SYNTHESIS OF XANTHINE OXIDASE BY RAT LIVER SLICES IN VITRO

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The influence of dietary protein on the activity of rat liver xanthine oxidase has been studied by several workers (1–5) and would point to a relationship between the availability of amino acids and the activity of liver xanthine oxidase (2). It has been demonstrated that the loss of liver xanthine oxidase activity in the rat greatly exceeds the loss of liver protein during acute inanition (1), revealing thereby the labile nature of this enzyme. This apparent functional lability of rat liver xanthine oxidase is further indicated in the results reported by Litwack *et al.* (5) and would suggest that the enzyme is capable of easy synthesis under favorable conditions. In accordance with the view of the participation of enzymes in the actively metabolizing pool of proteins, Anfinsen (6) isolated radioactive crystalline ribonuclease from bovine pancreas slices that had been incubated with $C^{14}O_2$. Very recently, Hokin (7) has reported on the synthesis *in vitro* of amylase in amylase-depleted pigeon pancreas slices by incubating the slices in media containing the essential amino acids. As far as is known, these are the only instances in which synthesis of an enzyme *in vitro* has been established. It was thought that a demonstrable increase in xanthine oxidase activity might result upon incubation of liver slices from protein-depleted rats in media containing essential nutrients; the stimulus to recovery of liver substance should be expected to be more potent the more severe its reduction (8, 9).

A prerequisite to this study was an accurate method for following xanthine oxidase activity. The manometric method of Axelrod and Elvehjem (10) presented difficulty owing to the high endogenous respiration as well as to inhibition of endogenous respiration by added xanthine. However, preliminary incubation for 40 minutes or dialysis at 0° for 18 hours could, as suggested by the authors, remove endogenous purine substrates without impairment of xanthine oxidase activity. Precipitation of the enzyme (11) to free it from endogenous substrates was not always satisfactory. The procedure of Dixon and Thurlow (12), in which anaerobic reduction of nitrate by xanthine oxidase is employed, was found to be non-specific. The coupling of the oxidation of xanthine to uric acid with the reduction of oxidized cytochrome *c* has been made the basis of a method for measuring xanthine oxidase activity through spectrophotometric study

of the rate of reduction of cytochrome *c* (13). This was satisfactory but did not offer any special advantage over the manometric procedure, which was therefore employed in the studies reported here.

An increase in xanthine oxidase activity has been observed when liver slices from protein-depleted animals are incubated in inactivated horse serum containing glucose. This increase is pronounced in serum which contains added methionine, glycine, riboflavin, and bicarbonate. The possible importance of methionine for xanthine oxidase activity has been reported (2, 5). The incorporation of formate and of bicarbonate into purines has been shown both *in vitro* (14) and *in vivo* (15, 16). Riboflavin addition may presumably aid in the synthesis of the flavin-adenine dinucleotide (FAD) moiety of xanthine oxidase.

That the increase in xanthine oxidase activity represented a true synthesis of the enzyme and not an activation was established by concomitant estimations of FAD, adenine, and protein nitrogen in slices incubated in supplemented serum. Unincubated slices removed from serum at zero time and slices incubated in physiological saline containing 0.2 per cent (weight by volume) glucose served as controls. Residual glycine in the medium was determined and its disappearance was calculated.

There was no increase in either uricase or D-amino acid oxidase activity as a result of incubation of slices in serum.

EXPERIMENTAL

Adult albino rats (inbred Wistar strain) were depleted of liver xanthine oxidase activity by initial fasting for 2 days, followed by feeding a nitrogen-free ration for 10 to 15 days. This diet was made up of (percentages) starch 75, sucrose 10, sesame oil 8, shark liver oil 2, and salt mixture (U. S. P. No. 2) 5, with the following vitamin additions (in mg. per kilo of diet): thiamine hydrochloride 2, pyridoxine hydrochloride 2, riboflavin 6, calcium pantothenate 10; nicotinic acid 20, inositol 200, α-tocopherol (Roche) 25, vitamin K (menadione) 5, choline chloride 500, folic acid (Lederle) 0.4, and vitamin B_{12} (Merck) 0.15. Animals when needed were sacrificed by decapitation and the livers were carefully removed and chilled in cracked ice. Slices, approximately 0.5 mm. thick, were prepared with the slicer of Stadie and Riggs (17) and transferred immediately to chilled and oxygenated physiological saline. A suitable number of slices were then incubated in the experimental media. Not more than 15 to 20 minutes elapsed from the time of killing until the flasks were ready for incubation; this was for 3 hours at 37°.

The following media were used.

Medium 1—Physiological saline containing 0.2 per cent (weight by volume) glucose, saturated with oxygen.

Medium 2—Horse serum,[1] inactivated by heating at 55° for 2 hours to destroy serum enzymes, containing 0.2 per cent (weight by volume final concentration) glucose and saturated with oxygen.

Medium 3—Medium 2 plus the following supplements: glycine 20 mg. per 100 ml., DL-methionine 20 mg. per 100 ml., riboflavin 250 γ per 100 ml., and sodium bicarbonate 10 mg. per 100 ml. The medium was saturated with oxygen.

Medium 4—Medium 1 plus the following additions: riboflavin 250 γ per 100 ml., adenine 100 γ per 100 ml., and potassium dihydrogen phosphate 10 γ per 100 ml. The medium was saturated with oxygen.

Medium 5—Medium 1 plus the following additions: riboflavin 250 γ per 100 ml., glycine 20 mg. per 100 ml., DL-methionine 20 mg. per 100 ml., potassium dihydrogen phosphate 10 mg. per 100 ml., and sodium bicarbonate 10 mg. per 100 ml. The medium was saturated with oxygen.

Horse serum contained 0.625 γ per ml. of folic acid assayed according to Sreenivasan *et al.* (18).

Treatment of Slices and Media after Incubation—Immediately after incubation the flasks were returned to a tray of ice. The slices were removed from the vessel. After adhering medium had been drained off, they were washed twice with chilled phosphate buffer of pH 7.4. The collected washings were returned to the medium. The washed slices were homogenized in a chilled glass homogenizer with 5 volumes of 0.01 M phosphate buffer. Suitable portions of this homogenate were used for measurement of enzyme activities and for estimations of adenine, riboflavin, flavin mononucleotide (FMN), FAD, and protein nitrogen. The medium with the washings was made to a convenient volume and saved for glycine determination.

Xanthine Oxidase Activity—Liver xanthine oxidase activity was determined by the method of Axelrod and Elvehjem (10) from the amount of oxygen consumed in a specified period in the Warburg manometric apparatus at 37° with xanthine as substrate and with air as the gas phase. Each reaction vessel had in the main chamber 1 ml. of phosphate buffer (0.01 M), pH 7.4, and 1 ml. of homogenate which contained from 20 to 25 mg. of dry weight liver. In the side arm was placed the substrate sodium xanthate equivalent to 100 γ of xanthine in 1 ml. of the buffer. The center well contained 0.2 ml. of 10 per cent KOH, making the total fluid volume 3.2 ml.

Interference due to endogenous purine substrates could be largely minimized either by preliminary dialysis of the homogenate overnight at 0° or by incubation of the flasks in the manometric bath for 40 minutes. The latter procedure was the one generally adopted in these studies. At the end of this period xanthine was tipped in, the stop-cocks closed, and

[1] Our thanks are due to Dr. A. K. Hazra and Dr. P. M. Wagle of the Haffkine Institute, Bombay, for supplies of horse serum.

readings taken at 20 minute intervals for a period of 120 minutes. In every experiment corrections were made for the endogenous respiration, *i.e.* oxygen consumption in the absence of added xanthine, so as to obtain the true rate of xanthine oxidation.

Uricase Activity—This was followed manometrically by essentially the same procedure as for xanthine oxidase. The side arm contained 100 γ of uric acid as sodium urate in 1 ml. of buffer; this was tipped into the main compartment after an initial 10 minute equilibration. Readings were taken at 20 minute intervals for 60 minutes.

D-*Amino Acid Oxidase Activity*—The procedure was the same as above except that pyrophosphate buffer of pH 8.6 was employed (19) and 1 ml. of 0.06 M DL-alanine was used as substrate in the side arm. Readings for oxygen uptake were taken every 20 minutes for 120 minutes after 10 minutes equilibration.

Adenine—The purines were fractionated from the liver homogenate by the method of Sutton *et al.* (16). Adenine was estimated in the purine solution by the method of Glazko and Wolf (20). This makes use of the fact that the reduction product of adenine when diazotized and coupled with the Bratton-Marshall reagent (21) forms a colored compound with absorption maximum at 505 mμ. With pure solutions of adenine hydrochloride subjected to the foregoing procedure, it was ascertained that the optical density was proportional to the concentration of adenine. With liver homogenate, recovery of added adenine was 90 to 95 per cent. A Coleman model 14 spectrophotometer was employed for the color measurements.

Riboflavin, FMN, and FAD—The flavin compounds were extracted from the liver homogenate with 5 per cent trichloroacetic acid at 0–5° for 15 minutes and determined by the differential procedure of Burch *et al.* (22), except that slightly larger volumes were used; fluorescence was measured in a Klett photofluorimeter.

Glycine was determined in the medium by the method of Alexander *et al.* (23). Colorimetric measurements were carried out in the Klett-Summerson photoelectric colorimeter with Filter 540.

Protein nitrogen was determined on 5 per cent trichloroacetic acid precipitate by a micro-Kjeldahl method (24).

The number of cells per gm. of liver was estimated by the method described by Price *et al.* (25). In this procedure, 0.002 per cent methyl green in 3 per cent acetic acid solution was used to stain specifically the desoxyribonucleic acid of the cell nuclei.

Desoxyribonucleic acid (DNA)—The nucleic acids were extracted by Schneider's procedure (26) and DNA was determined by the diphenylamine reaction (27) in suitable aliquots of the above extract.

Results

Feeding rats on the nitrogen-free diet greatly diminished liver xanthine oxidase activity. Thus, while the values for the normal stock rats were 480 to 525 μl. of O_2 per gm. of dry weight during 120 minutes, those for

TABLE I

Effect of Aerobic Incubation of Liver Slices on Xanthine Oxidase Activity

Rat No.	Xanthine oxidase activity, μl. O_2 per gm. dry liver per 2 hrs.			
	Medium 1	Medium 2	Medium 3	Unincubated Medium 3
83	227	324	350	220
87	224	288	311	
93	235		254	216
100H	286		358	
101H	0		200	0
102H	255		323	

TABLE II

Xanthine Oxidase Activity Based on Liver Nitrogen and Cell Nuclei

Experiment No.	Medium No.	Xanthine oxidase activity, μl. O_2 per 2 hrs.		
		Dry liver per gm.	Liver N per gm.	Liver nuclei per 10^9
1	1	324	3139	388
	3	433	4163	538
2	1	262	2240	256
	3	300	2777	292
3	1	258	2411	290
	3	400	3670	446
4	1	313	2989	340
	3	420	3962	456

animals on the protein-free diet for 10 to 15 days ranged from 224 to 324 μl. Occasionally complete absence of activity was observed.

Incubation of slices in serum increased xanthine oxidase activity, which was further increased if the serum was supplemented with glycine, methionine, riboflavin, and bicarbonate (Table I). Activity has also been expressed (Table II) as microliters of oxygen uptake per 2 hours per gm. of dry liver, per gm. of liver nitrogen, and per 10^9 liver cell nuclei (*cf.* (28)). This offers a means of calculating enzyme activity on a per cell basis and for comparison of activity with changes in liver nitrogen.

That the synthesis of xanthine oxidase on incubation of liver slices with supplemented serum is associated with an expected increase in FAD could be seen from the results of typical determinations presented in Table III. Estimations were carried out in slices incubated in saline (Medium 1), which served as control and in slices incubated in Medium 3. The rather

TABLE III

Riboflavin, FMN, and FAD Content of Liver Slices

Experiment No.	Medium No.	γ per gm. dry liver			Per cent increase in FAD
		Free riboflavin	FMN	FAD	
1	1	11	27	26	
	3	12	27	31	19.2
2	1	5	23	35	
	3	7.6	28	43	22.8

TABLE IV

Effect of Aerobic Incubation of Liver Slices on Xanthine Oxidase Activity and FAD Content

Experiment No.	Medium No.	Xanthine oxidase activity, μl. O_2 per 2 hrs.	γ per gm. dry liver		
			Free riboflavin	FMN	FAD
1	1	221	10.5	22.2	29.3
	3	466	10.6	25.5	33.9
	4	262	10.3	24.3	30.0
	5	248	9.9	25.1	32.5
2	1	318	8.2	16.6	32.8
	3	480	8.7	16.0	37.2
	4	289	8.6	18.4	37.6
	5	333	9.0	18.3	35.6

high values for FMN may possibly be due to involvement of the Kornberg enzyme system (29).

Incubating liver slices with the components of the flavin moiety but without serum (Table IV) showed no increase in xanthine oxidase activity, while an expected increase in FAD content (*cf.* (29)) was observed. This demonstrates that the increase in xanthine oxidase activity in the presence of serum is due not merely to the synthesis of FAD but also involves the synthesis of the protein component of xanthine oxidase.

The total synthesis of xanthine oxidase would also suggest synthesis *in vitro* of the purine base adenine and of protein nitrogen in the liver slices, which is demonstrated by the data set forth in Table V. The values for

protein synthesis should be taken as net, allowing for proteolytic reactions in the slices; this might explain to some extent the proportionately higher synthesis of xanthine oxidase compared to total protein. Biosyntheses *in vitro* of serum albumin by chick liver slices (30) and of ovalbumin by oviduct minces from hens (31) have been demonstrated.

As may be expected, the increase in xanthine oxidase activity per cell nucleus is greater than that per mg. of liver N, except with Experiment 2 (Table II). Activity has been expressed here on total liver N and not on liver protein N. It may be observed that there is no significant increase in liver N during incubation. It would be of interest to ascertain the extent to which the trichloroacetic acid-soluble proteolytic fragments contribute to the increase in protein N under favorable conditions of incubation, as in presence of serum of the liver slices.

Determinations of uricase activity were considered necessary, since xanthine oxidation represents the summation of xanthine oxidase and uricase activities (10). Hence the measurement of the over-all oxidation rate of xanthine to allantoin would have given a false criterion of xanthine oxidase activity if uricase activity was the limiting factor. However, it was observed that the oxidation of sodium urate was always very rapid and in no case limited xanthine oxidation (*cf.* (10)). Uricase activities ranged from 222 to 228 μl. of O_2. There was no significant change in uricase activity of slices as a result of incubation in serum with supplements (Medium 3).

TABLE V

Adenine and Protein Nitrogen in Liver Slices

Experiment No.	Medium No.	Adenine, mg. per gm. dry liver	Per cent increase	Protein N, mg. per gm. dry liver	Per cent increase	Protein N, mg. per mg. DNA
1	1	1.70		80.0		6.5
	3	1.81	6.6	90.2	12.7	7.33
2	1	1.66		84.3		6.85
	3	1.75	5.4	94.1	11.8	7.84

Data on D-amino acid oxidase activity also showed no change as a result of incubation for varying periods in different media. Thus, the values in one instance were 340, 355, and 350 μl. in Media 1, 2, and 3 respectively at the end of 3 hours incubation of liver slices from an animal deprived of protein for 10 days. It was observed, however, that the D-amino acid oxidase activity in protein-fasted animals was only 55 to 60 per cent that of animals on the stock diet. These data confirm the results of Seifter *et al.* (32) on the fall in D-amino acid oxidase activity during protein de-

pletion. Evidently this system is not as labile as the xanthine oxidase system which, though it undergoes pronounced fall in activity on protein depletion, is also rapidly regenerated on protein refeeding (5).

Data on glycine disappearance were not conclusive quantitatively on account of the rather high blanks obtained with the chromotropic acid reagent. Incubation of slices in Medium 3 decreased its glycine content on an average by 1.16 mg. per gm., dry weight, of the liver. Formate formation from the α-carbon of glycine and its incorporation into purines are well established (16); adenine synthesis in liver slices (Table V) and simultaneous disappearance of glycine from the medium are thus interrelated.

TABLE VI

Influence of Folic Acid on Xanthine Oxidase Synthesis in Vitro

Group	Rat No.	Leucocytes per c.mm.	Methionine, mg. per gm. liver	Choline, mg. per gm. liver	Liver weight, gm. per 100 gm. body weight	Liver N, mg. per gm.	μl. O_2 per gm. dry liver per 2 hrs.	
							Liver xanthine oxidase activity	Xanthine oxidase synthesis *in vitro*
FA-deficient	40	7,812	4.36	4.05	4.662	28.32	245	333
	42	6,562	4.37	3.70	6.368	28.98	266	400
FA-supplemented	41	10,312	4.91	5.01	3.796	29.04	306	306
	43	10,312	6.05	4.63	4.300	31.10	213	266

Influence of Folic Acid on Xanthine Oxidase Synthesis by Rat Liver Slices

The foregoing experiments were carried out with liver slices from protein-depleted animals. The results of certain preliminary observations are reported in Tables VI and VII to show that deficiencies other than protein deprivation also influence this synthesis. Specifically, these experiments related to the effect of dietary folic acid on the ability of rat liver slices to synthesize xanthine oxidase.

In the first experiment (Table VI) adult rats were fed the purified folic acid-free ration of Fatterpaker *et al.* (33) with supplementation of 0.15 mg. per kilo of vitamin B_{12} (Merck) and 0.5 per cent iodinated casein. The animals also received 0.04 mg. of L-thyroxine (Glaxo) subcutaneously once each week. In addition, 0.5 per cent nicotinamide was incorporated in the diet. This latter addition depletes the rat of its labile methyl reserve (34). Toxicity is reversed by folic acid (35), whose mediation in biological methylations is well known (33, 35). Folic acid deficiency was inferred at the end of 5 weeks from observations on a hemogram. The animals were then divided into two groups. One served as control and

the other received intraperitoneally 100 γ per day of folic acid. These were sacrificed after a further 32 and 38 days for determination of xanthine oxidase synthesis by liver slices *in vitro*. Typical values are reported (Table VI) along with other data on liver xanthine oxidase activity (obtained on incubation in Medium 1), liver weight, and liver nitrogen, methionine (36), and choline (37) content, and leucocyte count.

In the second experiment (Table VII) adult rats that had been fed the laboratory stock diet were used; this consisted of (percentages) wheat flour 59, casein 5, whole milk powder 4, peanut cake 13, yeast 4, wheat bran 4, dried fish meal 3, shark liver oil 2, sesame oil 2, and salt mixture (U. S. P. No. 2) 4. Folic acid deficiency was induced by daily subcutaneous injections of 12.5 γ of aminopterin (Lederle)[2] for 14 days. Some of the

TABLE VII

Influence of Aminopterin on Xanthine Oxidase Synthesis in Vitro

Group	Rat No.	Leucocytes per c.mm.	Liver weight, gm. per 100 gm. body weight	μl. O_2 per gm. dry liver per 2 hrs.	
				Liver xanthine oxidase activity	Xanthine oxidase synthesis *in vitro*
Stock diet	20	10,937	3.906	750	711
	14	10,623	4.161	739	720
" " + aminopterin	16	7,812	3.495	0	364
	18	8,062	3.717	212	546

values observed for liver xanthine oxidase activity and for xanthine oxidase synthesis *in vitro* are given, along with those for control animals fed the stock diet (Table VII).

The results clearly bring out the fact that the ability for xanthine oxidase synthesis *in vitro* is manifested only in the livers of the folic acid-deficient rats and not in the normal animals. Whether this could be attributed solely to the observed differences in methionine and choline contents, whose influence on xanthine oxidase activity has been shown (2, 5), cannot be stated at present. However, the results are in harmony with the observations of earlier workers that the ability for liver regeneration is in proportion to its deviation from the normal; this of course may not be true if necrotic changes set in. Further work is in progress.

[2] Obtained through the courtesy of Dr. T. H. Jukes and Dr. W. L. Williams of the Lederle Laboratories Division, American Cyanamid Company, Pearl River, New York.

DISCUSSION

The increase in xanthine oxidase activity measured under the conditions of these experiments are paralleled by a net increase in enzyme protein, in adenine as well as FAD, and in glycine metabolism. Our results therefore support the view that the increase in xanthine oxidase activity represents a true synthesis of the enzyme *per se* and is not merely an activation. However, the optimum cultural conditions for maximum elaboration of this enzyme have not been completely worked out, nor has it been ascertained whether the aerobic oxidase or the anaerobic dehydrogenase of the xanthine oxidase system (38) is preferentially synthesized. Meanwhile, it would seem that the observations reported here would be of value in the demonstration, more directly than hitherto, of the influence of folic acid on xanthine oxidation, on which there have been conflicting reports (39–41). Studies on the foregoing aspects and on the assessment of protein quality from determinations of xanthine oxidase synthesis *in vitro* are in progress.

SUMMARY

1. An increase in xanthine oxidase activity occurs when liver slices from protein-depleted rats are incubated aerobically in serum containing glucose.

2. Supplementation of the serum with glycine, methionine, riboflavin, and bicarbonate results in a further increase in enzyme activity.

3. That the observations represent a true synthesis *in vitro* of the enzyme and not merely an activation has been shown from determinations of FAD, adenine, and total protein and from glycine disappearance.

4. Preliminary results indicate that liver slices from folic acid-deficient rats show an ability to synthesize xanthine oxidase. Liver slices from normal rats fail to show this change. Folic acid deficiency was induced both by feeding a purified ration and by use of aminopterin in conjunction with the laboratory stock diet.

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