Biochem. J. (1968) **108**, 349
Printed in Great Britain

Short Communication

Regulation of Xanthine Oxidase in Rat Liver: Modifications of the Enzyme Activity of Rat Liver Supernatant on Storage at −20°

By E. Della Corte and F. Stirpe
Istituto di Patologia Generale dell'Università di Bologna, 40126 Bologna, Italy

(*Received* 26 *March* 1968)

The xanthine oxidase (xanthine–oxygen oxidoreductase, EC 1.2.3.2) of rat liver is an autoxidizable enzyme, i.e. can react with O_2 as electron acceptor, thus differing from the enzyme of bird organs, which reacts only with a dye [methylene blue (Richert & Westerfeld, 1951), 2,6-dichlorobenzenone-indo-3′-chlorophenol (Remy, Richert, Doisy, Wells & Westerfeld, 1955), tetrazolium salts (Schwartz & Litwack, 1957)] or with NAD (Morell, 1955) as electron acceptor, and which for this reason is considered to be a dehydrogenase (xanthine dehydrogenase). However, the xanthine oxidase activity of rat liver and of other mammalian organs is stimulated if it is assayed aerobically in the presence of methylene blue (Richert, Edwards & Westerfeld, 1949; Westerfeld & Richert, 1952). The activity measurable in the presence of O_2 has been interpreted as an oxidase activity, whereas the extra activity detectable on addition of methylene blue was interpreted as a dehydrogenase activity (Watts, Watts & Seegmiller, 1965).

During experiments on the regulation of rat liver xanthine oxidase, we observed that the enzyme activity of the supernatant (the only fraction containing the enzyme; Meikleham, Wells, Richert & Westerfeld, 1951) was enhanced, not only by addition of methylene blue, but also, to a smaller extent, by NAD. Xanthine oxidase activity measured with O_2 as acceptor was greatly increased on storage of the preparations at −20°, whereas only minor changes were observed if the enzyme was assayed with NAD or with methylene blue, though no formation of NADH occurred with stored preparations.

Experimental. Male rats of the Wistar Glaxo strain, about 3 months old and weighing 210–240g., were fed on a normal stock diet. Xanthine oxidase activity was assayed as described by Rowe & Wyngaarden (1966) with minor modifications. Livers were homogenized in 0·1M-tris–HCl buffer, pH 8·1 (1g. of liver + 5ml. of buffer), in a Potter–Elvhejem homogenizer with a Teflon pestle (A. H. Thomas, Philadelphia, Pa., U.S.A.) with 20 complete strokes at 1000rev./min. The homogenate was centrifuged at 600–800*g* at 0° for 20min. and the supernatant was centrifuged again at 105000*g* for 1hr. in the 8 × 25ml. rotor of an MSE 40 centrifuge. Samples of the clear supernatant were dialysed in Visking 20/32 tubes for 3hr. at 4° against three changes of 100vol. each of 0·1M-tris–HCl buffer, pH 8·1, or against 300vol. of the same buffer flowing continuously in 3hr. The enzyme activity was measured by following the increase in E_{292} due to formation of uric acid from xanthine at 25° in a Zeiss PMQII spectrophotometer equipped with a thermostatically controlled cuvette holder, automatic cuvette changer and recorder. Each preparation was assayed with O_2 (air), 0·67mM-NAD (Strittmatter, 1965) or 0·033mM-methylene blue as acceptor. Readings were taken at 1min. intervals for 5–15min. against blanks from which xanthine was omitted. All preparations were devoid of uricase activity. Enzyme activity is expressed as mμmoles of uric acid formed/min./100mg. of protein. Protein was determined by the method of Gornall, Bardawill & David (1949).

Results. Xanthine oxidase activity of freshly prepared supernatants was increased approximately four- and seven-fold respectively by the addition of NAD or of methylene blue (mean values of seven experiments, in mμmoles of uric acid formed/min./100mg. of protein: 86 ± 9 with O_2; 328 ± 18 with NAD; 600 ± 18 with methylene blue). If the determinations were repeated after storage of the supernatants at −20° for 24hr., the enzyme activity assayed with O_2 as acceptor was considerably increased, and became slightly but consistently higher than the activity in the presence of NAD. No significant changes were observed if the activity was measured in the presence of NAD or of methylene blue (mean values of the same supernatants of the above-mentioned experiments: 379 ± 39 with O_2; 342 ± 34 with NAD; 613 ± 57 with methylene blue). This change, referred to below as 'oxidase activation' or 'activation', began very shortly after the samples had frozen, and went to

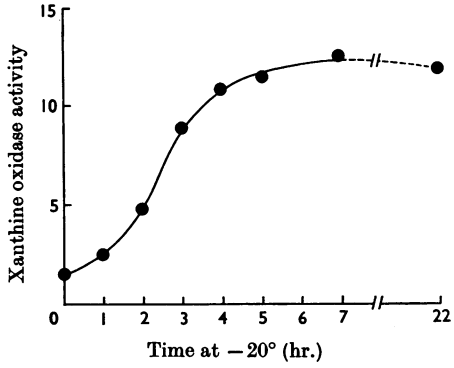

Fig. 1. Xanthine oxidase activity of rat liver supernatant stored at −20°. To avoid repeated thawing, separate samples were kept at −20° and thawed immediately before the assay. The assay mixture contained, in a final volume of 3·0ml., 0·1M-tris–HCl buffer, pH 8·1, 60μM-xanthine and 0·2ml. of rat liver supernatant. Xanthine oxidase activity is expressed as mμmoles of uric acid formed/min.

completion between 6 and 20hr., with considerable variation from a preparation to another, the activity increasing according to a sigmoidal curve (Fig. 1). The activity of the extracts kept at −20° then remained stable for several days.

Before 'activation' NAD was reduced during the oxidation of xanthine, as shown by the increased E_{340} of the reaction mixtures. The amount of uric acid formed in the presence of NAD was approximately the sum, in molar terms, of the uric acid formed in the presence of O_2 plus the amount of NAD reduced (mean values of three experiments, in mμmoles/min.: uric acid formed with O_2, 2·5, with NAD, 8·7; $NADH_2$ formed, 7·4). This seems to indicate that NAD is the acceptor for the extra amount of xanthine oxidized in its presence. After 'activation' almost no formation of $NADH_2$ was observed (values of the same samples as above, in the same order of results: 9·0, 8·2 and 0·5).

Attempts were made to obtain the 'activation' in other ways: rapid freezing and thawing of the extracts, repeated up to four times, caused rather a slight loss of enzyme activity. If the supernatants were kept at 0° for up to 5hr., or incubated at 37° for up to 1hr., or warmed at 50° for 4min., there was only a partial 'activation', which did not go to completion. The 'activation' obtained at −20° was not reversed if the 'activated' extracts were dialysed again as practised during their preparation. The enzyme activities of mixtures of equal parts of 'activated' and non-activated samples were consistently the average of individual values.

Similar experiments were performed with chick liver supernatants, but no modifications of the xanthine dehydrogenase activity were observed when these preparations were stored at −20°.

Discussion. The rate of the xanthine oxidase reaction with O_2 as acceptor increases when rat liver supernatants are stored at −20°, and from this it may be inferred that in freshly made preparations either the enzyme has a lower affinity for O_2, or only a minor part of it may react with O_2. Our experiments indicate also that rat liver xanthine oxidase may react with NAD. A difference exists, in this respect, between this enzyme and the xanthine dehydrogenase of chick liver, since the latter reacts at approximately the same (Morell, 1955) or at a higher rate (E. Della Corte & F. Stirpe, unpublished work) with NAD than with methylene blue, whereas in our non-activated preparations from rat liver the reaction rate with NAD is about one-half of that with methylene blue. The stoicheiometry of the reaction in the presence of NAD shows that before 'activation' xanthine is oxidized in part by O_2 and in part by NAD. Such a correspondence is not observed after 'activation'; this cannot be explained at the moment, but the lack of detectable $NADH_2$ under these conditions leads us to suppose either (i) that the reaction with O_2 interferes with that with NAD, or (ii) that the $NADH_2$ formed is reoxidized by xanthine oxidase ($NADH_2$ is a substrate for this enzyme; Corran, Dewan, Gordon & Green, 1939; Mackler, Mahler & Green, 1954), or (iii) that the capacity of the enzyme to react with NAD is lost.

Increases of enzyme activities with time at various temperatures have been reported. The activity of xanthine oxidase purified from pig liver is increased after short heating at 70° (Murray & Chaykin, 1966); the activity of commercial preparations of cream xanthine oxidase appears to be higher after storage of dilute solutions at 3° for 24hr. (Fried, 1966); the effect of similar treatments on our preparations is mentioned above. The glycogen synthetase activity of rat liver preparations is enhanced after preincubation at 20° (Gold & Segal, 1967), and this is believed to be the expression of a change that may play a role in the regulation of this enzyme *in vivo* (Mersmann & Segal, 1967).

We are indebted to Professor E. Bonetti for encouragement and interest in the work, and for criticism of the manuscript. The research was supported by a financial grant from Consiglio Nazionale delle Ricerche, Rome.

Corran, H. S., Dewan, J. G., Gordon, A. H. & Green, D. E. (1939). *Biochem. J.* **33**, 1694.
Fried, R. (1966). *Analyt. Biochem.* **16**, 427.
Gold, A. H. & Segal, H. K. (1967). *Arch. Biochem. Biophys.* **120**, 359.
Gornall, A. G., Bardawill, C. J. & David, M. M. (1949). *J. biol. Chem.* **177**, 751.
Mackler, B., Mahler, H. R. & Green, D. E. (1954). *J. biol. Chem.* **210**, 149.

Meikleham, V., Wells, I. C., Richert, D. A. & Westerfeld, W. W. (1951). *J. biol. Chem.* **192**, 651.

Mersmann, H. G. & Segal, H. L. (1967). *Proc. nat. Acad. Sci., Wash.*, **58**, 1688.

Morell, D. B. (1955). *Biochim. biophys. Acta*, **18**, 221.

Murray, K. N. & Chaykin, S. (1966). *J. biol. Chem.* **241**, 3468.

Remy, C. N., Richert, D. A., Doisy, R. J., Wells, I. C. & Westerfeld, W. W. (1955). *J. biol. Chem.* **217**, 293.

Richert, D. A., Edwards, S. & Westerfeld, W. W. (1949). *J. biol. Chem.* **181**, 255.

Richert, D. A. & Westerfeld, W. W. (1951). *Proc. Soc. exp. Biol., N.Y.*, **76**, 252.

Rowe, P. B. & Wyngaarden, J. B. (1966). *J. biol. Chem.* **241**, 5571.

Schwartz, H. G. & Litwack, G. (1957). *Nature, Lond.*, **180**, 761.

Strittmatter, C. F. (1965). *J. biol. Chem.* **240**, 2557.

Watts, R. W. E., Watts, H. E. M. & Seegmiller, J. E. (1965). *J. Lab. clin. Med.* **66**, 688.

Westerfeld, W. W. & Richert, D. A. (1952). *J. biol. Chem.* **199**, 393.