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STUDIES ON GLUCOSE DEHYDROGENASE OF *ASPERGILLUS ORYZAE*.

II. PURIFICATION AND PHYSICAL AND CHEMICAL PROPERTIES

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SUMMARY

A glucose dehydrogenase which catalyzes the oxidation by 2,6-dichlorophenolindophenol (DCIP) of glucose to gluconic acid was purified from induced mycelia of *Aspergillus oryzae*, and proved to be homogeneous upon ultracentrifugation and electrophoresis. The molecular weight was estimated to be approx. 118 000. The enzyme contained 1 mole of FAD as prosthetic group per mole of enzyme. The amino acid composition of glucose dehydrogenase is reported: it is rich in aspartic acid and glutamic acid. The purified enzyme is a glycoprotein containing 24% of carbohydrate consisting of glucose, mannose and hexosamines.

Glucose dehydrogenase was also partially purified from filtrates of induced culture and from non-induced mycelia. Although these preparations had lower specific activities than the highly purified preparation from induced mycelia, evidence was obtained that the glucose dehydrogenases from the three sources were identical with one another at least in molecular weight.

INTRODUCTION

A soluble glucose dehydrogenase, catalyzing the oxidation of glucose by certain redox dyes and quinones, occurs in *Aspergillus oryzae*[1,2]. This enzyme, being incapable of reacting with molecular O_2, differs from the flavoprotein, glucose oxidase (notatin) (EC 1.1.3.4), purified from several molds[3–5]. It also differs from NAD(P)-linked glucose dehydrogenase (EC 1.1.1.47) (refs. 6–8).

Working with rather crude preparations, OGURA[9–12] and KURASAWA AND IGAUE[2,13–15] concluded that this enzyme is a simple protein possessing no prosthetic groups. KUSAI[16] has also suggested that a similar glucose dehydrogenase of *Aspergillus niger* may not be a flavoprotein. Although this conclusion has been supported by our previous studies on a seeminlgy highly purified preparation[17], it was still felt desirable to reinvestigate the nature of the glucose dehydrogenase from *A. oryzae* with preparations of established purity.

Abbreviation: DCIP, 2,6-dichlorophenolindophenol.

In previous papers[18,19] we reported that the synthesis of glucose dehydrogenase in *A. oryzae* is specifically and markedly enhanced by hydroquinone or *p*-benzoquinone, and this induction of the enzyme formation is further augmented by the simultaneous presence of EDTA in the cultivation medium. These findings, which were of interest from the viewpoint of the regulation of enzyme synthesis, also enabled us to undertake the purification from starting materials containing 50–100 times as much glucose dehydrogenase as those employed in previous work[1,2,16,17].

In the present study we took advantage of this fact and induced the synthesis of glucose dehydrogenase by growing *A. oryzae* in shake cultures containing both hydroquinone and EDTA. By starting from the glucose dehydrogenase-rich mycelia thus obtained it was possible to purify this enzyme to an ultracentrifugally and electrophoretically homogeneous state. Contrary to the evidence presented previously[2,9,17], the purified enzyme was found to be a glycoprotein containing FAD as prosthetic group. The main purpose of this paper is to describe the purification of glucose dehydrogenase from induced mycelia and to report the physical properties as well as the chemical composition of the purified enzyme. Glucose dehydrogenase liberated into the medium during the shake cultivation was also purified from the culture filtrates. This latter preparation, though not completely pure, possessed properties similar to those of the enzyme purified from mycelia. Although a preparation of glucose dehydrogenase exhibiting no measurable flavin absorption bands could be obtained from the mycelia grown on the surface of standing media containing neither hydroquinone nor EDTA[17], the specific activity of this preparation was only 1–2% of that of the purest preparation obtained above.

MATERIALS AND METHODS

Cultivation

A strain of *A. oryzae*, which was employed in the previous work[18,19], was cultivated at 30–34° for 18 h under shaking conditions as described previously[19]; 1.2 l of the inoculated medium[19], containing 1 mM hydroquinone and 0.1 mM EDTA as inducers, was placed in a 5-l flask. About 10 g of wet mycelia ('induced mycelia') were obtained per flask. The cultivation medium from which mycelia were removed ('culture filtrate') was also saved for purification of glucose dehydrogenase excreted into the medium. Mycelia were also obtained from surface culture with and without the addition of inducers. For this purpose, conidiospores were sown on the surface of about 1 l of the above medium with and without 1 mM hydroquinone and 0.1 mM EDTA. The medium was placed, about 2 cm thick, in a flat, enameled iron vessel, and incubated at 28–30° for 42–48 h. About 40 g of mycelia were obtained per vessel. The mycelia obtained from cultures with and without the addition of inducers were called 'induced' and 'non-induced' mycelia, respectively. The mycelia harvested were washed with water, squeezed to remove the bulk of the water, and stored at −13° until use.

Enzyme assays

Glucose dehydrogenase activity was measured as described previously[18,19] with dichloroindophenol (DCIP) as hydrogen acceptor at room temperature (20–25°). The molar extinction coefficients used for DCIP were $21.0 \cdot 10^6$ at pH 7.6 (ref. 20)

and $18.5 \cdot 10^6$ cm$^2 \cdot$mole^{-1} at pH 6.5. The unit and the specific activity were defined in the preceding paper[19].

Glucose 6-phosphate and alcohol dehydrogenase activities were measured by following the reduction of $NADP^+$ and NAD^+, respectively, at 340 mμ in a final volume of 1.5 ml. The reaction mixture for glucose-6-phosphate dehydrogenase assay contained 0.1 M Tris–HCl buffer (pH 7.5), 0.32 mM $NADP^+$, 1 mM glucose 6-phosphate, 2 mM $MgCl_2$ and enzyme; that for alcohol dehydrogenase assay contained 0.1 M Tris–HCl buffer (pH 8.5), 70 mM ethanol, 0.4 mM NAD^+ and enzyme.

Glucose oxidase was measured by following the oxygen consumption in a Warburg manometer at 30° under air; the reaction mixture contained 0.1 M glucose, 0.1 M phosphate buffer (pH 6.5) and enzyme in a total volume of 2.0 ml.

Analytical methods

Sedimentation analysis was performed in a Hitachi model UCA-1 ultracentrifuge. Sedimentation coefficients were obtained as described by ISEMURA AND KAKIUCHI[21], and corrected for variation in the viscosity of solvent with temperature and for the difference in viscosity of the solvent and water. The sample was dialyzed overnight against 0.05 M potassium phosphate buffer (pH 6.5). The centrifuge was operated at 54 600 rev./min.

The molecular weight of the enzyme was determined by gel filtration through Sephadex G-100 as described by ANDREWS[22], in a column (2.5 cm × 52 cm) which had been equilibrated with 0.1 M phosphate buffer. The elution was conducted at 4° with the same buffer at a flow rate of 12 ml/h, and 2-ml fractions were collected. Samples of 1.0 ml containing 5–8 mg of protein were applied to the column. The column was calibrated with highly purified Penicillium glucose oxidase (EC 1.1.3.4; mol.wt., 154 000)[16], yeast glucose-6-phosphate dehydrogenase (EC 1.1.1.49; mol.wt., 110 000)[22] yeast alcohol dehydrogenase (EC 1.1.1.1; mol.wt., 126 000)[22], and yeast cytochrome *c* (mol.wt., 12 400)[23]. The elution rates of these enzymes were measured by determining their activities or the absorbance at 415 mμ (for cytochrome *c*).

The molecular weight of the enzyme was also determined by Archibald's[24] method of the approach to sedimentation equilibrium. In this experiment the ultracentrifuge was run at 15 500 rev./min at 19° with 5.0 mg of the enzyme protein per ml of 0.05 M phosphate buffer (pH 6.5). The molecular weight analysis was performed on the data obtained from the photographs taken at 16, 26, 36, 47, 57 and 67 min after reaching full speed. The calculation of the molecular weight was made according to EHRENBERG[25].

Free boundary electrophoresis was conducted in a Hitachi model HTB Tiselius-type apparatus. The sample was dialyzed overnight against 0.05 M potassium phosphate buffer (pH 6.5). With the same buffer as overlying solution, electrophoresis was run at 4°.

Absorption spectra were measured in a Cary spectrophotometer using fused silica cells (optical path, 1 cm). Extinction coefficients ($E_{1\,cm}^{1\%}$) were obtained on the dry weight of samples that had been dialyzed against distilled water.

Amino acid composition and hexosamine were determined in acid hydrolyzates of the lyophilized enzyme. Hydrolysis with 6 M HCl was carried out in evacuated sealed tubes at 110° for 24 h. Analysis was performed according to the method of MOORE, SPACKMAN AND STEIN[26] in a Beckman Spinco amino acid analyzer. The lyophilized

sample was oxidized by performic acid according to HIRS[27], and the oxidized sample was also analyzed for amino acids as above. Tryptophan was determined spectrophotometrically[28].

The anthrone color reaction was carried out according to SHETLAR AND MASTER[29], using D-glucose as the standard. Absorbance was read at 625 mμ (ref. 30). The neutral sugar components of the carbohydrate moiety were detected by means of gas chromatography[31]. The enzyme (about 4 mg) was hydrolyzed with 1 M H_2SO_4 in a sealed tube at 100° for 16 h. The hydrolyzate was neutralized with saturated $Ba(OH)_2$, and centrifuged. The supernatant thus obtained was passed through a Dowex50 X-2 (H^+-form) column (1.5 cm × 6 cm) and washed with 10 ml of distilled water. The eluate and washings were dried under vacuum, and treated with pyridine, hexamethyldisilane and trimethylchlorosilazane according to SWEELEY *et al.*[31]. The treated material was then subjected to gas chromatography as described by SWEELEY *et al.*[31] in a Shimadzu gas chromatograph CS-B at 150° using a column (0.3 cm × 200 cm) of 15% polyethyleneglycol succinate on chromosorb W. Authentic glucose, galactose and mannose were used as standards.

Protein was determined by the method of LOWRY *et al.*[32], with crystalline bovine serum albumin as a standard. In column techniques, the absorbance at 278 mμ was measured to estimate the distribution of protein among eluates.

Removal, identification and determination of enzyme-bond flavin

The procedure for removal of flavin from the enzyme protein was based on the method WARBURG AND CHRISTIAN[33]. Enzyme solution (1.0 ml, containing 1–2 mg of protein) was mixed with 600 mg of $(NH_4)_2SO_4$. The solution was cooled to 0° in an ice-bath and the pH of the solution was adjusted to 2.5 with 1 M HCl. After being allowed to stand for 10 min, the mixture was applied to a Sephadex G-100 column (0.9 cm × 8 cm), equilibrated with the same $(NH_4)_2SO_4$ solution (pH 2.5), and the column was treated with the same solution. The eluates were adjusted to pH 6.5 with 2 M ammonia. The eluates were used as apoenzyme of the dehydrogenase.

For identification and determination of the flavin, the enzyme was treated with acid $(NH_4)_2SO_4$ as described above, and the mixture was subjected to Sephadex G-25 gel filtration to separate the flavin from the protein moiety. The flavin thus separated was concentrated by lyophilization. The flavin in the concentrated sample was identified by paper chromatography, using Toyo Roshi No. 54 filter paper and 5.0% Na_2HPO_4 as solvent[34]. The flavin spots were located by observation of fluorescence under a Toshiba ultraviolet (366 mμ) lamp, model F 1-3, and identified by comparing with the spots of authentic FAD and FMN.

The flavin content was determined from the absorption spectrum of the preparation, assuming a molar extinction coefficient of $11.3 \cdot 10^6$ $cm^2 \cdot mole^{-1}$ for flavin at 450 mμ (ref. 35). The contents of FAD and FMN were determined according to the method of BURCH[36] by measuring the increment in fluorescence of neutralized trichloroacetic acid extracts before and after hydrolysis of FAD to FMN. To the enzyme solution (about 0.1 mg protein) trichloroacetic acid at 0° was added to a final concentration of 11%. Practically no precipitation occurred. After 15 min the sample was centrifuged, and 0.5 ml of the supernatant was immediately transferred to a glass tube containing 2 ml of 0.2 M K_2HPO_4. Another 0.5-ml portion of the supernatant was placed in a second glass tube, covered with a cap, and incubated in the dark at 37° for 18–20

h. The sample thus hydrolyzed was neutralized by mixing with 2 ml of 0.2 M K_2HPO_4. Authentic FAD and riboflavin were treated in exactly the same manner and used as standards. The contents of FAD and FMN were determined fluorimetrically with an Aminco Bowman spectrofluorimeter; the exciting wavelength was 470 mμ, and the emission wavelength 530 mμ (ref. 37).

Materials

Amberlite CG-50 was washed with 2 M NaOH and 0.2% NaClO, and then treated with 2 M HCl to convert it to the H^+-form according to the method of HAGIHARA *et al.*[38]. The resin in the H^+-form was equilibrated at pH 5.2 with 0.1 M ammonium acetate. DEAE-cellulose, obtained from Brown Co., was washed with 0.5 M NaOH and 0.5 M HCl, and equilibrated at pH 6.5 with 2 mM phosphate buffer.

Sephadex G-100 was purchased from AB Pharmacia. Glucose-6-phosphate dehydrogenase[39] and alcohol dehydrogenase[40] were purified from yeast. Glucose oxidase of *Penicillium amagasakiense*[16] and crystalline yeast cytochrome *c* were kindly supplied by Dr. I. SEKUZU and Sankyo Co. Ltd., respectively. FAD, FMN, riboflavin, NAD and NADP were obtained from Sigma Chemical Company.

RESULTS

Purification of glucose dehydrogenase from induced mycelia

Extraction

A. oryzae was cultivated under shaking conditions in a medium containing both hydroquinone and EDTA as described under MATERIALS AND METHODS. The mycelia thus obtained (1.5–2.0 kg) were ground mechanically for 30 min with half their weight of quartz sand, and treated 3 times with 1–2 l each of 0.2 M K_2HPO_4 containing 0.5 mM EDTA. This and all the subsequent manipulations were conducted at 4° unless otherwise stated. The mixture was centrifuged at 6000 × *g* for 20 min. About 10 l of brownish yellow supernatant ('mycelial extract') was obtained from 5 kg of mycelia. The pH of the extract was adjusted to 7.0 with 4 M NH_4OH.

$(NH_4)_2SO_4$ precipitation

To the mycelial extract finely powdered $(NH_4)_2SO_4$ was added to 90% saturation with mechanical stirring, and the mixture was adjusted to pH 6.5–7.0 with ammonia. After standing for 1 h, the mixture was filtered on large funnels in a room maintained at −10°; filtration was usually completed within 2 days. The precipitate was dissolved in a minimal amount of water, and dialyzed for 18 h against 15 l of 0.5 mM EDTA solution; the outer fluid was changed 3 times, and the cellophane dialysis tubing was replaced twice to avoid breakage due to the weak cellulolytic activity of the extract. Insoluble materials in the dialyzate were removed by centrifugation at 10 000 × *g* for 20 min, yielding 4–5 l of turbid reddish supernatant. If salts were not sufficiently removed by this dialysis step, unsatisfactory results would ensue in the following acetone treatment step.

Acetone precipitation

Cold acetone (1.5 vol. at −20°) was slowly added with mechanical stirring to the above dialyzate that had been chilled in an ice-salt bath. After standing for 10 min, the mixture was centrifuged at −10°. The precipitate was dissolved in a minimal amount of water and dialyzed for 18 h against 15 l of 0.1 M ammonium acetate buffer

(pH 5.2) containing 0.5 mM EDTA with two changes of the outer fluid. Insoluble materials were removed by centrifugation.

First Amberlite CG-50 treatment

The dialyzed solution (about 2 l) was applied to an Amberlite CG-50 type II column (6 cm × 25 cm), which had been equilibrated with 0.1 M ammonium acetate buffer (pH 5.2). The eluate contained a small amount of glucose dehydrogenase which seemed to be the partially modified enzyme. The column was washed with 4 l of the same buffer until the effluents became faintly yellow in color. The resin retaining most of the glucose dehydrogenase activity was scraped out into a beaker containing 1 M ammonium acetate solution, and treated with 4 M ammonia until the pH of the suspension became approx. 6.0. It was important to add ammonia very slowly to protect the enzyme from heat of neutralization. The resin suspension was filtered, and the residue on the filter paper was washed with 0.1 M potassium phosphate buffer (pH 6.5). The filtrate and washings were dialyzed for 18 h against 15 l of 2 mM potassium phosphate buffer (pH 7.0) with three changes of the outer fluid.

DEAE-cellulose treatment

The solution obtained above (about 2.6 l) was passed through a DEAE-cellulose column (4.5 cm × 16 cm), equilibrated with 2 mM potassium phosphate buffer (pH 6.5). While a large amount of brown impurities remained adsorbed at the top of column glucose dehydrogenase migrated rapidly on washing with the buffer and were eluted from the column. The eluate was dialyzed for 18 h against 0.1 mM ammonium acetate (pH 5.2) with three changes of the outer fluid.

Second Amberlite CG-50 treatment

The dialyzed solution (about 2 l) was applied to an Amberlite CG-50, type II column (3.1 cm × 20 cm), equilibrated with 0.1 M ammonium acetate buffer (pH 5.2). After the column had been washed with the same buffer (about 2.4 l) until the effluents became almost colorless, gradient elution was carried out using 1 l of 0.1 M ammonium acetate buffer (pH 5.2) in the mixing vessel and 1 l of 0.4 M ammonium acetate buffer (pH 5.8) in the resservoir, and 10-ml fractions were collected. The glucose dehydrogenase activity was thereby eluted out in association with the main protein peak which was yellow in color. The fractions containing the enzyme activity were combined. The combined solution was adjusted to pH 6.5 with 1 M KOH, dialyzed for 18 h against 15 l of 2 mM potassium phosphate buffer (pH 6.5), and then adjusted to pH 5.2 with 1 M HCl.

Third Amberlite CG-50 treatment

The solution was applied to an Amberlite CG-50 type II column (1.6 cm × 16 cm) equilibrated with 0.1 M ammonium acetate buffer (pH 5.2). The enzyme was then eluted with 0.8 M potassium phosphate (pH 6.5), and 2.0-ml fractions were collected. Yellow fractions were obtained at the early stages, followed by fractions that were brownish yellow in color. The yellow fractions (an $A_{460\ m\mu}/A_{380\ m\mu}$ ratio higher than 1.0) were collected (soln. 1 in Table I), and the brownish yellow fractions (an $A_{460\ m\mu}/A_{380\ m\mu}$ ratio lower than 1.0) were subjected again to the Amberlite column chromatography. The yellow fractions obtained in the second chromatography were combined (soln. 2 in Table I) and processed separately. The two yellow fractions were concentrated by dialysis against 1 l of saturated sucrose solution adjusted to pH 7.0, and were then dialyzed against 0.1 M potassium phosphate buffer (pH 6.5). Insoluble materials were removed by centrifugation.

TABLE I

PURIFICATION OF GLUCOSE DEHYDROGENASE FROM MYCELIA OF *A. oryzae* OBTAINED FROM SHAKE CULTURES CONTAINING HYDROQUINONE AND EDTA

Five kg of wet mycelia were used as the starting material. For soln. 1 and soln. 2, see text.

Purification step		*Protein (mg)*	*Total activity (units)*	*Specific activity (units/mg protein)*
Mycelial extract		105 000	2 100 000	20
$(NH_4)_2SO_4$ precipitation		31 600	1 900 000	60
Acetone fractionation		13 600	1 700 000	125
First Amberlite CG-50 treatment		9 400	1 500 000	160
DEAE-cellulose treatment		1 550	1 350 000	870
Second Amberlite CG-50 treatment		135	1 000 000	7 400
Third Amberlite CG-50 treatment	soln. 1	66	600 000	9 100
	soln. 2	60	270 000	4 500
Sephadex G-100 gel filtration	soln. 1	40	450 000	11 200
	soln. 2	9	99 000	11 000

Gel filtration

The concentrated enzyme solutions (soln. 1 and soln. 2; 25 ml each) were applied separately to Sephadex G-100 columns (5.2 cm × 21 cm) equilibrated with 0.1 M potassium phosphate buffer (pH 6.5), and the columns were treated with the same buffer, 10 ml fractions being collected. Glucose dehydrogenase was eluted in association with a symmetrical protein peak in both columns (Fig. 1). The fractions containing the enzyme were combined, concentrated by dialysis against saturated sucrose solution, then dialyzed against 0.1 M phosphate buffer (pH 6.5), and used as the purified enzyme.

Summary of purification

Table I summarizes the results of a typical purification experiment starting from 5 kg of wet mycelia. As will be seen, the final product represented a 550-fold purification over the mycelial extract, and the yield was 26%.

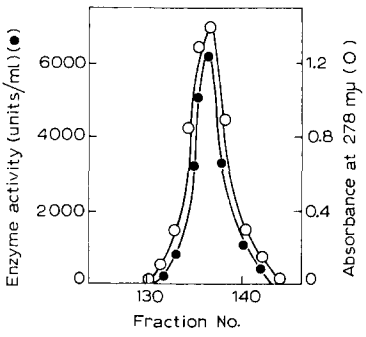


Fig. 1. Elution pattern of purified glucose dehydrogenase in gel filtration. The soln. 1 fraction obtained by the third Amberlite CG-50 treatment (66 mg protein, $60 \cdot 10^4$ units; see Table I) was applied to a Sephadex G-100 column (5.2 cm × 21 cm) which was equilibrated with 0.1 M potassium phosphate buffer (pH 6.5) and 10-ml fractions were collected at a flow rate of 10 ml/h.

Physical properties and flavoprotein nature

Homogeneity

That the enzyme preparation purified from induced mycelia was homogeneous was indicated by the chromatographic pattern shown in Fig. 1. Its homogeneity was confirmed further by ultracentrifugal and electrophoretic analyses. The sedimentation pattern obtained in the analytical centrifuge (Fig. 2) showed only one symmetrical

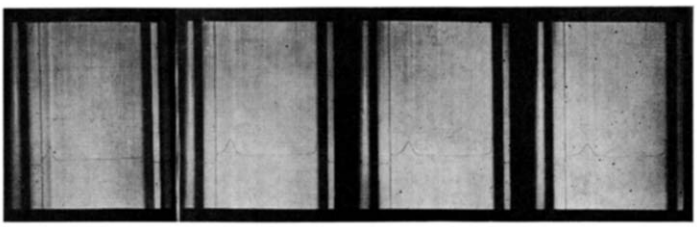


Fig. 2. Sedimentation patterns of purified glucose dehydrogenase. Protein concn., 7 mg/ml. 0.05 M potassium phosphate buffer (pH 6.5). Photographs (from left to right) were taken 15, 30, 40, and 48 min after reaching 54 600 rev./min.

boundary which was associated with the yellow color. The sedimentation coefficient was dependent on protein concentration (4.6, 5.6, and 5.7 S at 7.0, 3.0 and 2.2 mg protein/ml, respectively), and an $s^0_{20,w}$ value of 6.1 S was obtained by extrapolation. As shown in Fig. 3, the electrophoretic patterns of the purified enzyme also indicated its homogeneity. The enzyme migrated at pH 6.5 to the cathode showing only one boundary.

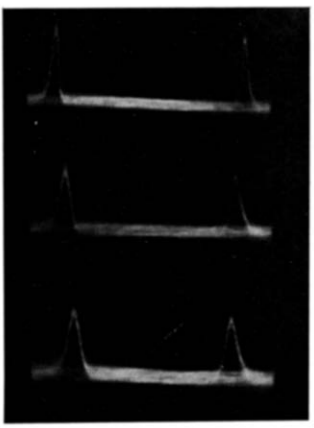


Fig. 3. Free-boundary electrophoresis of purified glucose dehydrogenase. Protein concn., 4.5 mg/ml. 0.05 M potassium phosphate buffer (pH 6.5). Current, 11 mA. Section area of the cell, 0.3 cm^2. Schlieren patterns (from top to bottom) were taken 20, 60 and 90 min after the passage of current. Left is ascending and right is descending.

Absorption spectra

Fig. 4 illustrates the absorption spectra of the purified enzyme measured at pH 6.5. In addition to an intense peak due to protein at 278 mμ, the spectrum of the oxidized enzyme showed two maxima at 380 and 460 mμ. These peaks clearly indicated the flavoprotein nature of the enzyme; the presence of prosthetic groups other than

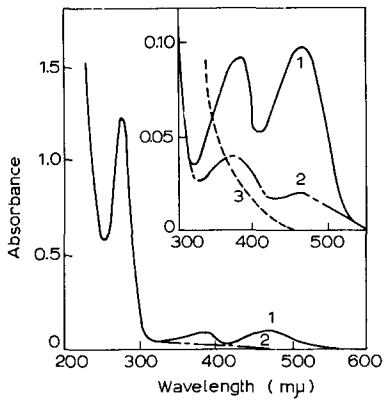
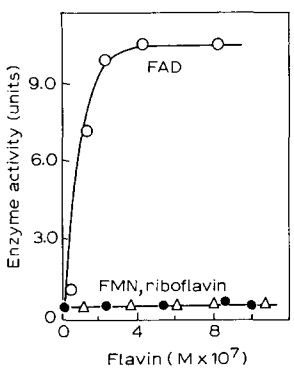


Fig. 4. Absorption spectra of purified glucose dehydrogenase (spec. activity, 11 000) from induced mycelia (shake culture). 0.1 M potassium phosphate buffer (pH 6.5). 0.84 mg of enzyme per ml. The insert shows the enlarged spectra in the wavelength region indicated. Curve 1, untreated, oxidized enzyme; Curve 2, after addition of 1 mM glucose; Curve 3, after further addition of dithionite.

flavin could not be inferred from this spectrum. The ratio of $A_{460\ m\mu}/A_{278\ m\mu}$ was 0.084, and $A_{380\ m\mu}/A_{460\ m\mu}$ was 0.93. The extinction coefficient ($E^{1\%}_{1\ cm}$) at 278 mμ was 14.9. Fig. 4 also shows that the addition of glucose caused partial bleaching of the flavin peaks at 460 and 380 mμ, suggesting the participation of the flavin in enzymic catalysis. Dithionite caused complete decolorization. From the spectrum and the protein content, it was calculated that 1 mole of flavin is contained per 90 300 g of enzyme protein.



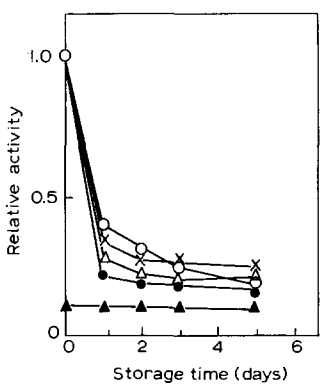


Fig. 5. Reactivation of apo-glucose dehydrogenase by flavins. The apoenzyme was prepared by acid $(NH_4)_2SO_4$ treatment followed by Sephadex G-100 gel filtration, and used immediately after preparation. The apoenzyme (1.3 μg protein/ml) was mixed with the indicated concentrations of flavins in 0.1 M potassium phosphate buffer (pH 6.5). The mixture was allowed to stand for 20 min at room temperature, and then the glucose dehydrogenase activity was measured. ○—○, FAD; ●—●, FMN; △—△, riboflavin.

Fig. 6. Inactivation of apo-glucose dehydrogenase during storage. The freshly prepared apoenzyme was dissolved in 0.05 M potassium phosphate buffer (pH 6.5) and stored at −18° in the presence of 0.2 M glucose (○), 0.5 μM FAD (●), 0.2 M glucose *plus* 0.5 μM FAD (×), and without any additions (△). After the indicated time of storage, the glucose dehydrogenase activity of each preparation was measured in the presence of 0.5 μM FAD. The activity of the preparation stored without additions was also measured in the absence of added FAD (▲).

Flavin prosthetic group

The flavin was released from the enzyme by acid $(NH_4)_2SO_4$ treatment. When examined by paper chromatography using 5% Na_2HPO_4 as a solvent, the dehydrogenase flavin showed an R_F value identical with that of FAD. The flavin was also liberated from the enzyme by trichloroacetic acid treatment and analyzed fluorometrically by BURCH's method[36]. It was thereby found that all the flavin was FAD, and its content corresponded to 1 mole of FAD per 90 000 g of protein.

The flavin-free apoenzyme prepared by acid $(NH_4)_2SO_4$ treatment and subsequent gel filtration through Sephadex G-100 showed only a negligible glucose dehydrogenase activity. Its activity was, however, restored by FAD, but not by FMN and riboflavin. Fig. 5 shows that half maximal reactivation occurred when the concentration of FAD was 0.084 μM. As shown in Fig. 6, the extent of reactivation by FAD decreased while the apoenzyme was stored at $-18°$; after 2 days of storage, almost no restoration was observed. This decrease was not appreciably prevented by the presence of either FAD or glucose or both. It is suggested that a certain irreversible change in conformation occurs during the resolution of the enzyme and this change is intensified during storage.

Molecular weight

The molecular weight of the glucose dehydrogenase was determined by both the Archibald sedimentation equilibrium method[24] and the Sephadex G-100 gel filtration method[22]. For the latter method, yeast cytochrome *c*, yeast glucose-6-phosphate dehydrogenase, yeast alcohol dehydrogenase and Penicillium glucose oxidase were used for calibration. The molecular weight determined by the Archibald method was 118 000, whereas that estimated by the gel filtration method was 120 000. A minimal molecular weight of 117 000 was also obtained by the determinations of the FAD content and dry weight of a purified preparation which had been thoroughly dialyzed against distilled water. On the other hand, it was stated above that 90 000 g of protein contains 1 mole of FAD. The significant discrepancy between the two groups of values (about 120 000 and about 90 000) suggested that the enzyme contains a considerable amount of non-protein components. As reported below, it was actually found that the enzyme is a glycoprotein. It may be concluded that *A. oryzae* glucose dehydrogenase is a glycoprotein having a molecular weight of 118 000 (average values obtained by three different methods) and that 1 mole of enzyme contains 1 mole of FAD.

Chemical composition and stability

Amino acid composition

Table II shows the results of the amino acid analysis of purified glucose dehydrogenase. As will be seen, 9 moles of histidine and 6 moles of half-cysteine residues were detected per mole of FAD. From the data it could also be calculated that one mole of FAD corresponds to 90 400 g of protein in close agreement with the values obtained above by different methods.

Sugar components

As mentioned above, glucose dehydrogenase was found to contain a significant amount of carbohydrate components. Thus, the purified enzyme gave a color reaction with anthrone in sulfuric acid. The color produced had a broad peak at 625 mμ, similar to that of the glucose-anthrone reaction[29,30]. After the blank absorbance had been subtracted, it was estimated from the color intensity and a standard curve drawn for

TABLE II

AMINO ACID COMPOSITION OF GLUCOSE DEHYDROGENASE OF *A. oryzae*

Amino acid	*Molar equiv per mole FAD*	*Amino acid*	*Molar equiv per mole FAD*
Lys	43	Cys*	6
His	9		
Arg	33	Val	58
Asp	104	Met	11
Thr	46	Ile	35
Ser	63	Leu	72
Glu	67	Tyr	32
Pro	46	Phe	25
Gly	80	Trp**	15
Ala	92		
Hexosamine***	20		

* Determined separately[27].
** Determined spectrophotometrically[28].
*** Extrapolation to zero time, assuming first order decay (T. IKENAKA, personal communication).

glucose that 28 g of sugars (as glucose) was contained per 100 g of enzyme protein. Since the enzyme contained such a large amount of carbohydrate in its molecule, its neutral components were detected by means of gas chromatography[31]. The chromatogram thus obtained indicated that the main components of the carbohydrate moiety of the enzyme were glucose and mannose (Fig. 7). The ratio of glucose to mannose was 4:1. Furthermore, it was revealed that the enzyme contained hexosamines in the

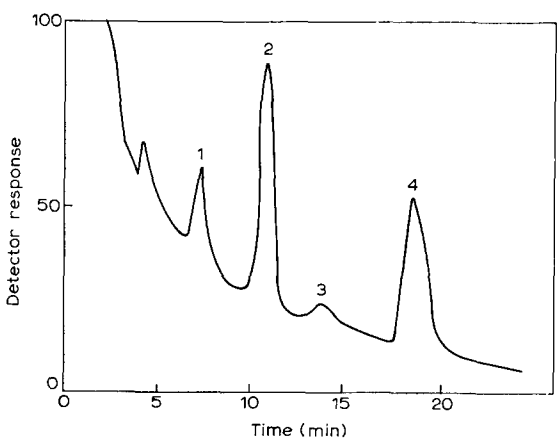


Fig. 7. Gas chromatogram of neutral sugars in the hydrolyzate of glucose dehydrogenase of *A. oryzae*. The enzyme was hydrolyzed and treated as described in METHODS AND MATERIALS. The hydrolysate was trimethylsilylated according to SWEELEY[31]. Analysis was performed at 150° under the conditions of flow rates of H_2, N_2 and air of 50 ml, 50 ml and 700 ml/min, respectively. 1, α-mannose; 2, α-glucose; 3, β-mannose; 4, β-glucose.

molecule, the contents of which were determined to be 20 moles per mole of FAD from the chart of amino acid analysis (Table II). These values, together with the FAD content per protein, gave a molecular weight of 119 000 for the enzyme, a value in reasonable agreement with those determined by the other methods.

Stability

Both crude and highly purified preparations of glucose dehydrogenase were fairly stable at −18° in 0.05 M potassium phosphate buffer (pH 6.5); only 10% of the activity was lost after 3 months, and 50% inactivation was observed after 6 months. The stability in the frozen state was not appreciably affected in the pH range from 5.2 to 7.0, but at pH 8.0 the enzyme was more labile. As mentioned above, the apoenzyme was unstable even at −18°. Fig. 8A shows that treatment of the enzyme at 45° for 15 min (at pH 6.5) caused some inactivation and the activity was completely

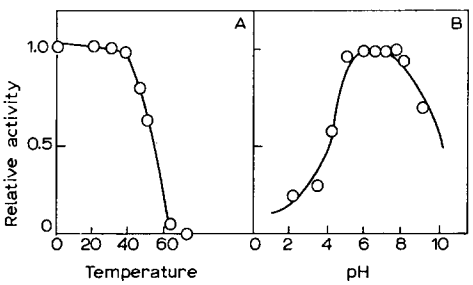


Fig. 8. Stability of glucose dehydrogenase to temperature and pH. A, The enzyme dissolved in 0.1 M potassium phosphate buffer (pH 6.5) was treated for 15 min at the indicated temperature and the residual activity was measured. B, The enzyme was dissolved in 0.2 M acetate, phosphate or Tris–HCl buffers of indicated pH, and the solution was maintained at 30° for 2 h. The residual activity was then measured.

lost by treatment at 60°. The presence of 0.2 M glucose during the heat treatment protected the enzyme to some extent. As shown in Fig. 8B, the enzyme activity was not appreciably affected by incubation for 2 h at 30° in the pH range from 5.0 to 8.0, but on both sides of this pH range the enzyme was rapidly inactivated.

Glucose dehydrogenase liberated into the medium

Purification

As reported previously[18,19], considerable amounts of the glucose dehydrogenase synthesized by *A. oryzae* in shake culture in the presence of hydroquinone and EDTA were liberated into the medium. The dehydrogenase thus liberated was purified from the culture filtrate. The filtrate was adjusted to pH 5.2 with HCl and passed through an Amberlite CG-50 type II column (5 cm × 10 cm) which had been equilibrated with 0.1 M ammonium acetate buffer (pH 5.2). The column was then washed thoroughly with the same buffer. The glucose dehydrogenase remaining on the column was eluted with a minimal amount of 4 M ammonia, and the eluate was immediately stored at −13°. When a sufficient amount of concentrated culture filtrate was obtained, this preparation (referred to as 'concentrated culture filtrate') was processed exactly as described for mycelial extracts, except that the acetone precipitation and third

TABLE III

PURIFICATION OF GLUCOSE DEHYDROGENASE FROM CULTURE FILTRATES

Purification step	*Protein (mg)*	*Total activity (units)*	*Spec. activity (units/mg protein)*
Concentrated culture filtrate	2940	190 000	65
$(NH_4)_2SO_4$ fraction	865	200 000	230
First Amberlite CG-50 treatment	266	210 000	790
DEAE-cellulose treatment	135	195 000	1370
Second Amberlite CG-50 treatment	40	105 000	2830
Sephadex G-100 filtration	11	60 000	5400

Amberlite CG-50 treatment steps were omitted. As summarized in Table III, 89-fold purification over the concentrated culture filtrate was achieved by these procedures with an overall yield of 31%. The specific enzyme activity of the final product (5400 units/mg protein) was, however, only about half that of the material purified from mycelia (11 000 units/mg protein).

Absorption spectrum

Fig. 9 shows the absorption spectrum of glucose dehydrogenase purified from the culture filtrate. This spectrum was essentially identical with that of the enzyme

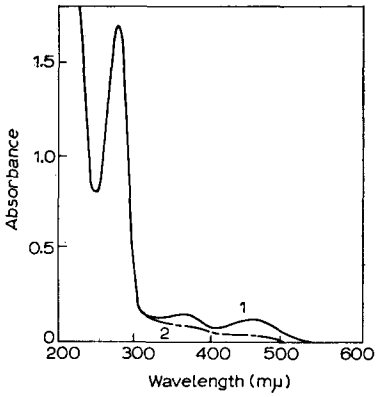


Fig. 9. Absorption spectra of glucose dehydrogenase (spec. activity, 5400) purified from culture filtration. 0.1 M potassium phosphate buffer (pH 6.5), 1.6 mg of enzyme per ml. Curve 1, untreated, oxidized enzyme; Curve 2, after addition of 1 mM glucose.

purified from mycelia, except that the ratio $A_{460\ m\mu}/A_{280\ m\mu}$ (0.065) was lower than that for the purified mycelial enzyme (0.084). This observation, together with the lower specific activity, suggested that the enzyme from the culture filtrate was still impure. As with the mycelial enzyme, the flavin absorption peaks at 380 and 460 mμ were also partially bleached by the addition of glucose (Fig. 9).

Glucose dehydrogenase from surface cultures

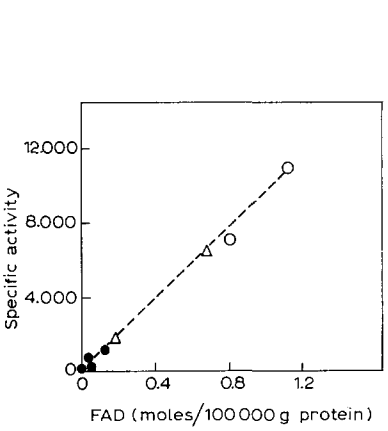
The enzyme occurring in non-induced mycelia obtained from surface cultures containing neither inducer nor EDTA was also purified by essentially the same

procedures as described above from 20 kg of mycelial pads[17]; but the purified product had a specific activity of only 170 as compared with 11 000 for the purified preparation from mycelia grown in shaking culture containing hydroquinone and EDTA. The absorption spectrum of this enzyme preparation showed only one absorption peak due to protein at 280 mμ, no other absorptions were observable in the wavelength range up to 650 mμ and no change in spectrum occurred on addition of either glucose or dithionite. However, many of the enzymic properties of this preparation were identical with those obtained from induced mycelia and culture filtrates.

When the same purification procedures were applied to induced mycelia grown on the surface of the medium containing both hydroquinone and EDTA, it was possible to obtain preparations of glucose dehydrogenase possessing a specific activity of up to 1100 units per mg protein. These preparations, in contrast to those from non-induced mycelia, showed a spectrum having feeble, but detectable, absorption peaks of FAD.

Comparison of enzyme preparations from different sources

Since purified glucose dehydrogenase preparations obtained from different sources were profoundly different from one another in absorption spectrum and specific activity, it was desirable to examine whether such differences were due to the differences in their properties or simply ascribable to differences in their purity. For this purpose, the relation between specific activity and FAD content in preparations purified to various extents from different sources was examined. Fig. 10 shows, that there



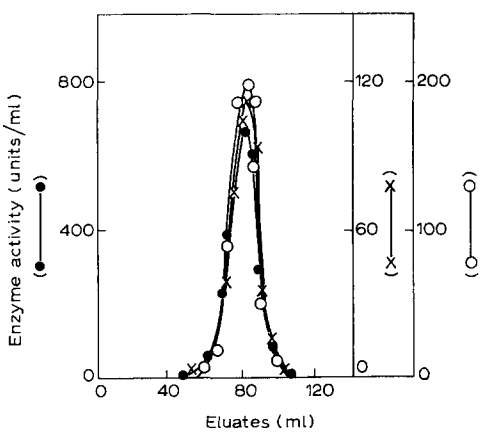


Fig. 10. Linear correlation between specific activity and FAD content in glucose dehydrogenase preparations from various sources and of various purities. ○, the enzyme from induced mycelia (shake culture); △, the enzyme from culture filtrate (shake culture); ●, the enzyme from induced mycelia (surface culture).

Fig. 11. Elution patterns in Sephadex G-100 gel filtration of three glucose dehydrogenase preparations obtained from different sources. The samples were separately subjected to gel filtration (column size, 2.5 cm × 52 cm; equilibrated with 0.1 M potassium phosphate buffer (pH 6.5)), and eluted with the same buffer; 2-ml fractions were collected. ●, the enzyme partially purified from induced mycellia (shake culture, spec. activity 5000); ○, the enzyme from culture filtrate (shake culture, spec. activity, 1250); ×, the enzyme from induced mycelia (surface culture, spec. activity, 250).

was a beautiful linear correlation, suggesting that the differences noticed were due to differences in purity. Furthermore, when three enzyme preparations from different sources were subjected to gel filtration through a Sephadex G-100 column, the glucose dehydrogenase activity was eluted at the same position (Fig. 11). It was therefore concluded that the enzymes obtained from different sources had the same molecular weight, despite pronounced differences in specific activity.

DISCUSSION

The discovery of an induction effect of *p*-benzoquinone or hydroquinone and EDTA on the biosynthesis of glucose dehydrogenase in *A. oryzae*[18,19] has enabled us to attempt the purification of this enzyme starting from induced mycelia as well as from the culture filtrate containing high concentrations of the dehydrogenase. It was thus possible to obtain highly purified preparations of glucose dehydrogenase from induced mycelia, which were homogeneous in ultracentrifugal and electrophoretical analyses. Application of essentially the same procedures to the culture filtrates yielded a glucose dehydrogenase preparation, which seemed to be about 50% pure judging from its specific activity. As previously reported[17], a preparation of this enzyme could also be obtained by similar procedures from mycelia grown on the surface of a standing medium containing no inducers. However, this last-mentioned preparation possessed a specific activity corresponding to only 1–2% of that of the homogeneous enzyme from induced mycelia. As will be discussed below, this low specific activity reflects the very low purity of the preparation.

Although it has long been believed that glucose dehydrogenase possesses no prosthetic group[9,13,17], the enzyme purified from induced mycelia is a flavoprotein and contains 1 mole of FAD per mole of enzyme. The enzyme partly purified from the culture filtrate is also a flavoprotein. No flavin could, however, be detected in the preparation obtained from non-induced mycelia, probably because of its very low purity. The failure of previous workers[9,13,17] to detect the flavin prosthetic group is undoubtedly also due to the low purity of their preparations. The FAD present in the purified enzyme is evidently essential for the catalytic activity, because the removal of FAD by acid $(NH_4)_2SO_4$ treatment leads to the inactivation of the enzyme, but the activity of the apoenzyme can be partly restored by the addition of FAD. Furthermore, there is a good correlation between the FAD content and the glucose dehydrogenase activity in preparations of widely varying purity.

The detection of FAD in *A. oryzae* glucose dehydrogenase raises anew the old question concerning the relation between this enzyme and the glucose oxidase of other fungi. When OGURA[1] reported the discovery of glucose dehydrogenase in *A. oryzae*, FRANKE[41] criticized this and claimed the identity of the two enzymes. However, glucose oxidase, though possessing FAD as prosthetic group, differs from glucose dehydrogenase in several important respects. The oxidase contains 2 moles of FAD per mole[16,42], whereas the dehydrogenase contains only 1 mole of FAD per mole as mentioned above. From this and other differences to be reported later, it may be concluded that the two enzymes differ from each other. However, it is an interesting fact that two glucose-oxidizing enzymes from fungi are both FAD-containing proteins.

The molecular weight of purified glucose dehydrogenase determined by the Archibald method and by the Sephadex G-100 gel filtration technique is 118 000,

whereas flavin analysis indicates that 1 mole of FAD is present per 90 000 g of protein. This discrepancy can be accounted for by the detection of 24% of carbohydrate components consisting of glucose, mannose and hexosamines in the enzyme molecule. Although the content of glucose in common glycoproteins is much lower than that of mannose, the reverse is true with *A. oryzae* glucose dehydrogenase. The reason for this anomaly is not yet known. Furthermore, the rate of release of the carbohydrate moiety from the enzyme molecule by acid hydrolysis is rather slow and is comparable to that of the cleavage of an ester bond. This fact, together with the high contents of aspartic and glutamic acids in the enzyme protein, suggests that the carbohydrate units are linked to the protein by ester bonds with the carboxyl groups of aspartic and glutamic acid residues.

The high content of carbohydrates, if present at the surface of the molecule, would tend to modify the properties of the enzyme protein. Some of the unusual properties of glucose dehydrogenase, such as slow precipitation on heat and trichloroacetic acid treatments, high solubility in water, and requirement of very high salt concentrations (90% saturated $(NH_4)_2SO_4$) for precipitation, may be related to the increased hydrophilic properties of the molecule conferred by the carbohydrate components. A similar suggestion has been made by SWOBODA AND MASSEY[43] for glucose oxidase of *Aspergillus niger*. Nothing more can be said about the significance of a large amount of carbohydrates in glucose dehydrogenase, but it is interesting to note that many enzymes of fungal origin, such as glucose oxidase[43,44], chloroperoxidase[45], α-amylase[44], glucamylases A and B (ref. 44), and cellulase[46], contain 5–30% of carbohydrate components.

As mentioned above, the preparation of glucose dehydrogenase purified from non-induced mycelia grown on the surface of a standing medium showed a very low specific activity and no spectral evidence for the presence of FAD. It has also been reported preliminary[17] that this preparation seemed to be rather homogeneous and to have a molecular weight of about 21 000. It seems possible, therefore, that glucose dehydrogenase produced in non-induced mycelia is a different entity from that produced in induced mycelia. However, the results shown in Fig. 11 indicate that the catalytically active portion of the two types of preparation possesses the same molecular weight. It is, therefore, concluded that the preparation obtained from non-induced mycelia contained only 1–2% of active glucose dehydrogenase (molecular weight 118 000) as a contaminant in a rather homogeneous protein of unknown nature and of much smaller size (molecular weight about 21 000).

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REFERENCES

1 Y. OGURA AND M. NAGAHISA, *Botan Mag. Tokyo*, 51 (1937) 599.
2 H. KURASAWA AND I. IGAUE, *Nippon Nôgei-Kagaku Kaishi*, 28 (1954) 954.
3 D. MÜLLER, *Enzymologia*, 10 (1941) 40.
4 C. E. COULTHARD, R. MICHAELIS, W. F. SHORT, G. SYKES, G. E. H. SKRIMSHIRE, A. F. B. STANDFAST, J. H. BIRKINSHAW AND H. RAISTRICK, *Biochem. J.*, 39 (1945) 24.
5 K. KUSAI, I. SEKUZU, B. HAGIHARA, K. OKUNUKI, S. YAMAUCHI AND M. NAKAI, *Biochim. Biophys. Acta*, 40 (1960) 555.
6 J. A. M. BACH AND H. L. SADOFF, *J. Bacteriol.*, 83 (1962) 699.
7 K. OKAMOTO, *J. Biochem. Tokyo*, 53 (1963) 348.
8 H. H. STRECHKER AND S. KORKES, *J. Biol. Chem.*, 196 (1952) 769.
9 Y. OGURA, *Acta Phytochim.*, 11 (1939) 127.
10 M. NAGAHISA AND Y. OGURA, *Botan. Mag. Tokyo*, 56 (1942) 137.
11 Y. OGURA, *J. Biochem. Tokyo*, 38 (1951) 75.
12 Y. OGURA, *J. Biochem. Tokyo*, 39 (1952) 31.
13 H. KURASAWA AND I. IGAUE, *Nippon Nôgei-Kagaku Kaishi*, 30 (1956) 553.
14 H. KURASAWA AND I. IGAUE, *Nippon Nôgei-Kagaku Kaishi*, 33 (1959) 532.
15 H. KURASAWA AND I. IGAUE, *Nippon Nôgei-Kagaku Kaishi*, 33 (1959) 536.
16 K. KUSAI, *Ann. Rept. Sci. Works, Fac. Sci. Osaka Univ.*, 8 (1960) 43.
17 T-G. BAK AND R. SATO, *Koso Kagaku Shimpozium*, 15 (1963) 180 .
18 T-G. BAK AND R. SATO, *Koso Kagaku Shimpozium*, 17 (1965) 234.
19 T-G. BAK AND R. SATO, *Biochim. Biophys. Acta*, BBA 65584.
20 A. H. PHILIPS AND R. G. LANGDON, *J. Biol. Chem.*, 237 (1962) 2652.
21 T. ISEMURA AND K. KAKIUCHI, *J. Biochem. Tokyo*, 51 (1962) 385.
22 P. ANDREWS, *Biochem. J.*, 91 (1964) 222.
23 E. MARGOLIASH, E. L. SMITH, G. KREIL AND H. TUPPY, *Nature*, 192 (1961) 1121.
24 H. K. SACHMAN, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. IV, Academic Press, 1957, p. 32.
25 A. EHRENBERG, *Acta Chem. Scand.*, 11 (1957) 1257.
26 S. MOORE, D. H. SPACKMAN AND W. H. STEIN, *Anal. Chem.*, 30 (1958) 1185.
27 C. H. W. HIRS, *J. Biol. Chem.*, 219 (1956) 611.
28 T. W. GOODWIN AND R. A. MORTON, *Biochem. J.*, 40 (1946) 628.
29 M. R. SHETLAR AND Y. F. MASTER, *Anal. Chem.*, 29 (1957) 402.
30 K. A. TRAYSER AND S. P. COLOWICK, *Arch. Biochem. Biophys.*, 94 (1961) 169.
31 C. C. SWEELEY, R. BENTLEY, M. MAKITA AND W. W. WEILS, *J. Am. Chem.Soc.*, 85 (1951) 265.
32 O. H. LOWRY, N. J. ROSEBROUGH, A. L. FARR AND R. J. RANDALL, *J. Biol. Chem.*, 193 (1951) 265.
33 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 298 (1938) 150.
34 G. L. KILGOUR, S. P. FELTON AND F. M. HUENNEKENS, *J. Am. Chem. Soc.*, 79 (1957) 2254.
35 O. WARBURG AND W. CHRISTIAN, *Biochem. Z.*, 298 (1938) 377.
36 H. B. BURCH, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 960.
37 V. MASSEY AND C. H. WILLIAMS, *J. Biol. Chem.*, 240 (1965) 4470.
38 B. HAGIHARA, I. MORIKAWA, I. SEKUZU AND I. OKUNUKI, *J. Biochem. Tokyo*, 45 (1958) 551.
39 A. KORNBERG AND B. L. HORECKER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 323.
40 E. RACKER, in S. P. COLOWICK AND N. O. KAPLAN, *Methods in Enzymology*, Vol. III, Academic Press, New York, 1957, p. 500.
41 W. FRANKE AND F. LORENZ, *Ann. Chem.*, 555 (1944) 111.
42 R. CECIL AND A. G. OGSTON, *Biochem. J.*, 42 (1948) 221.
43 B. E. P. SWOBODA AND V. MASSEY, *J. Biol. Chem.*, 240 (1965) 2209.
44 J. H. PAZUR, K. KLEPPE AND E. M. BALL, *Arch. Biochem. Biophys.*, 103 (1963) 515.
45 D. R. MORRIS AND L. P. HAGER, *J. Biol. Chem.*, 241. (1966) 1763.
46 T. IWASAKI, R. IKEDA, K. HAYASHI AND M. FUNATSU, *J. Biochem. Tokyo*, 57 (1965) 478.