XANTHOPTERIN OXIDASE*

by

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INTRODUCTION

In later years there has been a growing interest for the possible biological role of xanthopterin and allied substances, a group of compounds discovered by HOPKINS[1] and studied by means of organic chemical methods by the WIELAND School[2]. A few years ago WIELAND AND LIEBIG[3] undertook a study of the biological oxidation of xanthopterin made by synthetic methods[2]. They observed that crude xanthine oxidase preparations from milk and liver were capable of catalyzing an oxidation of xanthopterin. The oxidation product formed was assumed to be leucopterin.

The present paper deals with observations made on the enzymatic oxidation of xanthopterin by means of oxidase preparations subjected to various fractionation and purification procedures. The studies were performed by an enzymatic-optical technique (differential enzymatic spectrophotometry) which has been used previously for the study of purine metabolism[4]. The technique was used not only in a study of the properties of the enzyme but also as a tool in estimating the amount of xanthopterin present in filtrates from various biological sources.

TECHNIQUE

Spectrophotometry

For determination of ultraviolet spectra and differential spectrophotometric measurements a Beckman ultraviolet spectrophotometer was used.

Fluorometry

The majority of the fluorometric measurements was carried out in a Farrand electronic multiplier fluorometer.

MATERIALS

Xanthopterin

Synthetic samples of xanthopterin were kindly furnished by Dr E. L. R. STOKSTAD, Lederle Laboratories Division, American Cyanamid Company and by Dr G. HITCHINGS, Wellcome Research Laboratories.

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ENZYMES

Two different procedures for preparation of xanthopterin oxidase from milk were used.

1. The enzyme from whey was prepared by fractionation with ammonium sulphate. The whey is 0.75 saturated with ammonium sulphate. The precipitate is redissolved in pyrophosphate buffer pH 8.5 and reprecipitated by 0.25 to 0.38 saturation. The product is dissolved in pyrophosphate buffer pH 8.5. To this golden turbid solution additional amounts of ammonium sulphate are added in order to remove the last traces of turbidity. By further fractionation with ammonium sulphate a clear golden brown solution with high enzymatic activity is obtained, the golden brown colour which is apparently associated with the xanthopterin oxidase activity, being employed as a guide.

2. The enzyme from cream was prepared according to the method described by BALL[5] with two modifications.

(a) The cream was prepared from warm milk and special care was taken to prevent the fat fraction from cooling below 20° C prior to the elution of the fat globules. If this precaution is taken the yield of enzyme is 4 to 5 fold increased (cf. POLONOVSKY[6]).

(b) The addition of calcium chloride to the lipase digest of the phosphate eluate should be such as to remove the main part but not all of the turbidity. Repeated additions of calcium chloride give rise to undue losses of the enzyme. The remaining turbidity was removed during the ammonium sulphate fractionation.

ENZYMATIC OPTICAL DETERMINATION OF XANTHOPTERIN

It was noticed that addition of a milk xanthopterin oxidase preparation to a solution of xanthopterin brought about marked changes in the absorption spectrum. It is possible to obtain the ultraviolet absorption spectrum of the endproduct once the oxidation has run to completion. The oxidation product was obtained by enzymatic oxidation of xanthopterin. The ultraviolet spectra of the substrate and the endproduct are graphically illustrated in Fig. 1. The colourless endproduct is characterized by an extremely low solubility and by a faint fluorescence at strongly alkaline reactions[7,8]. These properties are characteristic of leucopterin.

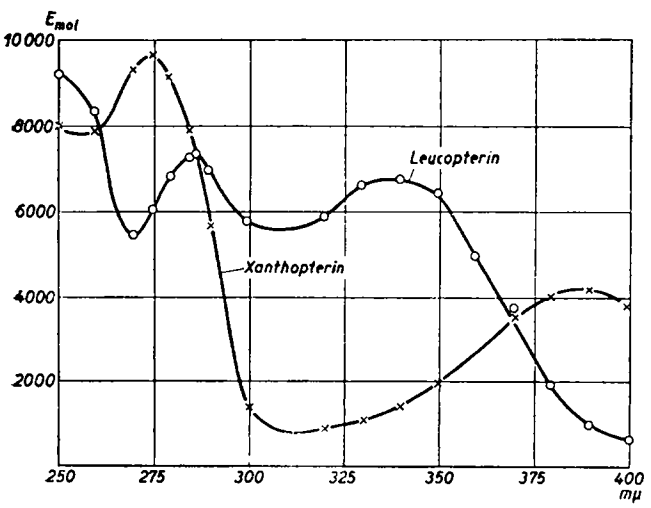

Fig. 1. Molecular extinction curves of xanthopterin and leucopterin at pH about 7

From Fig. 1 it appears that there is a maximum difference between the absorption of xanthopterin and that of leucopterin between $\lambda = 325$ and $\lambda = 330$ mμ, the wavelength $\lambda = 330$ mμ was, therefore, selected for quantitative estimations of xanthopterin. It is useful, in addition, to use

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the wavelength corresponding to the isobestic point of the two absorption curves (*i.e.* $\lambda = 371$ mμ) as well as that corresponding to the maximum decrease which occurs during oxidation (*i.e.* $\lambda = 400$ mμ). The characteristic changes in extinction in the three selected wavelengths which are brought about by the action of the oxidase on xanthopterin solutions have been used as a specific and sensitive method for detecting xanthopterin oxidase in various enzyme preparations as well as for a quantitative determination of xanthopterin in biological material.

Table I summarizes the extinction changes which take place at various wavelengths when xanthopterin is oxidized to leucopterin.

TABLE I

OPTICAL CONSTANTS OF THE OXIDATION OF XANTHOPTERIN TO LEUCOPTERIN AT p_H 7

To a phosphate buffer p_H 7.0 containing 10 μg of xanthopterin per ml is added xanthopterin oxidase. When the oxidation to leucopterin has gone to completion the following changes in density have taken place.

Wavelength in mμ	Density changes (per 10 μg per ml)
330	+ 0.310
371	0.00
400	— 0.175

Table II gives an example of a determination of the concentration of xanthopterin in phosphate buffer p_H 7.5 by the enzymatic differential spectrophotometric method mentioned above.

TABLE II

ENZYMATIC DIFFERENTIAL SPECTROPHOTOMETRY OF XANTHOPTERIN

The density changes at $\lambda = 330$ mμ of a xanthopterin-enzyme-mixture is measured at p_H about 7.

Concentration of xanthopterin μg per ml	Density changes at $\lambda = 330$ mμ	Density changes at $\lambda = 330$ mμ per μg per ml
5	0.158	0.032
10	0.309	0.031
15	0.464	0.031

If xanthopterin is kept for one hour at p_H 3 to 4 (acetate or phosphate buffer) and an aliquote of this solution is added to a phosphate buffer at p_H about 7 the ultraviolet

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spectrum undergoes a distinct change. The extinction maximum at $\lambda = 390$ mμ is markedly lowered whereas the extinction at $\lambda = 330$ mμ is unaltered. After some hours of incubation at pH 7 the shape of the ultraviolet spectrum approaches that of the original spectrum at this pH. SCHOU[9] has found that the changes in the absorption spectrum of a xanthopterin solution brought about by changing the pH value of the solution is due to a shift in the equilibrium between the enol and ketone forms of xanthopterin. The rate of this shift is catalyzed by hydrogen or hydroxyl ions[9]. For a closer analysis of this phenomenon the reader is referred to Dr SCHOU's article.

ENZYMATIC FLUOROMETRIC DETERMINATION OF XANTHOPTERIN

The enzymatic oxidation of xanthopterin to leucopterin is accompanied by a disappearance of the blue fluorescence. This phenomenon has been used as a basis for a specific and highly sensitive assay method for xanthopterin in biological fluids.

Table III gives an example of a fluorometric analysis of pure xanthopterin solutions. It can be seen that as small an amount as $22 \cdot 10^{-12}$ mole per ml can be estimated.

TABLE III

FLUORESCENCE OF PURE XANTHOPTERIN IN PHOSPHATE BUFFER SOLUTIONS pH 8.04

Concentration of xanthopterin mole/ml	Fluorescence readings
$2.2 \cdot 10^{-11}$	7½
$4.4 \cdot 10^{-11}$	15
$8.8 \cdot 10^{-11}$	29
$17.6 \cdot 10^{-11}$	56
$35.2 \cdot 10^{-11}$	116
$70.4 \cdot 10^{-11}$	221
$140.8 \cdot 10^{-11}$	446
$281.6 \cdot 10^{-11}$	856
Quinine standard $1.8 \cdot 10^{-11}$ mole/ml in 0.1 N H_2SO_4	50

In the experiment recorded in Table IV the same amount of xanthopterin is added to urine in various dilutions. It can be seen that the fluorescent substances which occur in a fresh urine sample are not identical with xanthopterin since addition of the enzyme gives rise to a constant decrease of the fluorescence (corresponding to $8 \cdot 10^{-11}$ mole per ml, cf. Table III) independent of the dilution of urine.

An eluate obtained by adsorption of the urine on superfiltrol and elution with 5% aqueous pyridine is also devoid of xanthopterin. However, as will be described in

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TABLE IV

DIFFERENTIAL FLUOROMETRIC DETERMINATION OF XANTHOPTERIN ADDED TO DILUTED URINE

To human urine diluted with phosphate buffer pH 7.7 xanthopterin is added to make the concentration $8 \cdot 10^{-11}$ mole per ml.

Dilution of urine	Fluorescence readings	Fluorescence after addition of xanthopterin	Increase in fluorescence due to the addition of xanthopterin	Fluorescence after incubation of the urine-xanthopterin mixture with xanthopterin oxidase	Decrease in fluorescence due to the incubation with xanthopterin oxidase
400 fold	14	38	24	13½	24½
200 fold	27	52	25	26½	25½
133 fold	40½	64	23½	39	24
100 fold	53	78	25	53	24

another place, illumination of the pyridine eluate by visible light releases appreciable amounts of xanthopterin. Moreover, if fresh urine is adsorbed on superfiltrol and eluted at 100° with buffers more alkaline than pH 9 an instantaneous release of xanthopterin occurs. It therefore seems that fresh urine and pyridine eluates contain a precursor of xanthopterin. This precursor which has been shown to be non-identical with the ketoform (cf. SCHOU[9]) has been called proxanthopterin[10]. A more rigid proof that xanthopterin can be liberated in urine under certain conditions is afforded by differential enzymatic spectrophotometry at the three selected wavelengths.

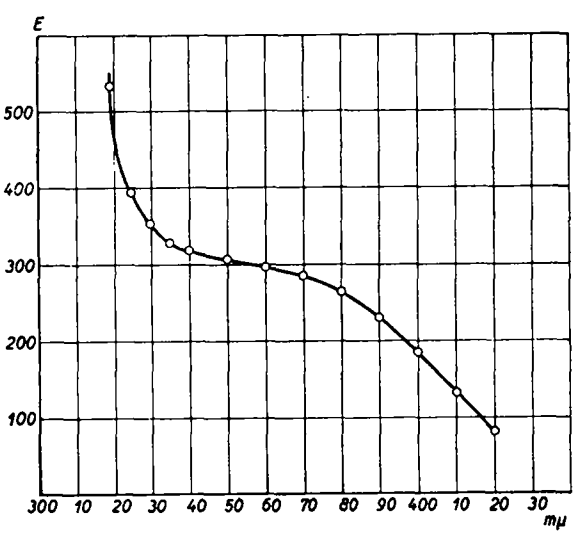

Fig. 2. Ultraviolet absorption spectrum of an alkaline eluate (of norite) prepared from human urine

Fig. 2 shows a spectrum of an alkaline eluate (of norite) prepared from human urine. Table V shows an enzymatic optical analysis of this eluate. The agreement between the data obtained from this highly impure eluate and those obtained from a pure solution of xanthopterin are quite satisfactory. The amount of xanthopterin present in the undiluted norite eluate was calculated to be 15.4 μg or $6.4 \cdot 10^{-8}$ mole per ml.

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TABLE V

DIFFERENTIAL SPECTROPHOTOMETRY WITH XANTHOPTERIN OXIDASE UPON AN ELUATE PREPARED FROM URINE

Fresh human urine is treated with Norite. The Norite is eluated with an alkaline aqueous solution. The p_H of an aliquote of the eluate is adjusted to about p_H 7.5 with phosphate buffer. After addition of xanthopterin oxidase the following density changes take place.

Wavelength mμ	Density changes
330	+ 0.174
371	+ 0.003
400	— 0.110

Calculated from Table II $\frac{D_{330}}{D_{400}} = 1.7$

Found from this Table $\frac{D_{330}}{D_{400}} = 1.6$

The concentration of xanthopterin per ml of eluate-buffer mixture, calculated from Table II, is found to be $3.1 \cdot 10^{-8}$ mole or, per ml of undiluted eluate, $6.4 \cdot 10^{-8}$ mole, *i.e.* 1.15 mg per liter of urine.

ASSAY OF ENZYMES

The enzyme preparations from cream as well as those from whey oxidize the following substrates:

a. *Xanthopterin.* The estimation of xanthopterin oxidase activity is based upon the described spectral changes brought about by enzymatic oxidation of xanthopterin. If the enzyme preparations to be assayed were clear solutions only one wavelength was selected for routine assays. The increase in absorption at $\lambda = 330$ mμ per unit of time is proportional to the amount of enzyme added. This holds true provided the substrate is added in excess in respect to the substrate and provided the determinations are restricted to the initial part of the reaction.

b. *Hypoxanthine and Xanthine.* The test is essentially the same as described previously[4]. The wavelength $\lambda = 293$ mμ has been selected instead of the previously used $\lambda = 290$ mμ because the maximum of the uric acid spectrum at p_H 7.5 to 8 is located at $\lambda = 293$ mμ. As stated previously a prerequisite for estimating xanthine oxidase activity in the ultraviolet (at $\lambda = 293$ mμ) is the complete removal of uricase. The requirement is readily satisfied when working with oxidase preparations from milk as milk does not contain any uricase.

c. *Aldehydes.* The milk xanthine oxidase is capable of catalyzing the oxidation of various aldehydes besides purines and pterines. Formaldehyde, acetaldehyde and salicylic aldehyde, for instance, are rapidly oxidized by addition of the oxidase and 2-amino-4-hydroxy-6-formyl-pteridine aldehyde (6-aldehyde) undergoes a slow oxidation in the presence of the enzyme. Since the spectral changes which accompany the oxidation of the aldehydes most often are small or non-existent within the wavelength range generally

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applicable for biological materials, the enzymatic oxidation of this group of compounds must be followed by other methods. The standard techniques most commonly used are determinations of oxygen consumption or rate of reduction of methylene blue. Since the latter method is simple and sensitive it has been used in the present study. The methylene blue tubes used were of such dimensions as to fit into the macro- or micro-adapters of the Coleman Junior Spectrophotometer. A side-arm into which enzyme solutions should be pipetted was attached on the methylene blue vessels. The decrease of density at $\lambda = 660$ mμ was followed in the Coleman spectrophotometer.

THE SPECIFICITY OF XANTHINE OXIDASE FROM MILK

The enzyme from milk rapidly oxidizes hypoxanthine and xanthine to uric acid. Guanine is not oxidized nor are the nucleosides or nucleotides of any of the purines or pyrimidines. According to BOOTH[11] milk contains an enzyme which oxidizes adenine directly by a two step oxidation. Such a type of oxidation has recently been described[12] in a series of studies on rats fed ^{15}N labeled adenine. The oxidation product was shown to be 2, 8-dihydroxyadenine. One of us (H. K.) has investigated the oxidation of adenine with fractionated xanthine oxidase from cream. These studies indicate strongly that adenine is being oxidized to 2, 8-dihydroxyadenine (E_{max} 305 mμ). The absorption at 290 mμ is not altered upon addition of purified uricase. LORZ AND HITCHINGS[13] have shown that xanthine oxidase catalyzes the oxidation of 2-aminopurines to guanines. 1-Methylxanthine is oxidized faster than xanthine[13]. Xanthopterin is oxidized at a rate which is about 25 to 30 times slower than that of the oxidation of hypoxanthine or xanthine regardless of whether the enzyme is prepared from whey or from cream. The rate of the oxidation of xanthine is reduced by further addition of xanthopterin[14, 15]. Reduced diphosphopyridine nucleotide is slowly oxidized by the enzyme[5]. All of the aldehydes just mentioned with the exception of 6-aldehyde are rapidly oxidized in the presence of the enzyme from whey as well as the enzyme from cream. The oxidation of some of the substrates mentioned is greatly inhibited by small amounts of 6-aldehyde[16]. These observations point to the view that the enzymatic oxidation of the purines and aldehydes is due to a common enzyme. It has further been shown[17] that the enzyme from cream is able to oxidize 2-amino-4-hydroxypteridine to isoxanthopterin and that this oxidation is also markedly inhibited by 6-aldehyde.

OXIDATION OF XANTHOPTERIN BY LIVER ENZYME

Liver aldehyde oxidase prepared from beef liver according to HARRISON[18] does not only catalyze the oxidation of aldehydes and hydroxypurines (hypoxanthine and xanthine) but also of xanthopterin (cf.[3]).

The occurrence of such an oxidation in human liver, if existing, would, as stressed by previous authors[3], pose the question of accounting for the absence of leucopterin in human urine.

REDUCTION AND OXIDATION OF THE ENZYME BY THE SUBSTRATE

If a concentrated solution of xanthine oxidase from cream is investigated in the spectrophotometer an extinction curve like that illustrated in Fig. 3 is obtained. This

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corresponds closely to that described by BALL[5]. BALL also found that addition of hypoxanthine to the enzyme under anaerobic conditions brings about a marked decrease in the extinction. The difference in the extinction curves between oxidized and reduced enzyme corresponds rather closely to the extinction curve of a flavin nucleotide. The enzyme which we have prepared shows the same extinction changes when incubated with xanthine, xanthopterin and acetaldehyde as substrates (see Fig. 4). When xanthopterin is used as the substrate it is necessary to confine the analysis to a wavelength at $\lambda = 470$ mμ since xanthopterin exhibits an appreciable absorption at shorter wavelengths.

Fig. 5 illustrates the density changes at $\lambda = 470$ mμ of xanthopterin oxidase when this enzyme is reduced in the presence of xanthopterin under anaerobic conditions. Furthermore, the figure illustrates the density changes which result when the reduced enzyme is reoxidized by shaking with oxygen. The density changes were followed not only at $\lambda = 470$ mμ but also at $\lambda = 620$ mμ at which wavelength the density remains unaltered by reduction and oxidation of the enzyme. After a larger proportion of the enzyme has become reoxidized the leucopterin is formed in large amounts and it gradually precipitates. The accompanying turbidity manifests itself as an increase in density at $\lambda = 620$ mμ as well as $\lambda = 470$ mμ.

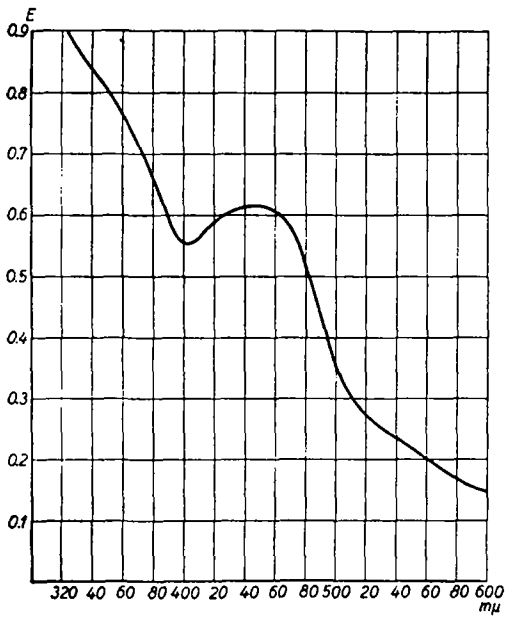

Fig. 3. Absorption spectrum of a solution of xanthopterin oxidase from cream containing about 5.1 mg per ml of protein, pH 8. Measured in the Beckman Spectrophotometer in 1 cc layer

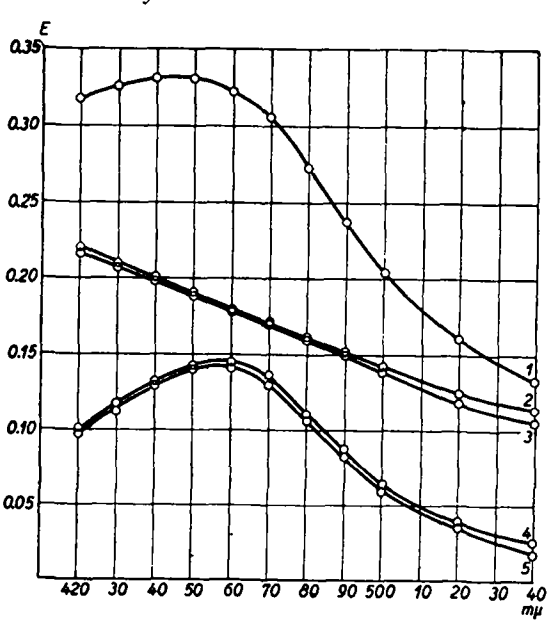

Fig. 4. Absorption spectra of a solution of xanthopterin oxidase from cream containing about 5.1 mg per ml of protein, pH 8.

1. Xanthopterin oxidase in the oxidized state
2. Xanthopterin oxidase after reduction under anaerobic conditions in the presence of xanthine
3. Xanthopterin oxidase after reduction under anaerobic conditions in the presence of acetaldehyde
4. Curve 2 substracted from curve 1
5. Curve 3 substracted from curve 1

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The following anaerobic controls were run: (1) xanthopterin oxidase without xanthopterin and (2) xanthopterin with traces of oxidase. The maximum decrease in density at $\lambda = 470$ mμ in these two controls amounted to 0.020 at the most and no increase in density took place upon introduction of oxygen.

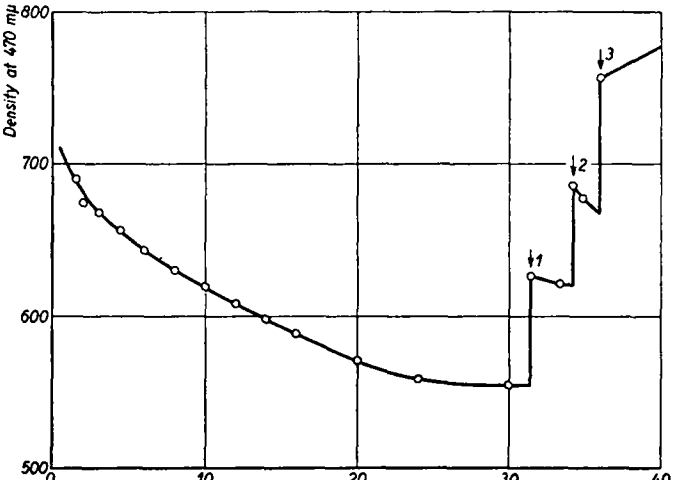

Fig. 5. 250 μl of concentrated oxygen-free xanthopterin oxidase containing about 2 mg of protein were pipetted into a WARBURG vessel containing 2.25 ml of 0.2 *M* oxygen-free phosphate buffer p_H 8. The side vessel contained 500 μg of xanthopterin dissolved in 200 μl oxygen-free 0.3 *N* NaOH. At time zero the xanthopterin was added to the enzyme, and 2 ml of the mixture were transferred as quickly as possible to a silica cuvette which was subsequently stoppered. The first reading in the spectrophotometer was usually taken about 90 sec after the mixing of xanthopterin and enzyme.

A diaphragm with a hole of 3 mm in diameter was used in order to cut out the upper part of the solution which is apt to absorb small traces of oxygen from the environment.

Ordinate: density of enzyme-substrate mixture at 470 mμ.

Abscissae: minutes after mixing. ↓1: admission of air. ↓2: 2 sec shaking with air. ↓3: 2 sec shaking with oxygen.

Light source: tungsten lamps. Slit width: 0.08 mm.

PROPERTIES OF DIALYZED ENZYME

BALL reported[5] that xanthine oxidase preparations, subjected to two weeks dialysis in distilled water, lost their activity but could be reactivated by addition of a filtrate from heat-inactivated enzyme. We have been able to observe a 4 to 5 fold increase in activity of dialyzed xanthine oxidase (10 days of dialysis) upon addition of a filtrate from undialyzed xanthine oxidase inactivated and denaturated at 80° C. It was noticed, however, that this reactivation was only faintly pronounced during the first few minutes after the addition of 'cofactor' and then became increasingly manifest. Inasmuch as the filtrate used still contained protein it was thought advisable to check whether the observed effect could be brought about by addition of sulfhydryl containing substances. It turned out that reduced glutathione possesses an activating effect similar to that of the filtrate. From Table VI it can be seen that glutathione promotes as marked an effect as does the filtrate, and the combination of filtrate and glutathione does not give rise to any further augmentation. Undialyzed xanthine oxidase is not influenced by addition of glutathione. It was further observed that addition of pyrophosphate, glycine, alanylglycine and cyanide to the dialyzed enzyme had similar reactivating effect on the catalytic activity as had filtrate and glutathione. Since the substances just mentioned are all known to form complex compounds with heavy metal ions[19] it is reasonable to ascribe the reactivation to the removal of free ions of heavy metals through a formation of complex compounds.

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If it is assumed that complex-formers, present in the crude enzyme preparation, are lost during dialysis then it is reasonable to suppose that traces of heavy metal ions liberated from the glass vessel during prolonged dialysis lead to the low activity of the dialyzed enzyme. This interpretation may also apply to previous observations[5]. It should be added that storage of the dialyzed enzymes at 0° C quite often brings about a partial reactivation. It was also found that addition of tris(hydroxymethyl)aminomethane buffer usually gives rise to an augmentation of the activity. It is not possible to state with certainty that the latter effect is due to binding of heavy metal.

TABLE VI

ACTIVATION WITH FILTRATE AND GLUTATHIONE OF DIALYZED XANTHINE OXIDASE

To 3 ml of a phosphate buffer p_H about 7 containing $3 \cdot 10^{-7}$ mole per ml of xanthine is added 10 μl of a concentrated solution of xanthine oxidase which has been subjected to dialysis for 14 days. The rate of oxidation of xanthine is measured in this solution and in the same solution supplied with 1. filtrate, 2. glutathione and 3. both filtrate and glutathione.

Enzyme-xanthine mixture	ΔE_{293} per minute	Moles of xanthine oxidized per ml per minute after the lapse of 20 minutes after addition of enzyme
1. No addition	+ 0.003	$6 \cdot 10^{-10}$
2. Addition of glutathione Concentration: 0.025 mg per ml of the mixture	+ 0.014	$28.2 \cdot 10^{-10}$
3. Addition of 15 μl of filtrate	+ 0.014	$28.2 \cdot 10^{-10}$
4. Addition of both 2 and 3	+ 0.014	$28.2 \cdot 10^{-10}$

SUMMARY

An enzyme which catalyzes the oxidation of xanthopterin to leucopterin has been isolated from cream and also from whey. An enzymatic differential spectrophotometric method is given for the estimation of xanthopterin. The enzyme reaction could also be followed fluorometrically. These methods have been used as assay methods for xanthopterin in biological fluids.

The specificity of the enzyme is discussed. The enzyme is supposed to be identical with the xanthine oxidase described by BALL.

The reactivation of the dialyzed enzyme was studied.

RÉSUMÉ

Nous avons isolé, à partir de la crème et aussi du petit lait, un enzyme catalysant l'oxydation de la xanthoptérine en leucoptérine. Nous décrivons une méthode spectrophotométrique différentielle pour l'évaluation de la xanthoptérine. D'autre part, la réaction enzymatique peut aussi être suivie fluorométriquement. Ces méthodes ont été utilisées pour déterminer la xanthoptérine dans les liquides biologiques.

Nous avons discuté la spécificité de l'enzyme; nous supposons qu'il est identique avec la xanthine-oxidase décrite par BALL.

La réactivation de l'enzyme dialysé a aussi été discutée.

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ZUSAMMENFASSUNG

Ein, die Oxydation von Xanthopterin zu Leukopterin katalysierendes Enzym wurde aus Rahm und auch aus Molke isoliert. Xanthopterin kann mittels einer spektrophotometrischen enzymatischen Differential-Methode bestimmt werden. Die enzymatische Reaktion kann auch fluorometrisch verfolgt werden. Diese Methoden wurden zur Bestimmung von Xanthopterin in biologischen Flüssigkeiten verwendet.

Die Spezifizität des Enzyms wurde erörtert; es scheint, dass es mit der Xanthinoxidase von BALL identisch ist.

Auch die Reaktivierung des dialysierten Enzyms wurde untersucht.

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