels at the time of death are shown in Table 4. Clearly, there was a large difference between the riboflavin-restricted animals and their respective controls, but no detectable difference between the two groups of riboflavin-restricted animals.

Table 4. *Milk riboflavin in riboflavin-restricted and pair-fed rats*

(Mean values with their standard errors; no. of animals in parentheses)

Treatment	Riboflavin added to maternal diet (mg/kg)	Riboflavin concentration (μg/g milk)	
		Mean	SE
Riboflavin-restricted (group 1)	0·12	0·61***	0·15 (4)
Pair-fed to group 1	15	7·96	0·69 (5)
Riboflavin-restricted (group 2)	0·25	0·56***	0·15 (5)
Pair-fed to group 2	15	8·77	0·99 (6)

Mean values were significantly different from pair-fed controls: *** $P < 0.001$.

DISCUSSION

Despite numerous early studies of the effect of diets deficient in riboflavin on the development of congenital malformations in rats (Warkany & Schraffenberger, 1943, 1944; Noback & Kupperman, 1944; Leimbach, 1949; Gilman *et al.* 1952; Grainger *et al.* 1954; Giroud & Boisselot, 1947; Giroud *et al.* 1950, 1951; Shepherd *et al.* 1968) there is surprisingly little information about the minimum amount of riboflavin needed to support reproduction in the rat, when supplied with an abundance of all other known required nutrients. In the present study, the casein component of the diet supplied about 0·40 mg riboflavin/kg when present at 210 g/kg, and this was insufficient to support gestation. However, a very small increment yielding a total of 0·51 mg/kg was sufficient, in several experiments, to support reproduction with nearly the same efficiency as a control diet which supplied 15 mg riboflavin/kg. The amount needed for saturation of the tissues and optimum biochemical functions was not determined in the present study, but from the observations of Leclerc (1979a) it is likely to be about 2–2·5 mg/kg. This four- to fivefold difference between minimum and optimum intakes in the rat is comparable to the difference between unsupplemented pregnant or lactating women in a West African village, and a group of supplemented mothers in the same community who were receiving the minimum riboflavin increment which was needed to prevent biochemical deficiency (Bates *et al.* 1981).

In contrast to observations by Coward *et al.* (1942), Segaloff & Segaloff (1944) and Esch *et al.* (1981), an impairment in fertility resulting from interference with the oestrous cycle was not apparent from the response of the riboflavin-deficient animals receiving no added riboflavin in the present study. The extent of riboflavin restriction generally did not prevent conception, but resulted in early fetal resorption. It will be of interest to determine whether interference with the oestrous cycle requires additional dietary or environmental insults, which act in combination with a marginal riboflavin intake.

At intermediate intakes of riboflavin, which were just sufficient to support reproduction, there was little evidence that the sucking offspring were protected from the effect of maternal deficiency as a result of sequestering mechanisms operating in the mammary glands. Indeed, the milk levels of riboflavin from the riboflavin-restricted dams were extremely low, and the hepatic flavin levels of the riboflavin-deficient pups were somewhat lower than those of the corresponding dams. A similar conclusion, that the offspring are not protected against the effects of maternal deficiency, was reached for severely-deficient rats by Giroud *et al.* (1951), although in more recent studies by Leclerc (1979b) it was shown that when the maternal-diet riboflavin content during lactation was increased from about 4 to about 8 mg/kg, there was a small but definite increase in maternal hepatic riboflavin levels without

any accompanying increase in pups' tissue levels, suggesting that the pups reached a maximum tissue riboflavin level at a lower maternal intake than was needed for maternal tissue saturation. There may, of course, be a minimum intake below which this protective mechanism is inoperative, and the fetus (or sucking offspring) then becomes at least as severely deficient as the mother. Some evidence from human studies (Bamji, 1976; Clarke, 1971; van den Berg *et al.* 1978; Bates *et al.* 1982) suggests that in riboflavin-deficient human populations the newly-born infant may generally be less deficient than its mother, and studies in West Africa (Bates *et al.* 1982) suggest that some protection may continue through the period of sucking until weaning. This conclusion is, however, derived entirely from studies of blood, i.e. EGRAC and riboflavin levels, and the picture may be different in other tissues. In addition, of course, it is possible that human infants have the ability to sequester riboflavin more efficiently than rat fetuses and pups, perhaps partly because the numbers of dependent offspring are fewer, and the demand for nutrients is thus less intense.

Clearly there is still much to be learned about the interrelation between mother, fetus and sucking offspring with respect to their requirements for riboflavin and the effect of a marginal deficiency, and it is also essential to assess the extent to which studies on laboratory animals can clarify the interrelations which exist in human reproduction. This is an important area for study, since a high proportion of the world's human population exists on diets which are deficient in riboflavin, and it is possible that a marginal riboflavin deficiency may interact with other environmental and genetic factors to upset the delicate processes of fetal and neonatal development.

The accompanying paper (Duerden & Bates, 1985) describes functional changes in the brown adipose tissue of rat pups born to riboflavin-deficient dams, and thus provides evidence of the sensitivity of particular vital metabolic pathways towards moderate degrees of riboflavin deficiency.

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