The Determination of Orotic Acid in the Blood Serum by Means of the Spectrophotometric Method

Besides polarographic detection[1], the enzymatic spectrophotometric method is used[2], which needs a very particular preparation of reagents used for the transformation of orotic acid to uridylic acid. Orotic acid can be further isolated from other compounds by paper chromatography[3], active coal[4], ion-exchanger Dowex 1-X10[5], and can also be established on the basis of its strong absorption of UV-light. BANCHI and LOMUTO[6] described several unspecific reactions for the detection of the pure substance. Besides the reaction with ferric chloride, which is rather insensitive, they perform the estimation of oxidation products of orotic acid.

In 1961 TSUJI[7] published a new method which is based on the bromination of orotic acid, removal of the abundant bromide by sodium thioglycolate, and colour reaction with *p*-dimethylaminobenzaldehyde. The coloured product which appeared was shaken into *n*-butyl acetate.

ADACHI et al.[8] used the described procedure in their own modification for the detection of orotic acid in multivitamin preparations. They also detected the optimal conditions for the determination. Instead of sodium thioglycolate, L-ascorbic acid was used for the debromination and thus, the unfavourable influence of different concentrations of the reduction agent on absorbance and sensitivity of the described method was eliminated.

This short survey shows that the described procedures for the establishment of orotic acid are very pretentious in regards to time and material, and are also unspecific and little sensitive. That is why we tried to use ADACHI's method[8] with our own modification for the determination of orotic acid in the blood serum.

Methods. Reagents: (1) 0.4 *M* buffered solution of sodium citrate with hydrochloric acid – pH 2.5. (2) Saturated solution of bromine in water. (3) 5% solution of L-ascorbic acid in the distilled water. (4) 2.5% solution of *p*-dimethylaminobenzaldehyde p.a. in *n*-propanol. (5) Deproteinating agent: 1.65 g uranyl acetate p.a. ($UO_2(OCOCH_3)_2 \cdot 2H_2O$) is dissolved in 100 ml of distilled water. (6) *n*-butyl acetate p.a. (7) The standard solution of orotic acid – 2 mg%.

The standard procedure: 3.0 ml of distilled water are added into centrifuge tubes with 1.0 ml of the blood serum or 1.0 ml of the standard solution. For the blank test, 4.0 ml of distilled water are put into a test tube. 1.0 ml of a solution of uranyl acetate is given in drops to all test tubes during the final shaking. After 10 min the coagulated proteins are removed by centrifugation. Then to 2.5 ml of supernatant, 2.0 ml of 0.4 *M* buffer and 0.5 ml of saturated bromine water are added, followed by 1.0 ml of a solution of ascorbic acid after 60 sec. The period of 60 sec is not critical, as similar results even after an interval of 20 sec up to 5 min are obtained. After subsequent shaking, the test tubes are left for 5 min in water bath of 40 °C. After the debromination 2.0 ml of *p*-dimethylaminobenzaldehyde solution are pipetted and the colouring develops for 10 min in a water bath of 40 °C. After cooling, 4.0 ml of butyl acetate are added for the extraction of the 5-(*p*-dimethylaminobenzylidene)-barbituric acid formed. Measuring is performed with a wave-length of 458 nm by spectrophotometer Unicam SP 800. If the layer of butyl acetate is turbid, centrifugation for 2–3 min is advised with subsequent measuring of the clear solution. The resulting colour is stable for several hours.

Calculation:

$$\frac{\text{E of the sample}}{\text{E of the standard}} \times 20 = \mu\text{g of orotic acid in 1.0 ml of serum.}$$

$$\frac{\text{E of the sample}}{\text{E of the standard}} \times 2 = \text{mg\% of orotic acid in the blood serum.}$$

Error in the method, after a careful performance of the analysis, does not exceed ± 3.0% when 10.0 µg of orotic acid in the sample is established.

Results and discussion. Special attention must be devoted to the buffered solution the pH value of which must be exactly 2.5. At this value, the sensitivity of the method is at its maximum. ADACHI et al.[8] made the pH of the sample 2–3 at the beginning of the procedure and then added 0.2 *M* citric acid-potassium citrate buffer (pH 2.5). To avoid tedious manipulations and eventual mistakes during the first adjustment of the pH of the reacting mixture, we tried to use the described buffer at a higher molarity. However, we observed that the sensitivity of the method was thereby decreased. After many tests, the 0.4 *M* buffered solution of sodium citrate-hydrochloric acid at pH 2.5 was found to be the most suitable.

As the resulting colour is dependent on the pH values of the mixture, the greatest problem was the deproteination. After unsuccessful experiments with trichloroacetic, perchloric, metaphosphoric acids, procedures after FOLIN-WU or SOMOGY, the most suitable compound was found to be uranyl acetate. The essential advantage of this kind of deproteination is that the pH value of the reaction

Table I. Comparison of values of extinctions in the determination of sodium orotate using uranyl acetate

µg of sodium orotate	Extinction with $UO_2(OCOCH_3)_2$		without $UO_2(OCOCH_3)_2$	
2.5	0.08	0.075	0.08	0.07
5.0	0.16	0.17	0.16	0.16
10.0	0.31	0.32	0.32	0.315
20.0	0.62	0.635	0.62	0.64

Table II. Determination of various amounts of sodium orotate added to the blood serum. Values expressed in µg

Added amount of sodium orotate to the serum	Determined	Recovery %
2.5	2.6 (2.5– 2.64)	104
5.0	4.8 (4.75– 4.9)	96
10.0	10.0 (9.85–10.15)	100
20.0	19.5 (19.4 –19.7)	97.5

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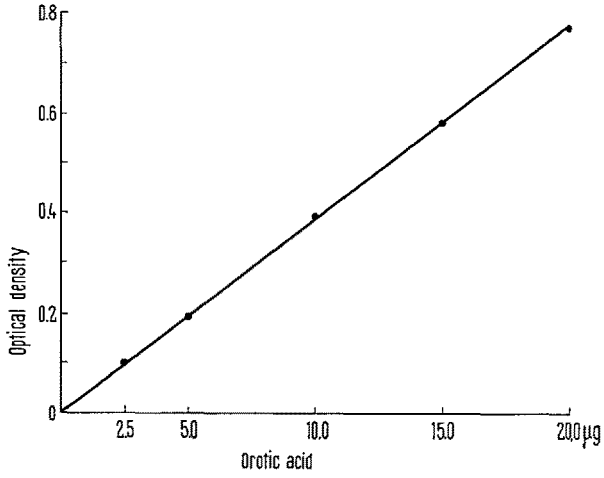
mixture is not generally influenced and therefore the preliminary adjustation of the sample need not be performed.

The question of whether or not uranyl acetate influences the course of the reaction, was answered by the determination of various amounts of orotic acid (Na salt) in the presence or absence of uranyl acetate. Table I shows that uranyl acetate does not influence the course of the reaction.

The dependence of the extinction on the amount is linear only up to the 20 μg concentration of orotic acid (Figure). If we suppose that the amount of orotic acid will be higher, we can use less supernatant after the deproteination, or even dilute the serum to a certain degree with water. Table II presents a good agreement between the doses of orotic acid (Na salt) added to the blood serum and the observed values.



Dependence of extinction on the amount of the determined orotic acid.

The method described is also very successful for the following of orotic acid in the blood serum and in some organs of rats, which was proved in several of our model experiments.

The usefulness of this method was proved also in healthy persons as well as in patients with higher levels of bilirubin, biliary acids, lipidic substances, uric acid etc. No falsely increased values were observed in any cases.

In experiments performed in vitro it was found out that other substances, namely 4-aminoantipyrine, antipyrine, amidopyrine, indol derivates, β-naphtyl-amine as well as barbituric acid, thiobarbituric acid and tryptophan react under the same experimental conditions.

Zusammenfassung. Das Prinzip des Orotsäurenachweises beruht auf der Deproteination des Serums mittels Uranylessigester und der Verwandlung der Orotsäure durch Bromation in 5, 5-Dibrombarbitursäure und nachfolgende Debromation in Barbitursäure. Letztere ergibt mit *p*-Dimethylaminobenzaldehyd ein intensiv gefärbtes Produkt, 5-(*p*-dimethylaminobenzyliden)-Barbitursäure.

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Amylase and Polyphenol Oxidase Production by Germinating Conidia of *Colletotrichum falcatum* Went

In an earlier publication Singh[1] has reported the secretion and location of sucrase during germination of conidia of *Colletotrichum falcatum* Went, the red rot organism of sugarcane. The present investigations deal with the production of amylase and polyphenol oxidase by the germinating conidia of a light type highly sporing virulent isolate and a dark type sparsely sporing less virulent one. The presence of amylase is presumed in view of the utilization of starch by different isolates of *C. falcatum* during germination (Singh[2]). However, the existence of polyphenol oxidase is presumed on the basis of the reddening of canes infected with the red rot fungus and which is apparently comparable to the reddening or darkening of several mushrooms (Agarics and Polypores, etc.), apples, potatoes and other iron- or copper-containing plant parts on injury. This sort of reddening is known to be due to the oxidation of mono- and O-di-hydric phenols.

Actively growing (12–16 days old), single-conidium cultures of 2 *C. falcatum* isolates (Nos.[3] 78 and 244, the dark and the light type, respectively) were used in the present investigations. Amylase: Secretion of amylase by germinating conidia has been detected both qualitatively and quantitatively by the following 2 methods (Bernfeld[4]): (1) Change of the Iodine-staining properties of the substrate. (2) Increase in the reducing power of soluble starch. Both these methods are characteristic for the action of α-amylases. However, the second method can only be used for the assay of β-amylase.

(1) Change of iodine-staining property of the substrate: Qualitative detection of amylase was achieved by germinating conidia, in suspension drops, on oat-meal agar plates containing 1.0% soluble starch (B.D.H.) at 27 °C[5]. After 18 h[5], the plates were flooded with 0.5% iodine solution. A central unstained area became evident in the deep violet agar plates in both the isolates. The unstained area marked a zone of amylase activity as a result of the germination of the conidia and utilization of starch in the medium. This area was slightly bigger in the light type isolate (Figure I 1, 2) than in the dark type one (Figure I 3, 4), thereby indicating greater enzyme activity of the former.

(2) Increase in the reducing power of soluble starch: This method of assaying the amylase activity is based on the increase in the reducing power of soluble starch, and is applicable to both α- and β-amylases. The activity was measured through an enzyme extraction, obtained by crushing to fineness 0.2 g of germinated conidia of each

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[5] Optimum temperature and incubation period (Singh[2]).