Ten embryos were explanted at 7·5 days of gestation and grown in the circulators for 4 days. At explantation egg cylinders were about 0·6 × 0·15 mm and all expanded in culture, eight forming embryos with beating hearts and six reaching the ten to fifteen somite stages with yolk sacs more than 2·5 mm in diameter.

Only three of the explanted embryos—from the group explanted at 8·5 days—developed a functioning blood circulation, although the yolk sac in nearly all explants could be seen to have a capillary network with conspicuous masses of erythrocytes. This circulatory failure seemed to be associated with abnormal heart development in most of the embryos. The two lateral heart primordia, instead of fusing into a single heart, developed into two more or less separate hearts. Often these paired hearts beat independently of each other. This result recalls the experiments of Goss[3], who obtained double hearts in rat embryos explanted at 9·5 days, by applying pressure to prevent the heart primordia from uniting. It seems likely that a similar mechanism may have operated in our embryos. *In vivo* the yolk sac at 9·5 days of gestation is oval with the embryo forming at one end, but in our cultures it was approximately spherical by this stage and probably exerted abnormal mechanical stresses on the embryo.

Although the frequency of abnormalities, particularly of heart formation, was greater than in embryos explanted at later stages, there was clearly much growth and development of the embryo and its membranes. This seems to be the first time that extensive growth has been obtained in culture from rat embryos as young as 1–2 days' post-implantation, and it is interesting that rabbit embryos of similar stages have also recently been successfully cultivated using circulating medium[4]. Such methods have many applications for mammalian experimental embryology, and the present studies are being continued.

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Diminished Responsiveness to Thyroid Hormone in Riboflavin-deficient Rats

The hepatic activity of mitochondrial α-glycerophosphate dehydrogenase can be up to 100-fold greater in hyperthyroid rats than thyroidectomized rats[1]. Both the thyroid hormones, thyroxine or triiodothyronine, or their analogues, enhance enzyme activity and enhancement is blocked by simultaneous administration of actinomycin *D* and/or puromycin[2,3]. The enhancement has been suggested as a test for thyroid function[4]. Here we report that both the basal activity of the enzyme and the maximal activity induced by triiodothyronine are dependent upon adequate dietary intake of riboflavin.

Male Wistar weanling rats were divided into three groups. The first group was placed initially on a low iodine diet for 2–4 weeks, and then received a thyroid-ablating dose of 300 μCi ^{131}I carrier free/100 g body weight in a single intraperitoneal injection. The thyroid gland is virtually completely destroyed[5] and the animals become athyreotic within 7 weeks after irradiation. The animals, after treatment with ^{131}I, remained on the iodine-deficient diet, with a normal riboflavin intake. The second group, individually caged, was fed *ad libitum* a diet deficient in riboflavin (Nutritional Biochemicals Corporation of Cleveland, Ohio). The third group served as controls and was fed *ad libitum* a diet specially prepared by Nutritional Biochemicals Corporation, which was identical in composition to the riboflavin-deficient diet except for the addition of 22 mg of riboflavin/kg diet. Results obtained with these animals were the same as those with rats fed ordinary Purina Chow.

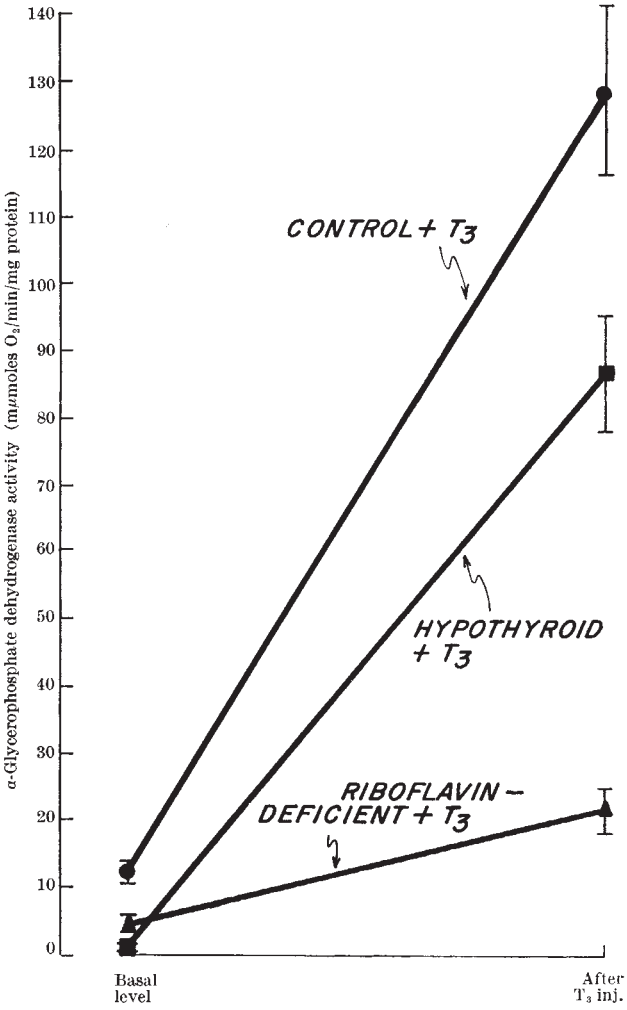

Fig. 1. Hepatic activity of mitochondrial α-glycerophosphate dehydrogenase before and 48 h after intraperitoneal injection of triiodothyronine (100 μg/100 g body weight) to normal, hypothyroid and riboflavin-deficient rats. Data are means of ten to fifteen animals with *S.E.*

Hypothyroid, riboflavin-deficient and control animals were killed both before and after a single intraperitoneal injection of L-triiodothyronine (100 μg/100 g body weight). Animals were studied 48 h after treatment with L-triiodothyronine by which time α-glycerophosphate dehydrogenase reaches maximal activity[6]. The livers were removed and mitochondria isolated following homogenization in sucrose and differential centrifugation. All assays were performed on fresh liver.

Measurements were made of mitochondrial α-glycerophosphate dehydrogenase with an oxygen electrode

(Yellow Springs Instrument Company, Yellow Springs, Ohio) to record oxygen uptake by the preparations and with phenazine methosulphate as the electron acceptor[7]. Enzyme activity in normal animals is increased ten-fold by triiodothyronine (Fig. 1)[1]. In hypothyroid rats, basal enzyme activity is reduced to less than one-fifth of normal; following triiodothyronine injection, activity is elevated some seventy-five-fold above basal levels. As others have found, the maximal activity recorded in hypothyroid animals treated with T_3 is somewhat less ($P < 0{\cdot}05$) than that of normal animals similarly treated[6].

In animals fed a diet deficient in riboflavin for more than 2 months, basal hepatic enzyme activity is reduced substantially below normal levels (Fig. 1). These data extend the findings of previous reports that activities of flavoprotein enzymes, such as glycolic acid oxidase, TPN cytochrome *c* reductase, D and L amino-acid oxidase, and xanthine oxidase, are reduced in riboflavin deficiency[8,9], and that reductions in activity occur in both hypothyroidism and riboflavin deficiency[10,11]. Mitochondrial α-glycerophosphate dehydrogenase is unusual among flavoprotein enzymes, however, in having lower hepatic activity in hypothyroid than in riboflavin-deficient rats.

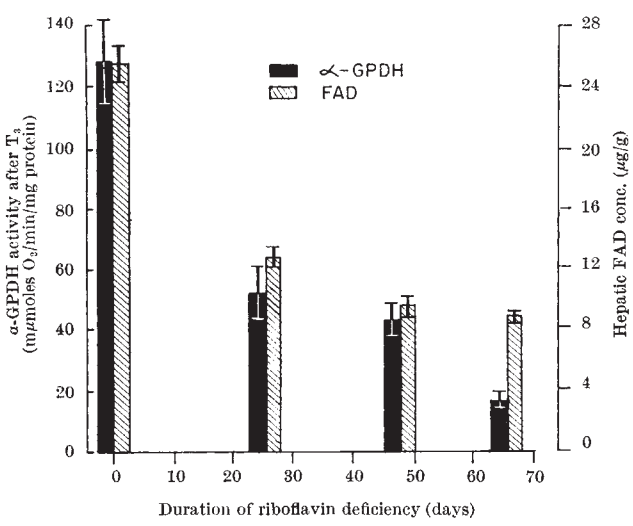

Fig. 2. Relations of mitochondrial α-glycerophosphate dehydrogenase activity after triiodothyronine injection to hepatic levels of FAD in rats with progressive riboflavin deficiency. Data are means of six to nine animals with *S.E.*

When riboflavin-deficient rats are treated with triiodothyronine, only a five-fold increase in activity occurs, reaching levels which only approximate those of normal animals without triiodothyronine. Thus the response to triiodothyronine is markedly diminished in deficient rats. Experiments to determine the effect of calorie restriction on enzyme activity showed that normal rats which had been starved for 3–5 days are not less responsive to triiodothyronine.

In subsequent experiments, animals were killed at intervals after being fed riboflavin-deficient diets, and simultaneous measurements were made of mitochondrial α-glycerophosphate dehydrogenase activity following triiodothyronine stimulation and of hepatic concentrations of flavin adenine dinucleotide (FAD)[12]. Comparisons were made of enzyme activity with the basal levels of FAD, because no change in FAD concentrations occurred with triiodothyronine injection in these circumstances. The induction of the enzyme by thyroid hormone and the hepatic FAD content were both decreased in deficient rats (Fig. 2). The magnitude of the decrease in enzyme induction and in FAD concentration was remarkably similar. Only in animals deficient for the longest time, 65 days, was induced enzyme activity reduced more than FAD concentration. The lack of further decrease in FAD is consistent with previous observations[8] that the tissue concentrations of FAD in deficient animals have a lower limit necessary for survival.

The decreased enzyme activity of deficient rats was not increased by incubation of liver extracts *in vitro* with either FAD or riboflavin (Table 1). This suggests that the measured activity probably represents true loss of apoenzyme rather than deficiency of the cofactor. Normal responsiveness of deficient animals could be restored by giving riboflavin orally and parenterally for 4 to 6 days.

Table 1. LACK OF EFFECT OF FAD AND RIBOFLAVIN ADDED *in vitro* ON ACTIVITY OF HEPATIC MITOCHONDRIAL α-GLYCEROPHOSPHATE DEHYDROGENASE FROM RIBOFLAVIN-DEFICIENT RATS

Source of enzyme	Additions	Enzyme activity (mμmoles O_2/min/mg protein)
Normal rats	No incubation	128·6 ± 12·8
Riboflavin-deficient rats	No incubation	50·6 ± 11·9
Riboflavin-deficient rats	None	49·3 ± 11·9
Riboflavin-deficient rats	+ Riboflavin	40·8 ± 7·4
Riboflavin-deficient rats	+ FAD	50·4 ± 9·8

Mitochondrial preparations in 0·6 M potassium phosphate buffer were incubated at 37° C for a 1 h period with and without the addition of riboflavin (10^{-3} M) and FAD (2×10^{-4} M). All data are means with *S.E.* of seven experiments. All animals received L-triiodothyronine (100 μg/100 g) 48 h before death. Riboflavin-deficient rats were on the test diet for 25 days.

Our results indicate that restriction in the supply of dietary riboflavin has profound effects on hepatic enzyme activity: both the basal levels of mitochondrial α-glycerophosphate dehydrogenase and particularly the levels induced by triiodothyronine are markedly diminished. Flavoprotein enzymes are in general stabilized against inactivation by binding to their coenzymes, FMN and FAD[10]. Lack of FMN and FAD may account in part for the reductions in enzyme activity observed both in hypothyroidism and in riboflavin deficiency.

When the rate of apoenzyme synthesis in riboflavin-deficient animals is markedly accelerated by excess thyroid hormone, the deficiency of FMN and FAD would be expected to intensify, and a greater limitation would thereby be imposed on the accumulation of apoenzymes. We propose that the diminished response of liver mitochondrial α-glycerophosphate dehydrogenase to thyroid hormone may be related to the restricted supply of FAD available for stabilizing newly synthesized enzyme protein.

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