Regulation of Xanthine Dehydrogenase in Chick Liver

EFFECT OF STARVATION AND OF ADMINISTRATION OF PURINES AND PURINE NUCLEOSIDES

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1. The xanthine-dehydrogenase activity of chick liver, expressed per mg. of nitrogen, is increased during starvation. 2. Administration of inosine and possibly of adenine has a comparable effect on the xanthine dehydrogenase, and also induces an elevation of the total quantity of enzyme. Hypoxanthine, xanthine, guanine, xanthosine, guanosine and adenosine are ineffective. Cortisone is equally ineffective. 3. The administration of puromycin abolishes the effect of inosine and reduces that of starvation. It is concluded that inosine induces an increased synthesis of xanthine dehydrogenase, whereas during starvation the enzyme is spared with respect to other liver proteins. 4. The hypothesis is formulated that chick-liver xanthine dehydrogenase is an adaptive enzyme, its activity being regulated by inosine or by one of its metabolites.

The xanthine-oxidase activity of mammalian organs and the xanthine dehydrogenase of avian organs are influenced by a variety of dietary changes. (The enzyme of bird tissues is called 'dehydrogenase' since it is not autoxidizable and a hydrogen carrier must be added to allow the reaction to proceed; Richert & Westerfeld, 1951; Ricceri, 1953.) A discrepancy exists between mammalian and avian species in the response to starvation. This condition causes a great impairment of the xanthine-oxidase activity in the rat (Miller, 1948; Westerfeld & Richert, 1951; Allard, de Lamirande & Cantero, 1957; Stirpe & De Stefano, 1957), whereas an increase in xanthine-dehydrogenase activity (on the basis of nitrogen content) was observed in the kidney of starved pigeons (Stirpe & De Stefano, 1957); a similar enhancement was observed in the heart but not in skeletal muscles of starved pigeons (Livrea & Ricceri, 1958).

Dietrich (1954) and Feigelson, Feigelson & Wood (1954) reported independently that the xanthine-oxidase activity of mouse and rat liver was increased by administration of xanthine. On this basis, Stirpe & De Stefano (1957) formulated the following working hypothesis of an adaptive response to starvation of the avian xanthine dehydrogenase. Since pigeon is a uricotelic species, the increased protein catabolism caused by starvation would increase the production of uric acid and of its

precursors hypoxanthine and xanthine, which could induce the changes observed in the xanthine-dehydrogenase activity.

The experiments described in this paper were designed with the aim of testing this hypothesis. Since pigeon liver is devoid of xanthine dehydrogenase and since the enzyme activity of kidney preparations is very variable (Richert & Westerfeld, 1951; Stirpe & De Stefano, 1957), chick liver has been used for present work. It has been observed that the activity of xanthine dehydrogenase in chick liver is affected by starvation and by administration of inosine.

EXPERIMENTAL

Chemicals. Xanthine (sodium salt) was purchased from Sigma Chemical Co. (St Louis, Mo., U.S.A.); other purines, purine nucleosides and puromycin hydrochloride were products of Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Cortisone acetate was obtained from Merck, Sharp and Dohme Italia (Pavia). All other reagents were of analytical grade and were products of E. Merck A.-G. (Darmstadt, W. Germany). Solutions were prepared in deionized water.

Animals. One-month- and 3-4-day-old white Leghorn chicks were obtained from a local dealer and were kept in the animal room for about a week before being used for the experiments. They are referred to below as 1-month- and 10-day-old chicks. They were fed on a commercial diet (diet PAMAR 6, Mulini Muratori, Siena) containing 24% of protein (manufacturer's data), and were housed in cages with constant illumination throughout the night, to allow

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continuous consumption of food. Chicks were isolated in individual cages for starvation experiments, and three or four were kept per cage for other experiments. Free access to water was always allowed.

Determination of xanthine-dehydrogenase activity. The adaptation by Schwartz & Litwack (1957) of the method of Litwack, Bothwell, Williams & Elvehjem (1953) was modified as follows. Chicks were decapitated and bled, the liver was removed, separated from the gall bladder and washed in ice-cold 39 mM-sodium phosphate buffer, pH 7.4. The liver was then blotted on filter paper, weighed and homogenized in buffer (1g. of liver + 5 ml. of buffer) in a Potter-Elvehjem homogenizer with Teflon pestle (A. H. Thomas Co., Philadelphia, Pa., U.S.A.), driven at 920 rev./min. The homogenate was centrifuged for 10 min. at 24000g and 0° in a Martin-Christ Junior III KS refrigerated centrifuge fitted with multi-speed attachment and rotor no. 5060. At the end of the run care was taken to discard as much as possible of the 'fatty fluffy layer' at the top of the supernatant. The latter was decanted and used for enzymic assays with the minimum of delay. Samples (1 ml.) of the supernatant were incubated at 37° in a Dubnoff shaker in 50 ml. flasks containing 4 ml. of buffer and 0.5 ml. of 0.01 M-methylene blue. After preincubation for 20 min., 0.6 ml. of 38 mM-xanthine was added; 0.5 ml. samples were taken within 10–15 sec. ('zero time') and again after 5, 10 and 15 min. of incubation. These samples were added to centrifuge tubes containing 3.5 ml. of 40% (w/v) sodium tungstate–2 N-sulphuric acid–water (1:1:5, by vol.). After 15 min. the volume was adjusted to 6 ml. with water and the tubes were centrifuged. Xanthine was determined in 1 ml. portions of this supernatant as described in the original method. A Unicam SP.600 spectrophotometer was used for the determinations. Replacement of triphenyl-tetrazolium used in the original method by methylene blue resulted in higher activities. Methylene blue did not interfere in the colorimetric determination of xanthine because it was absorbed by the protein precipitate, leaving a colourless supernatant. It was not necessary to run blanks without substrate, since after the 20 min. preincubation variations in the endogenous xanthine values were negligible.

Xanthine-dehydrogenase activity is expressed as μ moles of xanthine consumed/mg. of N/10 min., or as 'total activity' (μ moles of xanthine consumed/total liver/10 min.). Because birds of equal weight had been distributed amongst the various groups it was unnecessary to express total activity on the basis of body weight.

Nitrogen was determined by a micro-Kjeldhal method.

RESULTS

Experiments with 1-month-old chicks showed that starvation caused a significant enhancement of liver xanthine-dehydrogenase activity, expressed per mg. of N (Table 1). A similar effect of starvation was observed with 10-day-old chicks and with these the effect of starvation was already well marked after 24 hr. On the basis of these results 10-day-old chicks and the 24 hr. period were chosen for further experiments, unless otherwise stated.

Effect of administration of purines and of purine nucleosides. Xanthine was administered to chicks under the experimental conditions (dosage, length

Table 1. *Effect of starvation on liver xanthine-dehydrogenase activity*

Chicks were 1 month old in experiment 1 and 10 days old in other experiments. Experimental conditions are described in the text. Numbers of animals used are given in parentheses and results are expressed $\pm$ S.E.M.

Expt. no.	Treatment	Xanthine-dehydrogenase activity (μ moles of xanthine consumed/mg. of N/10 min.)
1	None (4)	1.44 $\pm$ 0.10
	Starvation for 4 days (5)	2.10 $\pm$ 0.19*
2	None (4)	1.17 $\pm$ 0.31
	Starvation for 1 day (4)	2.28 $\pm$ 0.11†
	Starvation for 2 days (6)	2.53 $\pm$ 0.15†
	Starvation for 3 days (4)	2.26 $\pm$ 0.21†
3	None (6)	1.07 $\pm$ 0.07
	Starvation for 1 day (4)	1.51 $\pm$ 0.23‡
4	None (5)	1.26 $\pm$ 0.13
	Starvation for 1 day (5)	2.59 $\pm$ 0.18†

* Significantly different ($P < 0.05$) from control.

† Significantly different ($P < 0.001$) from control.

‡ 0.1 > $P > 0.05$.

of time, route) used by Dietrich (1954) and by Feigelson *et al.* (1954) for mice and rats. Administration of xanthine did not cause any enhancement of the xanthine-dehydrogenase activity above the value observed in controls injected with 0.9% sodium chloride solution, even after 2 weeks of treatment. Short-term injection of hypoxanthine suspensions was equally ineffective (Table 2). Since Dietrich (1954) reported that cortisone enhanced the xanthine-oxidase activity of mouse liver, the possibility that a similar effect could occur in chicks was checked, but no enhancement was observed (Table 2).

From Tables 1 and 2 it appears that in most of the experiments the xanthine-dehydrogenase activity was higher in chicks injected with saline. Consequently it was thought that the effects of administered substances, which being poorly soluble must be given as suspensions, could be disturbed by the consequences of the injections themselves. Therefore purines and purine nucleosides have been given mixed with the diet (2%) for 24 hr. This dosage was arbitrary, but the period of administration was chosen having in mind that the effects of starvation were manifest after 24 hr., and consequently the causes of the enzymic changes had to be searched for within this period. Results are summarized in Table 3. Only inosine produced a statistically significant enhancement of xanthine-dehydrogenase activity, which reached values 80–120% above normal. Addition of purines or nucleosides to the diet did not reduce food intake.

Table 2. *Liver xanthine-dehydrogenase activity after injection of xanthine, hypoxanthine and cortisone*

Chicks were 10 days old at the beginning of treatment. Xanthine, hypoxanthine and cortisone acetate were given in saline suspension by intraperitoneal injection. Controls received the same volume of saline. Other details are as given in Table 1.

Expt. no.	Treatment	Xanthine-dehydrogenase activity (μ moles of xanthine consumed/mg. of N/10 min.)
1	Saline (3)	1.98 $\pm$ 0.33
	Xanthine* (4)	1.45 $\pm$ 0.23
	Saline (2)	2.31
	Xanthine† (3)	2.09 $\pm$ 0.20
2	Saline (4)	1.46 $\pm$ 0.19
	Hypoxanthine‡ (4)	1.40 $\pm$ 0.21
3	Saline (6)	1.85 $\pm$ 0.21
	Cortisone§ (2)	1.44
	Cortisone (2)	1.27

* One daily injection of 15 mg./100 g. body wt. for 1 week.

† One daily injection as above for 2 weeks.

‡ Two daily injections of 13.4 mg./100 g. body wt. for 2 weeks. This amount corresponds in moles to 15 mg. of xanthine (monosodium salt).

§ 1 mg., 4 hr. before death.

|| 1 mg., 24 and 12 hr. before death.

Table 3. *Effect of administration of purines and of purine nucleosides on liver xanthine-dehydrogenase activity*

The substances were given mixed with the diet (2%) for 24 hr. Other details are as given in Table 1.

Expt. no.	Treatment	Xanthine-dehydrogenase activity (μ moles of xanthine consumed/mg. of N/10 min.)
1	None (7)	1.51 $\pm$ 0.21
	Hypoxanthine (5)	1.34 $\pm$ 0.29
	Xanthine (2)	0.82
2	None (5)	1.32 $\pm$ 0.13
	Hypoxanthine (2)	1.50
	Guanine (4)	1.16 $\pm$ 0.18
	Inosine (3)	2.89 $\pm$ 0.35*
3	None (6)	1.51 $\pm$ 0.13
	Adenine (4)	2.03 $\pm$ 0.20†
	Inosine (4)	2.71 $\pm$ 0.39*
	Guanosine (4)	1.56 $\pm$ 0.10
4	None (4)	1.61 $\pm$ 0.15
	Xanthosine (4)	1.27 $\pm$ 0.08
	Adenosine (4)	1.21 $\pm$ 0.14
5	None (6)	1.07 $\pm$ 0.07
	Inosine (10)	1.91 $\pm$ 0.17‡

* Significantly different ($P < 0.001$) from controls.

† Not significantly different ($P = 0.45$) from controls.

‡ Significantly different ($P < 0.01$) from controls.

Table 4. *Effect of puromycin on the liver xanthine-dehydrogenase activity of normal and starved chicks and of chicks receiving inosine*

Puromycin was given dissolved in saline by intraperitoneal injections of 2 mg./100 g. body wt. each, one 24 hr. and one 12 hr. before death. Inosine was given as described in Table 4. Other details are as given in Table 1.

Treatment	Xanthine-dehydrogenase activity (μ moles of xanthine consumed/mg. of N/10 min.)
No puromycin	
None (5)	1.26 $\pm$ 0.13
Starvation for 1 day (5)	2.59 $\pm$ 0.18*
Inosine (2)	2.76*
Injected with puromycin	
None (4)	1.40 $\pm$ 0.19
Starvation for 1 day (4)	1.98 $\pm$ 0.16†
Inosine (4)	1.14 $\pm$ 0.30

* Significantly different ($P < 0.001$) from control.

† Not significantly different ($P > 0.1$) from the normally fed, puromycin-injected group.

Table 5. *Total liver xanthine-dehydrogenase activity of normal and starved chicks and of chicks receiving inosine*

Data are mean values and were calculated from the enzymic activity and nitrogen content of the supernatant used in the experiments reported in the preceding Tables. The numbers of chicks are given in parentheses.

Treatment	Total xanthine-dehydrogenase activity (μ moles of xanthine consumed/total liver/10 min.)	Total liver nitrogen (mg./total liver)
1-month-old chicks		
None (4)	360.1	247.9
Starvation (5)	356.9	173.3
10-day-old chicks		
None (26)	65.8	49.6
Starvation (13)	72.2	33.8
Inosine (19)	124.8	52.8
Puromycin-treated chicks		
None (4)	64.8	48.3
Starvation (4)	65.3	32.7
Inosine (4)	56.1	47.4

Effect of inhibition of protein synthesis. Ethionine, azaguanine and puromycin, given intraperitoneally, were tested in a preliminary screening, and puromycin was selected because it did not affect the xanthine-dehydrogenase activity in normal chicks. This inhibitor was used at a dosage similar to that found by Greengard, Smith & Acs (1963) to inhibit protein synthesis in rat liver. It

was assumed that this dosage was equally effective on protein synthesis in chick liver, but it was technically impossible to check this point. Puromycin did not affect food intake. Puromycin did not affect the xanthine-dehydrogenase activity in normally fed, untreated chicks, but abolished completely the enhancement due to inosine administration, and reduced that due to starvation (Table 4).

Total xanthine-dehydrogenase activity. A comparison of the total activity of the dehydrogenase as well as of the total nitrogen content (calculated in the supernatant used for the enzymic assays) of the liver of chicks of the various groups is given in Table 5. In starved chicks the total enzymic activity is not above the normal, whereas the total quantity of nitrogen is considerably decreased. In inosine-treated chicks there were no substantial changes in the amount of nitrogen, whereas the total xanthine-dehydrogenase activity was much more than in controls. Puromycin prevented the increase in the total xanthine-dehydrogenase activity in chicks that received inosine.

DISCUSSION

According to Dietrich (1954) and to Feigelson *et al.* (1954) mammalian xanthine oxidase is an adaptive enzyme, its concentration being increased after injections of substrate xanthine. Dietrich (1954) interpreted the increase in xanthine oxidase as due only in part to stress. The experimental conditions of these investigations were repeated exactly in our work, but no enhancement of the dehydrogenase was observed in chick liver. Among all the purines and nucleosides tested only inosine and possibly adenine caused an elevation of xanthine-dehydrogenase activity. According to Dixon (1926), Booth (1938) and Klenow (1952) adenine may be a substrate for xanthine oxidase. A possible reason for the unique behaviour of inosine is that it was the most soluble substance tested, and thus it could be the only one absorbed in sufficient amount to be effective. The higher activity of xanthine dehydrogenase observed after inosine administration seems to be an adaptive response to this substance or to one of its metabolites. Inosine affects both the activity per mg. of nitrogen and the total activity of the liver. An 'increased total enzyme activity' could be due either to increased quantity of the enzyme, i.e. higher number of enzyme molecules, or to increased activity of the enzyme, the number of molecules remaining unchanged ('synthetic response' versus 'kinetic response'; Greengard, 1963). A larger amount of enzyme could be the result of increased synthesis or of decreased degradation of the enzyme. A definite answer to some of these questions could

be given by titration of the enzyme with an anti-serum; however, experiments with puromycin indicate that the inosine-induced elevation of the xanthine-dehydrogenase activity is due to increased amount of newly synthesized enzyme, since inosine has no effect when protein synthesis is inhibited by puromycin. Inosine seems to affect specifically the synthesis of xanthine dehydrogenase, since the changes in the total quantity of this enzyme are not accompanied by changes in the amount of liver nitrogen. However, a stimulating effect of inosine on other enzymes cannot be excluded.

The xanthine-dehydrogenase activity appeared greater during starvation only when it was expressed per mg. of nitrogen; puromycin reduced only in part this effect of starvation. The total activity remains unchanged during starvation, and this would exclude an adaptation of xanthine dehydrogenase under these conditions, if it is assumed that there is adaptation only when synthesis of an enzyme occurs. However, the concept of adaptation in higher animals has been enlarged to include the cases in which the enzyme is 'spared' with respect to other proteins by a decrease in the rate of enzyme degradation (Bojarski & Hiatt, 1960), and this mechanism has been called 'preferential maintenance' (Weber, 1963) or 'enzyme stabilization' (Schimke, Brown & Smallman, 1963; Schimke, Sweeney & Berlin, 1964). In the liver of starved chicks the total amount of liver nitrogen is considerably decreased, whereas the total xanthine-dehydrogenase activity is unchanged. If the total enzyme activity is the expression of the quantity of enzyme present in the liver, it can be concluded that the xanthine dehydrogenase has been spared during the consumption of liver protein caused by starvation. This could be considered as an example of enzymic adaptation operating through decrease in the rate of enzyme degradation, and is the opposite of what occurs in starved (Miller, 1948) and in protein-depleted rats (Litwack, Williams, Feigelson & Elvehjem, 1950), in which xanthine oxidase is consumed more rapidly than liver protein. This difference could be accounted for by the different protein metabolism in mammalian and avian species. Starvation causes an accelerated protein breakdown both in mammals and birds; this results in increased formation of urea in the former (Schimke, 1962b) and presumably in increased production of uric acid in the latter animals. The enhanced synthesis of urea is accompanied by an increased activity of the enzymes of the urea cycle, which has been interpreted as an adaptive response (Schimke, 1962a,b, 1963). Since the role of xanthine dehydrogenase in uric acid formation in birds may be considered as corresponding to that of the urea-forming enzymes in ureotelic species, the maintenance of the xanthine-dehydrogenase

activity in the liver of starved chicks can be interpreted as the equivalent to the response of the urea-cycle enzymes in starved rats.

The hypothesis of an adaptation to overload of substrate originating from accelerated turnover of nucleic acids has been formulated to explain the increased xanthine-oxidase activity observed in the deficiency of vitamin E in rabbits (Young & Dinning, 1951; Dinning, 1953; Richert & Westerfeld, 1953), in the deficiency of animal protein factor in rats (Rabbi, Marchetti & Viviani, 1956; Marchetti & Viviani, 1956) and during liver regeneration in mice (Feigelson, Feigelson & Gross, 1957).

Other possible mechanisms should be considered: an alteration of cell permeability seems to be out of the question, since cell-free preparations were used; however, the possibility of a better extractability of the enzyme cannot be entirely excluded, nor can one eliminate the possibility that reactions which can be coupled with the oxidation of xanthine (Morell, 1955) may operate at an increased rate in starved chicks or in those receiving inosine. Cortisone is known to play a role in the adaptation of several enzymes, including xanthine oxidase (Dietrich, 1954). Furthermore, cortisone stimulates the xanthine-oxidase activity in rats on a protein-deficient diet (Clark & Pesch, 1956), whereas the enzyme is depressed in adrenalectomized rats (Reid, O'Neil & Lewin, 1956). The lack of response observed in chicks may be peculiar to this species or it may be due to differences between our experiments and those of other investigators.

The question remains whether inosine itself acts or as a precursor of hypoxanthine or xanthine. Inosine (or inosine phosphate), being the first intermediate in hypoxanthine formation (Greenberg, 1951; Schulman & Buchanan, 1952), could be the regulator of the subsequent reaction(s) in the pathway leading to uric acid. This phenomenon in enzymic adaptation is well known in micro-organisms, but not, to our knowledge, in higher animals. The 'mirror image' of this is the feedback control of arginine-glycine transaminidase by creatine in chick liver (Walker, 1960) and in rat kidney (Fitch, Hsu & Dinning, 1960). Should inosine act as a precursor of xanthine or hypoxanthine, these being the really effective substances, the lack of response of the xanthine dehydrogenase to xanthine or hypoxanthine administration may be due to their inadequate absorption.

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