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Effect of Dietary Protein on Liver Riboflavin Levels and on Inactivation of Estradiol by Liver.*

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In the course of a study on the effect of vitamin deficiencies on the metabolism of estrogenic hormones,[1] it was found that the livers of thiamine- as well as of riboflavin-deficient rats failed to inactivate estradiol whereas the liver of animals depleted in pyridoxine, pantothenic acid, biotin, or vitamin A retained the ability to metabolize the hormone. Furthermore, determinations of thiamine and riboflavin levels in the liver of deficient animals demonstrated a direct relationship between the vitamin content of this organ and its ability to inactivate estradiol. In continuation of these studies the influence of the protein intake upon the riboflavin content of the liver and the ability of liver tissue to inactivate estradiol was investigated.

Weanling rats of both sexes were placed on a basal diet containing casein as the only source of protein. The diet had the following composition: casein, vitamin free, 8%; dextrose, 78%; salt mixture, U.S.P. XI No. 1, 4%; hydrogenated vegetable oil (Crisco), 8%; and cod liver oil, 2%. Several groups of animals maintained on this low protein diet received a daily supplement of 25 mg of *dl*-methionine or of 25 mg *l*-cystine. An additional group of rats was placed on an adequate protein intake by increasing the casein content of the basal diet to 18%, compensating for the change by a corresponding decrease in dextrose. The diets were fed *ad libitum*. All animals received by stomach tube a daily supplement of B vitamins consisting of 40 γ each of thiamine and pyridoxine; 80 γ of riboflavin; 200 γ of calcium pantothenate; 500 γ of nicotinamide and 5 mg of choline.

Animals of the various groups were sacrificed at intervals simultaneously with litter mates from the control group maintained on 18% casein. The riboflavin content of the liver was determined by the method of Hodson and Norris[2] and expressed in γ per gram fresh tissue. The inactivation of estradiol by 100 mg of liver slices was tested by an assay method based on increase in weight of the immature rat uterus.[3]

Results obtained on a total of 59 rats are summarized in Table I. The low protein diet retarded markedly the growth of the animals; the addition of methionine allowed for a moderate increase in growth. The growth promoting effect of 25 mg *l*-cystine under these conditions was about the same as that of 25 mg *dl*-methionine.

Whereas the riboflavin concentration in the liver of the rats maintained on 18% casein remained practically unchanged throughout the period of 3 months, a gradual decline was observed in rats maintained on 8% casein; after 3 months the riboflavin concentration in their livers was almost half of that of the control animals. The supplementation with methionine tended to offset the decrease in riboflavin. On the other hand, the animals receiving cystine, in spite of their somewhat superior growth rate, showed, after 3 months, riboflavin values in the same low range as those without any supplement.

All rats maintained on the low protein diet for a period of 3 months failed to inactivate estradiol. Supplementation with methionine enabled these animals to maintain their ability to inactivate the hormone, whereas with

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1 Singher, H. O., Kensler, C. J., Taylor, H. C., Jr., Rhoads, C. P., and Unna, K., *J. Biol. Chem.*, in press.

2 Hodson, A. Z., and Norris, L. C., *J. Biol. Chem.*, 1939, **131**, 621.

3 Lauson, H. D., Heller, C. G., Golden, J. B., and Sevringhaus, E. L., *Endocrinology*, 1939, **24**, 35.

TABLE I.
Effect of Dietary Protein on Concentration of Riboflavin in the Liver and the Ability of Liver Tissue to Inactivate Estradiol.

Dietary protein	Days on diet	No. of animals	Body wt, g		Riboflavin, γ/g		Estradiol	
			Avg	Range	Avg	Stand. error	No. of tests	Inactivation
18% casein	37-57	6	178	126-227	23.6	1.3	4	4+
8% "	37-57	9	72	57-87	17.1	1.8	6	6+
8% " + 25 mg methionine daily	37-57	9	92	74-106	18.6	1.5	6	5+; 1±
18% Casein	89-98	8	281	218-330	22.5	0.8	8	8+
8% "	89-98	10	114	97-137	12.5	0.8	9	9—
8% " + 25 mg methionine daily	89-98	12	136	101-165	15.6	0.9	11	11+
8% " + 25 mg cystine daily	98	5	141	125-186	12.9	1.4	5	2+ ;3—

cystine, only 2 out of 5 animals were able to inactivate estradiol.

Discussion. Evidence of an interrelationship between protein metabolism and certain vitamins of the B-complex has recently been obtained. Riboflavin deficiency in chicks decreases the utilization of protein.[4] Sarett *et al.*[5] reported that the urinary excretion of riboflavin in rats bears an inverse relationship to the level of protein intake. Later, Sarett *et al.*[6] found that the concentration of riboflavin in the liver varies directly with the level of protein in the diet and is independent of the vitamin intake. Our results confirming those of Sarett likewise demonstrate a relationship between dietary protein intake and retention of riboflavin in the liver. In spite of the ingestion of liberal amounts of the vitamin the livers of rats maintained on a low protein diet are unable to retain riboflavin. Our data indicate that methionine plays a role in correcting this defect.

Prolonged protein deficiency, which is known to damage the liver in a variety of functions, was found to produce failure in the inactivation of estradiol by liver tissue. This dysfunction can be prevented by the feeding of methionine. The concomitant decrease in riboflavin concentration of the liver alone can not explain these findings. Three animals of the group maintained on 8% casein for 3 months had riboflavin concentrations above 14 γ per gram (14.3, 16, and 17 γ respectively), and 3 animals of the group receiving methionine in addition showed values below 14 γ per gram (11, 11.7, and 12.5 γ respectively). The livers of the rats in the first group failed to inactivate estradiol, whereas these supplemented with methionine did inactivate the hormone. On the other hand, in a previous study on a large number of riboflavin-deficient rats maintained on 18% casein, a concentration of 14 γ riboflavin per gram liver was found to be a critical level; none of the livers with less than 14 γ riboflavin per gram inactivated estradiol, and all livers with a riboflavin concentration above 14 γ per gram invariably inactivated the hormone.[1]

Therefore, the function of liver tissue in inactivating estradiol appears to depend on an adequate dietary intake, not only of thiamine and of riboflavin,[1] but also of protein, in which the sulfur-containing amino acids may be essential. In this respect our findings bear an analogy to the protective influence of dietary ingredients against the tumor producing effects of butter yellow. The pathologic changes in the liver following the ingestion of butter yellow in animals maintained on diets inadequate both in vitamins and in protein could be prevented to a large extent by the feeding of riboflavin and casein.[7] The effect of riboflavin *per se* could be demon-

[4] Kleiber, M., and Jukes, T. H., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 34.

[5] Sarett, H. P., Klein, J. R., and Perlzweig, W. A., *J. Nutr.*, 1942, **24**, 295.

[6] Sarett, H. P., and Perlzweig, W. A., *J. Nutr.*, 1943, **25**, 173.

[7] Kensler, C. J., Sugiura, K., Young, N. F., Halter, C. R., and Rhoads, C. P., *Science*, 1941, **93**, 308.

strated on animals maintained on adequate levels of protein[8] and that of casein and especially of the amino acids methionine and cystine on rats receiving adequate amounts of vitamins.[9]

[8] Antopol, W., and Unna, K., *Cancer Research*, 1942, **2**, 694.

[9] György, P., Poling, E. C., and Goldblatt, H., PROC. SOC. EXP. BIOL. AND MED., 1941, **47**, 41.

Summary. Rats maintained on a low protein diet were unable to retain normal amounts of riboflavin in the liver. Methionine tended to counteract the decrease in the riboflavin concentration of the liver. The livers of rats maintained on a low protein diet for a period of 3 months failed to inactivate estradiol. Supplementation with methionine maintained the ability to inactivate estradiol.

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Acetylcholine Content of the Myenteric Plexus and Resistance to Anoxia.

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It has been pointed out in an earlier paper[1] that the order of increasing resistance of the parts of the mammalian nervous system to anoxia and hypoglycemia is essentially the same as the order of parts arranged according to increasing amounts of acetylcholine (ACh) per unit weight. Those parts which are least resistant (cerebellum and cortex) are low in ACh and those which are among the more resistant (spinal nerves and autonomic ganglia) are high in ACh.

Cannon and Burket[2] have shown the cells of the myenteric plexus to be highly resistant to oxygen lack, enduring 3 hours of complete anemia. Van Liere[3] has found the alimentary canal to be affected significantly only by a strong degree of anoxia. It is of interest, therefore, to know the normal level of ACh in the myenteric plexus. Dikshit[4] has shown that the rate of formation of ACh by pieces of intestine *in vitro* is as high as that of equivalent amounts of nervous tissue but, so far as we are aware, no one has attempted to estimate the ACh-content of the enteric plexuses. This we have done for Auerbach's plexus in the rabbit and guinea pig and it will be seen that, in these species, the ACh-content of this plexus is extraordinarily high.

Methods. Tissue for assay was obtained by stripping off the longitudinal muscle and serosa of the entire small intestine of adult guinea pigs and rabbits. Most of Auerbach's plexus adheres to the longitudinal muscle. Assays were also made on pieces of whole intestine. The tissues were rinsed, carefully blotted to remove excess moisture, and weighed. They were ground, with a small amount of a phosphate medium plus eserine 1:5,000, acidified to pH 3-4 with normal HCl, and allowed to stand for one hour or more. They were then neutralized with normal NaOH and the volume was adjusted to give 100 mg tissue/cc fluid. After centrifuging, the supernatant fluid was further diluted for assay. This method gives the "total" ACh. Assays were made using the ventricle preparation ("heart") from Venus mercenaria, or the frog's rectus abdominis muscle. An estimate of the relative proportion of nervous to non-nervous tissue in the removed outer layers was obtained by projecting a stained preparation of the myenteric plexus of the rabbit and making a tracing. Cutting out and weighing the parts gave an estimate of the area of the plexus and the thickness was obtained from cross sections. This method indicated that

[1] Welsh, J. H., and Hyde, J. E., *J. Neurophysiol.*, 1944, **7**, 41.

[2] Cannon, W. B., and Burket, I. R., *Am. J. Physiol.*, 1913, **32**, 347.

[3] Van Liere, E. J., *Anoxia*, Univ. Chicago Press, 1942.

[4] Dikshit, B. B., *Quart. J. Exp. Physiol.*, 1938, **28**, 243.