Regulation of Xanthine Dehydrogenase in Chick Liver

FURTHER EXPERIMENTS ON THE EFFECTS OF INOSINE, ACTINOMYCIN D AND OTHER FACTORS

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1. It has been confirmed that the xanthine-dehydrogenase activity of chick liver is enhanced by starvation and by administration of inosine; the effects of these treatments are not additive. 2. Inosine has no effect when given to chicks depleted of the enzyme by feeding a low-protein diet. 3. Actinomycin D prevents the effect of inosine, but itself enhances the activity of xanthine dehydrogenase. 4. The xanthine-dehydrogenase activity is unchanged after addition of orotic acid to the diet, and is stimulated by injection of inorganic iron.

Enhancement of the specific activity of xanthine dehydrogenase (activity/mg. of nitrogen) is caused by starvation in pigeon kidney (Stirpe & De Stefano, 1957) and heart (Livrea & Ricceri, 1958) and in chick liver (Stirpe & Della Corte, 1965). Administration of inosine, but not of other purines or purine nucleosides, including the substrates xanthine and hypoxanthine, causes an increase of the specific activity of chick-liver xanthine dehydrogenase to an extent that is comparable with that caused by starvation. However, calculation of the total enzyme activity of the liver, of the total amount of liver nitrogen, and the effect of puromycin led to the conclusion that during starvation there is a loss of liver nitrogen, but that xanthine dehydrogenase is spared, whereas inosine stimulates new synthesis of the enzyme (Stirpe & Della Corte, 1965).

In the present paper we give further information on the effect of inosine: thus it does not occur in chicks on a low-protein diet, and its action is prevented by administration of actinomycin D. Actinomycin itself enhances the activity of the xanthine dehydrogenase. Orotic acid, which stimulates purine biosynthesis, has no effect on xanthine-dehydrogenase activity, but an enhancement was observed after administration of inorganic iron.

EXPERIMENTAL

Chemicals. NAD and orotic acid were obtained from Sigma Chemical Co. (St Louis, Mo., U.S.A.), actinomycin D was from Merck, Sharp and Dohme (Rahway, N.J., U.S.A.) and ferric ammonium citrate (green) was from C. Erba S.p.A. (Milan, Italy). The ingredients from the diets came from Laboratorio D.ri Piccioni (Brescia, Italy), who also prepared the vitamin and salt mixtures. The sources of other

chemicals were as indicated previously (Stirpe & Della Corte, 1965). Solutions were prepared in glass-redistilled water.

Animals and diets. Chicks (Cobb's × Cobb's) were obtained from Incubatoio Fortini (Bologna, Italy) on the day of hatching, and were kept for a week in the animal room before being used. They were maintained on a commercial starter diet containing 22% of protein (dry-weight basis, manufacturer's data) under the conditions described previously (Stirpe & Della Corte, 1965). Batches of 20–30 chicks were purchased: since some variability between batches was observed in the xanthine-dehydrogenase activity, as well as in the response to a given treatment, a group of controls was run for each batch. The composition of the semi-purified diets is given in Table 1.

Determination of xanthine-dehydrogenase activity. Liver extracts were prepared as described by Strittmatter (1965) for studies on enzyme activities in tissues. Xanthine-

Table 1. *Composition of semi-synthetic diets*

	Composition (%)	
	25%-casein diet	5%-casein diet
Enriched casein*	25	5
Cellulose	3	3
Hydrogenated cotton-seed oil	3	3
Briggs' salt mixture A†	5	5
Vitamin mixture‡	0.75	0.75
Choline chloride	0.25	0.25
Sucrose	63	83

* 220g. of casein, 21g. of glycine, 9g. of L-arginine hydrochloride and 6g. of L-cysteine.

† Briggs (1952), with selenium and molybdenum (Fox & Briggs, 1960).

‡ Bird (1947).

Table 2. *Effect of inosine and of starvation on liver xanthine-dehydrogenase activity*

The reaction mixture contained, in a final volume of 3.0 ml.: phosphate buffer ($\text{Na}_2\text{HPO}_4\text{--KH}_2\text{PO}_4$), pH 7.5, 0.03M; xanthine (monosodium salt), 0.135M; NAD, 0.67mM; 0.1 ml. of suitably diluted enzyme preparation. NAD was omitted from the reference cuvette. Results are given as mean values $\pm$ S.E.M. The numbers of chicks are given in parentheses.

Expt. no.	Treatment	Xanthine-dehydrogenase activity	
		($\mu\text{moles/min./mg.}$ of liver)	($\mu\text{moles/min./100 g.}$ body wt.)
1	Controls (9)	0.319 $\pm$ 0.045	1.21
	Starved for 24 hr. (3)	1.351 $\pm$ 0.346	
	Inosine (2%) in the diet:		
	For 24 hr. (6)	0.865 $\pm$ 0.099	3.45
	For 48 hr. (4)	0.587 $\pm$ 0.164	2.23
2	For 72 hr. (4)	0.815 $\pm$ 0.038	3.14
	Controls (saline-injected) (6)	0.260 $\pm$ 0.040	1.01
	Inosine (100 mg./chick) intraperitoneally 4 hr. before death (4)	0.831 $\pm$ 0.044	2.93
	Starved for 24 hr., saline-injected (5)	0.723 $\pm$ 0.072	
	Starved for 24 hr., inosine as above (6)	0.816 $\pm$ 0.046	

dehydrogenase activity was assayed spectrophotometrically by the procedure of Strittmatter (1965), but the concentration of xanthine in the reaction mixture was reduced by one-half to decrease the basal E_{290} ; this modification did not affect the rate of oxidation of xanthine. The determinations were performed at about 20° in a Beckman DU spectrophotometer. The enzyme activity is expressed as μmoles of uric acid formed/min./mg. of liver (wet wt.), or as $\mu\text{moles/min./100 g.}$ body wt.

RESULTS

Effect of inosine under various conditions. Enzyme assays by the present method confirmed that starvation and administration of inosine enhanced the activity of liver xanthine dehydrogenase (Table 2). Prolonged administration of inosine over 24 hr. caused no further increase in activity. A comparable enhancement of enzyme activity was obtained when inosine was injected intraperitoneally 4 hr. before death instead of being given with the diet. This permitted study of the effect of inosine in starved chicks, but no stimulation of activity was observed beyond that produced separately by starvation or inosine injection.

The activity of mammalian xanthine oxidase (see review by Knox & Greengard, 1965) and of avian xanthine dehydrogenase (Remy & Westerfeld, 1951) is greatly decreased by dietary protein deficiency. On the other hand, Mangoni, Pennetti & Spadoni (1955) were able to maintain the amount of liver xanthine oxidase in protein-depleted mice by repeated injections of xanthine, a treatment that induces the enzyme in this species (Dietrich, 1954; Feigelson, Feigelson & Wood, 1954). However, in our experiments with protein-depleted chicks, enzyme activity was not restored when inosine was

injected after some days of protein depletion, nor was it maintained when inosine was administered continuously with the low-protein diet (Table 3).

Effect of actinomycin D on xanthine-dehydrogenase activity. We observed (Stirpe & Della Corte, 1965) that puromycin completely prevented the effect of inosine on xanthine dehydrogenase and concluded that inosine enhances the activity of xanthine dehydrogenase by stimulating the synthesis of the enzyme. However, the possibility remained that inosine could act as a 'stabilizer' of the enzyme, i.e. by decreasing the rate of its degradation, as has been demonstrated for tryptophan in the case of tryptophan pyrrolase (Schimke, Sweeney & Berlin, 1965). The enhancement of tryptophan-pyrrolase activity owing to stabilization by tryptophan is also prevented by puromycin, but not by actinomycin (Greengard, Smith & Acs, 1963). We were unable to find reports of experiments with actinomycin in chicks, and therefore administered a dosage that was effective in inhibiting RNA synthesis in rat liver (Schwartz, Sodergren, Garofalo & Sternberg, 1965) and in mouse liver (Trakatellis, Axelrod & Montjar, 1964). The effect of inosine was completely prevented when the injection of actinomycin D was given 30 min. before inosine (Table 4). The antibiotic itself caused a significant enhancement of enzyme activity but this was lower than that caused by inosine.

Effect of other substances. Orotic acid, which stimulates purine biosynthesis in the rat (Windmueller & Spaeth, 1965), does not affect the activity of xanthine dehydrogenase when given to chicks for 48 hr. (Table 5). A significant enhancement of the enzyme activity was observed after injection of iron, but no effect was seen when iron

Table 3. *Ineffectiveness of inosine on liver xanthine-dehydrogenase activity of protein-depleted chicks*

Experimental conditions were as given in Table 2.

Expt. no.	Treatment	Xanthine-dehydrogenase activity	
		(μ mole/min./mg. of liver)	(μ moles/min./100g. body wt.)
1	25%-casein diet for a week (6)	0.346 $\pm$ 0.042	1.95
	5%-casein diet for a week (6)	0.061 $\pm$ 0.029	0.19
	5%-casein diet for a week, + inosine (2%) for 24 hr. (6)	0.030*	
	5%-casein diet for a week, 100 mg. of inosine intraperitoneally 4 hr. before death (6)	0.071†	0.26
2	5%-casein diet for 4 days (4)	0.102 $\pm$ 0.019	0.69
	5%-casein diet + inosine (2%) for 4 days (4)	0.132 $\pm$ 0.022	0.66
	5%-casein diet for a week (4)	0.055 $\pm$ 0.012	0.38
	5%-casein diet + inosine (2%) for a week (4)	0.026 $\pm$ 0.003	0.16

* Activity was detectable in only one chick (0.180 μ mole/min./mg. of liver).† Activity was detectable in only two chicks (0.271 and 0.156 μ mole/min./mg. of liver).Table 4. *Effect of actinomycin D on the stimulation of liver xanthine-dehydrogenase activity by inosine*

Inosine was given by intraperitoneal injection (100 mg./chick) 4 hr. before death. Actinomycin D was given by the same route (100 μ g./100 g. body wt.) 4½ hr. before death. Results are given as mean values $\pm$ s.e.m.; each group contained four chicks. Experimental conditions were as given in Table 2.

Treatment	Xanthine-dehydrogenase activity	
	(μ mole/min./mg. of liver)	(μ moles/min./100g. body wt.)
Controls (saline-injected)	0.202 $\pm$ 0.019	0.86
Inosine	0.537 $\pm$ 0.087	1.99
Actinomycin D	0.368 $\pm$ 0.050*	1.36
Actinomycin D + inosine	0.395 $\pm$ 0.079	1.37

* Significantly different from saline-injected controls ($P < 0.025$).Table 5. *Effect of orotic acid and of iron on liver xanthine-dehydrogenase activity*

Orotic acid was given in the diet (1%, w/w) for 2 days. Ferric ammonium citrate was given either in the diet (5%, w/w) for a week, or by intraperitoneal injection (1.5 mg. of Fe/kg. body wt.) 4 hr. before death. Other details were as given in Table 2. Results are given as mean values $\pm$ s.e.m. The numbers of chicks are given in parentheses.

Treatment	Xanthine-dehydrogenase activity	
	(μ mole/min./mg. of liver)	(μ moles/min./100g. body wt.)
Controls (8)	0.416 $\pm$ 0.038	1.49
Orotic acid (6)	0.379 $\pm$ 0.034	1.45
Ferric ammonium citrate in the diet (6)	0.372 $\pm$ 0.042	1.67
Ferric ammonium citrate injected (6)	0.564 $\pm$ 0.041*	2.25

* Significantly different from controls ($P < 0.025$).

was incorporated in the diet, in spite of the longer period of administration (Table 5).

DISCUSSION

With the exception of the results obtained in a small group of starved chicks (Expt. 1, Table 2), the

maximum increase of xanthine-dehydrogenase activity was approximately the same in all experiments, suggesting a limit to the capacity for enhancement of the enzyme activity. It has been demonstrated that xanthine dehydrogenase is not an 'indispensable' enzyme for chicks, since when it is inhibited by allopurinol the products of nitrogen

catabolism are excreted as unidentified compounds that are different from oxypurines and are probably produced through different metabolic channels (Krakoff & Meyer, 1965).

Orotic acid did not affect the activity of xanthine dehydrogenase, although Marchetti, Puddu & Caldarera (1964) observed an increase of xanthine-oxidase activity in rats receiving an orotic acid-supplemented diet for 4 weeks. Apart from differences in experimental conditions, the main one being the length of the treatment, the difference in results may perhaps be explained by the different roles of this enzyme in rats and chicks. Thus in mammals purines are essentially the product of nucleic acid catabolism, whereas in birds they are also the end product of protein metabolism. Consequently, any variations of nucleic acid metabolism caused by orotic acid would be expected to produce a relatively greater change in xanthine-oxidase substrates in mammals than in birds.

The inability of inosine to maintain xanthine-dehydrogenase activity in protein-depleted chicks may indicate that the lack of substrate(s) is not the only cause of the decrease of this enzyme during protein deficiency in the chick, as it seems to be in the mouse (Mangoni *et al* 1955), or that inosine is utilized in a different way under these dietary conditions. It is also possible that the amino acids provided by the 5%-casein diet were inadequate for a normal rate of synthesis on the enzyme: according to Litwack & Fisher (1957) the lack of only one essential amino acid markedly depresses the xanthine dehydrogenase of chick liver. Folic acid deficiency is another condition enhancing chick-liver xanthine dehydrogenase only when the diet contains adequate protein (Remy & Westerfeld, 1951).

The stimulating effect of iron on xanthine dehydrogenase was observed only when iron salt was injected. No substantial changes in the iron content of liver were observed in animals receiving ferric ammonium citrate in the diet for 2 weeks at the same level given in our experiments (Powell & Emmerson, 1966). The injection of various substances, for example ethanol (Kenney, Greenman, Wicks & Albritton, 1965), may stimulate enzyme activities, but the stimulation of xanthine dehydrogenase by iron may be a consequence of the metal being an integral part of the enzyme molecule. Iron stimulates ferritin synthesis (Fineberg & Greenman, 1955; Loftfield & Eigner, 1958), and Strittmatter (1965) has observed an accelerated development of xanthine dehydrogenase in newly hatched chicks receiving molybdenum, which, like iron, is a constituent of this enzyme. Further, iron is reduced from the ferric to the ferrous form by xanthine oxidase (Green & Mazur, 1957) and is accumulated in the liver of rats when

xanthine oxidase is inhibited by allopurinol (Powell & Emmerson, 1966). Therefore it is possible that a load of iron stimulates xanthine dehydrogenase by a substrate-inducer type of mechanism.

The effect of inosine is completely inhibited by actinomycin D. On the basis of the current interpretation of the effect of this antibiotic on induced changes of enzyme activities, and considering also the effect of puromycin (Stirpe & Della Corte, 1965), it is concluded that inosine enhances the xanthine-dehydrogenase activity by stimulating the synthesis of the enzyme, and that normal RNA synthesis is required. Actinomycin itself enhances the activity of xanthine dehydrogenase. Other examples of stimulation of protein synthesis or of enzyme activity (usually interpreted as enhanced synthesis) by this antibiotic have been reported (Pollock, 1963; Moog, 1964; Eagle & Robinson, 1964; Rosen, Raina, Milholland & Nichol, 1964; Garren, Howell, Tomkins & Crocco, 1964; Scarano, De Petrocellis & Augusti-Tocco, 1964; Goodwin & Sizer, 1965; Drysdale & Munro, 1965; De Matteis, 1966). Our results do not indicate whether one of the several hypotheses formulated to interpret these effects is applicable in the case of xanthine dehydrogenase. However, it may be noted that puromycin does not affect the basal activity of xanthine dehydrogenase (Stirpe & Della Corte, 1965; Strittmatter, 1965). Lippe & Szego (1965) have shown that actinomycin stimulates the secretion of corticosterone in the rat; however, it seems unlikely that the effects of actinomycin on chick-liver xanthine dehydrogenase arise from adrenocortical hyperactivity, since administration of cortisone has no effect (Stirpe & Della Corte, 1965).

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