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Preparation of UDP-Glucuronic Acid by Bacterial UDP-Glucose Dehydrogenase

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The distribution of UDPG dehydrogenase in microorganisms, especially *Bacillus* species, was investigated and it was found that several strains of *Bacillus licheniformis* possessed this enzyme and were most suitable for the enzymatic preparation of UDPGA from UDPG since these organisms scarcely showed decomposition of both UDPG and UDPGA. Disintegrated cells such as sonicated ones can easily oxidize UDPG to UDPGA with a high conversion rate. The reaction product was purified by a Dowex 1 (Cl^- form) column and was identified as UDPGA by chemical and physical analyses and by biological functions such as glucuronide formation with *o*-aminophenol by the homogenate of mouse liver.

UDPG dehydrogenase of *B. licheniformis* required NAD for it's activity. The optimum pH is 9.5 and the optimum temperature is 40°C. Under aerobic conditions the NADH oxidizing system in this organism coupled with the dehydrogenation of UDPG facilitated the completion of UDPG-UDPGA conversion. In the presence of 0.5 mM NAD, 6 mM UDPG was dehydrogenated to UDPGA at a conversion rate of 87% in 17 hr at 30°C by the sonicate of *B. licheniformis* IAM 11054.

UDPGA was isolated in 1955 by Dutton and Storey[1] from liver extract as a factor participating in detoxification through conjugation with glucuronic acid. UDPGA is the donor of the glucuronic acid moiety of glucuronide in detoxification and of acidic mucopolysaccharide.[2] It is also the precursor of UDP-xylose,[3~5] UDP-galacturonic acid[6,7] and UDP-iduronic acid.[8]

In animals, plants and microorganisms, UDPGA is formed by the NAD-linked dehydrogenation of UDPG.[9] The occurrence of UDPG dehydrogenase in microorganisms has already been presumed in some bacteria[10~13] and yeast,[14] since these organisms excrete acidic polysaccharides containing glucuronic acid. It was also demonstrated that *Bacillus licheniformis*[15] and *B. subtilis* var. *niger*[16] contained glucuronic acid as a component of cell walls in the form of teichuronic acid and that with the former species, a teichuronic acid-like substance was synthesized by the 30,000×*g* particulate fraction prepared from early logarithmic phase cells in the presence of UDPGA and UDPGalNAc.[17] However, there have been no descriptions of UDPG dehydrogenase of these organisms.

The authors investigated the distribution of UDPG dehydrogenase in *Bacillus* species and found the enzyme in several strains of *B. licheniformis*. Since these organisms scarcely decomposed both UDPG and UDPGA, it was concluded that the crude extract was suitable for the enzymatic preparation of UDPGA from UDPG.

Abbreviations used: UDPG, uridine diphosphate glucose; UDPGA, uridine diphosphate glucuronic acid; UDPGalNAc, uridine diphosphate *N*-acetylgalactosamine.

Materials and Methods

Microorganisms Microorganisms belonging to the genus *Bacillus* were obtained from the JFCC (The Japanese Federation of Culture Collection of Microorganisms).

Chemicals UDPGA was purchased from Sigma Chemical Co. UDPG was prepared according to the method of Tochikura *et al.*[18]

Cultivation method Microorganisms were cultivated in a liquid medium containing 10 g of glucose, 5 g of peptone, 2 g of yeast extract, 2 g of $(NH_4)_2SO_4$, 1.8 g of $Na_2HPO_4 \cdot 12H_2O$, 0.5 g of K_2SO_4, 0.2 g of $MgSO_4 \cdot 7H_2O$ and 2 mg of $FeSO_4 \cdot 7H_2O$ per liter. Two hundred ml of the medium in a 2 *l* flask was inoculated with 20 ml of a 20 hr culture and incubated at 30°C for 6 hr on a rotary shaker (200 rpm). The cells were harvested by centrifugation and put in 0.05 M phosphate buffer (pH 7.0) to make a 20% (w/v) cell suspension.

Preparation of enzyme solution The cell suspension (20%) was sonified in an ice bath at 20 KHz for 5 min with a Super Sonic Vibrator UR-150, Tomy Seiko Co., Ltd., followed by centrifugation at $10,000 \times g$ for 20 min. The supernatant solution was used in the experiment.

Assay of UDPG dehydrogenase UDPG dehydrogenase was assayed by determining the UDPGA formed from the UDPG. The reaction mixture contained 1.5 μmole of UDPG, 2 μmole of NAD, 25 μmole of glycine buffer (pH 9.5) and 0.1 ml of enzyme solution in a total volume of 0.25 ml. After incubation for 3 to 20 hr at 30°C, the reaction was stopped by the addition of 0.05 ml of 0.6 M perchloric acid. The resulting precipitate was centrifuged off and the formed UDPGA and residual UDPG in the supernatant were separated by paper electrophoresis under the conditions described in Fig. 1. After drying in an oven, the ultraviolet-absorbing spots corresponding to UDPGA and UDPG were cut out and extracted with 0.01 N HCl. The optical density at 262 nm was measured with a Spectrophotometer QV-50, Shimadzu Seisakusho Ltd. and the amounts of UDPGA and UDPG were calculated based on the molar absorption coefficient, $\varepsilon_{262}=10,000$.

Analytical methods Phosphorus was determined by the method of Fiske-SubbaRow.[19] Glucuronic acid was identified by paper chromatography[20] and the specific color reaction of Dische.[21,22] The amount of glucuronic acid was determined by the color reaction with carbazole-H_2SO_4[23] or naphthoresorcinol.[24]

Glucuronide formation UDPGA was examined in glucuronide formation with *o*-aminophenol according to the method of Dutton and Storey,[25] using the liver from a freshly killed mouse. A 10% (w/v) liver homogenate as a source of UDP-transglucuronylase was prepared with a Universal Homogenizer, Nihon Seiki Seisakusho Co. and submitted to the examination under the conditions described in Table 3.

Results and Discussion

Distribution of UDPG dehydrogenase in genus *Bacillus* As can be seen in Table 1, UDPG dehydrogenase was detected in strains of *B. licheniformis* and also distributed in several other species (*B. subtilis* var. *niger*, *B. megaterium* and *B. cereus*). Among the organisms tested, *B. licheniformis* IAM 11054 showed the highest yield of UDPGA through the reaction period. Figure 1 shows the formation of UDPGA from UDPG by a cell-free extract of *B. licheniformis* IAM 11054. It is noteworthy that most of the substrate (UDPG) was converted into the product (UDPGA) after 20 hr incubation with no significant decomposition of UDPG, UDPGA or NAD. On account of these results, *B. licheniformis* IAM 11054 was used for further studies.

Culture age had a remarkable influence on cell activity and the highest activity was obtained from cells cultivated for 6 hr. This relationship in *B. licheniformis* IAM 11054 is shown in Fig. 2. The cultivation time of 6 hr corresponded to the early to middle exponential phase.

Isolation and identification of the reaction product Two liters of the reaction mixture containing 10.8 mmole of UDPG, 1 mmole of NAD, 200 mmole of glycine buffer (pH 9.5), and 800 ml of enzyme solution prepared from *B. licheniformis* IAM 11054 were supplemented with toluene and incubated at 30°C. After 17 hr, the UDPGA formed was isolated according to the procedure shown in Fig. 3. The profile of ion exchange column chromatography using Dowex 1 (Cl^- form) is shown in Fig. 4. Substrate, product and

Table 1. Formation of UDPGA from UDPG by microorganisms belonging to the genus *Bacillus*.

Strains	UDPGA formed (μmole/ml) Reaction time (hr)		
	3	7	20
B. aneurinolyticus AKU 0201	0	0	0
B. firmus IFO 3330	0	0	0
B. natto AKU 0204			0
B. mesentericus var. *flavus* IFO 3028			0
B. subtilis IFO 3007	0	0	0
B. subtilis IFO 3026	0	0	0
B. subtilis IAM 1193			0
B. subtilis var. *aterrinus* AKU 0216	0	0	0
B. subtilis var. *niger* IFO 3214	0.68	1.44	3.08
B. sphaericus IFO 3525	0	0	0
B. brevis IFO 3331	0	0	0
B. pumilus IFO 3030	0	0	0
B. circulans IFO 3329	±	±	0
B. megaterium AHU 1240	±	0.48	1.08
B. cereus IFO 3132	0.92	1.12	0
B. polymyxa NIAH 474	±	±	±
B. licheniformis IAM 11054	2.60	4.10	5.30
B. licheniformis NIAH 157	2.44		4.32
B. licheniformis NIAH 215	0.88		1.68
B. licheniformis AHU 1531	2.44		3.80
B. licheniformis AHU 1532	1.88		3.28

byproducts were clearly separated under the conditions described. The product (UDPGA) was converted into its sodium salt by passing it through the cation exchanger, a Dowex 50W (Na^+ form) column and precipitated by adding four volumes of methanol together with two volumes of acetone. After repeated precipitation, the product was washed with acetone, followed by ether, and desiccated over $CaCl_2$. About 5 g of an amorphous white powder were obtained. This isolate was regarded as the trisodium salt of UDPGA.

The isolate showed the ultraviolet absorption spectrum characteristics of uridine nucleotide, and gave UMP on hydrolysis in 0.1 N HCl for 10 min at 100°C. As shown in Table 2, the results of the chemical analyses indicate that the isolate contains two atoms of phosphorus and one mole of glucuronic acid per one mole of uracil moiety, and one of the phosphorus atoms was acid-labile, which was liberated in 1 N H_2SO_4 at 100°C for 15 min. These facts support that the isolate is uridine diphosphate glucuronate bearing both a pyrophosphate bond and a sugar phosphate bond. The infrared absorption spectrum of the isolate is shown in Fig. 5.

Elemental analysis showed: C, 27.40; H, 3.27 and N, 4.21%. Calculation for the trisodium salt of UDPGA ($C_{15}H_{19}N_2O_{18}P_2Na_3 \cdot 2H_2O$) gave: C, 26.39; H, 3.37 and N, 4.11%. Both results are in good accordance.

The isolate was further investigated on its biological function, glucuronide formation. After incubation of the product with mouse liver homogenate in the presence of *o*-aminophenol, the formation of a conjugate, *o*-aminophenyl glucuronide, was confirmed by the

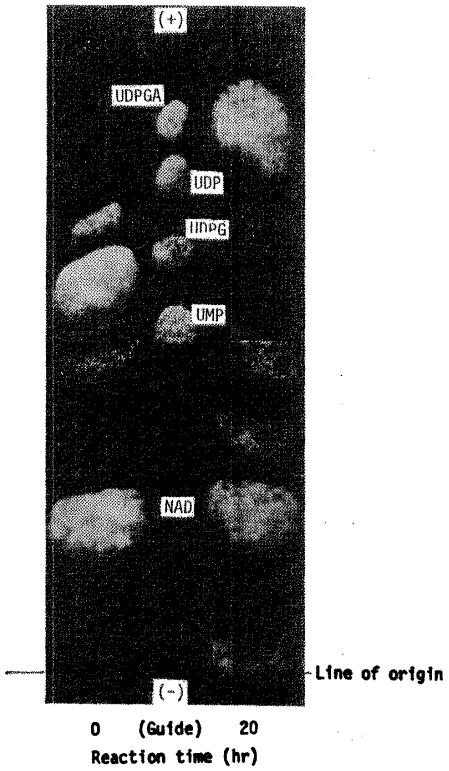

Fig. 1. Paper electrophoresis of reaction mixture. Paper electrophoresis was carried out for 90 min at 2.5 mA/cm using 0.1 M ammonium acetate buffer (pH 5.2).

characteristic pink-purple color after diazotization and coupling with *N*-1-naphthylenediamine dihydrochloride at pH 2.25~2.50. The results are shown in Table 3.

Effects of several factors on UDPGA formation Several factors affecting the formation of UDPGA were studied with a cell-free extract of *B. licheniformis* IAM 11054. Since UDPG dehydrogenase of this organism requires NAD as shown in Table 4, 8 or 16 mM of NAD was added to all the reactions except for the studies on NAD concentration.

1. *Time course* The reaction was attempted for 25 hr (Fig. 6). After 17 hr the reaction was stationary, and the maximal conversion rate of UDPG to UDPGA was approximately 87%.

2. *Enzyme concentration* As shown in Fig. 7, the reaction was proportional to enzyme concentration.

3. *Optimum temperature* The effect of temperature on the reaction was investigated. As shown in Fig. 8, the reaction was most accelerated at 40°C.

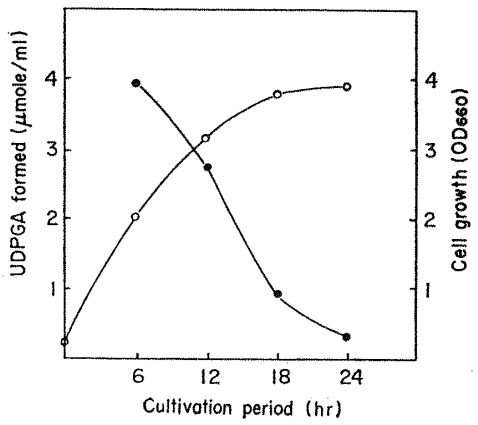

Fig. 2. Effect of culture age on the activity of UDPG dehydrogenase of *B. licheniformis* IAM 11054. Reaction time: 5 hr.
●, UDPGA formed; ○, Cell growth.

Reaction mixture 2 /
- pH was adjusted to 3 with HCl
- centrifuged

Supernatant
- uracil derivatives were adsorbed on charcoal
- stirred for 30 min

Charcoal
- suspended in 50% methanol containing 0.05% NH_4OH
- uracil derivatives were eluted from charcoal
- filtrated

Eluate
- concentrated *in vacuo* (to several hundred ml)

Chromatography on Dowex 1 (Cl^- form) column
- UDPGA was eluted with 0.01 N HCl containing 0.1 M NaCl after UDPG and UDP

UDPGA fraction
- UDPGA was adsorbed on charcoal, eluted and concentrated *in vacuo* to 250 ml

Dowex 50W (Na^+ form) column
- UDPGA·Na_3 was collected, concentrated, and precipitated from the mixture of four volumes of methanol and two volumes of acetone

UDPGA·Na_3

Fig. 3. Purification procedure of UDPGA from reaction mixture.

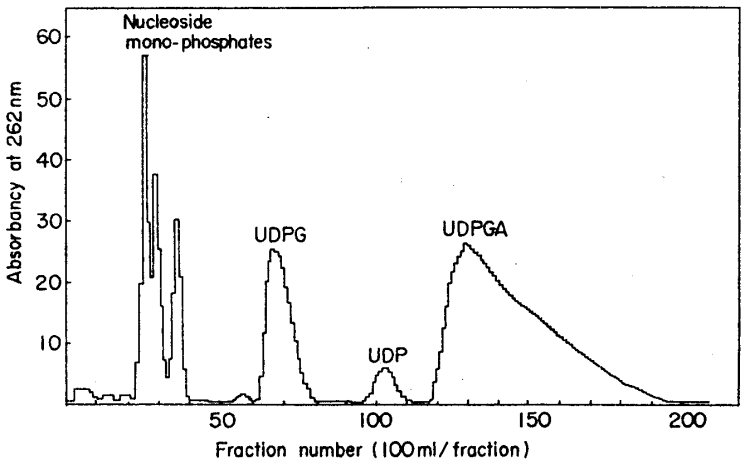

Fig. 4. Separation of UDPGA by ion exchange column chromatography.
Ion exchanger: Dowex 1 (×4), 200~400 mesh, Cl^- form.
Column: 4×40 cm. Eluant: 0.1 M NaCl-0.01 N HCl

Table 2. Analysis of isolated UDPGA.

	μmole/μmole uracil	
	Found	Calculated
Inorganic phosphate	0	0
Acid-labile phosphate	0.99	1.00
Organic phosphate	2.14	2.00
Glucuronic acid	1.02	1.00

4. *pH optimum* As shown in Fig. 9, the maximal activity was obtained at pH 9.5 with glycine buffer.

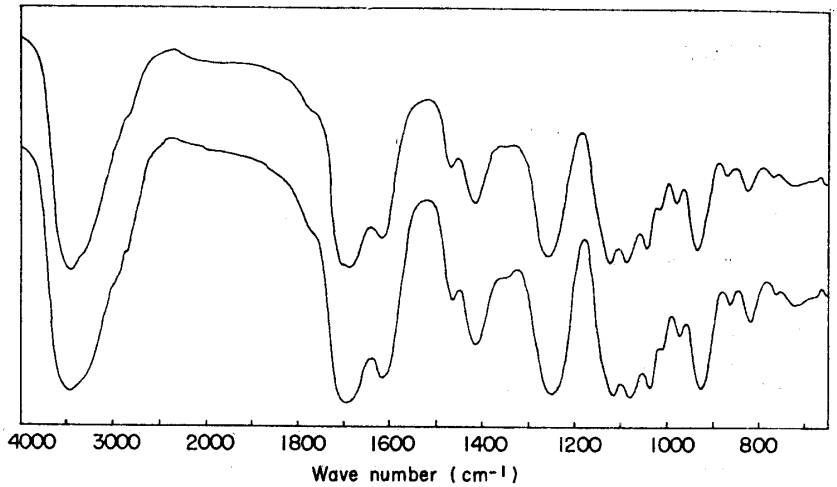

Fig. 5. Infrared spectra of isolate (upper) and authentic UDPGA (lower) (KBr method).

Table 3. Formation of *o*-aminophenyl glucuronide.

	Amount used (μg)	Conjugation activity (Absorbancy at 550 nm)
Isolated UDPGA	258	0.175
Authentic UDPGA	248	0.189
Authentic UDPG	2038	0

The reaction mixture containing UDPGA, 0.2 ml of 0.5 M Tris buffer (pH 7.4), 0.2 ml of 2.2 mM *o*-aminophenol and 1 ml of mouse liver homogenate in a total volume of 3 ml was shaken for 20 min at 37°C. Three ml of precipitant (a mixture of equal volumes of 2 M phosphate and 2 M trichloroacetate, each adjusted to pH 2.10) was then added. To 3 ml of the supernatant, 1 ml of 0.05% $NaNO_2$ was added, followed by 1 ml of 0.5% ammonium sulfamate, and by 1 ml of 0.1% naphthylethylenediamine dihydrochloride. The conjugate product, *o*-aminophenyl glucuronide, was detected as a pink-purple color after incubation at 25°C for 2 hr. The color was read at 550 nm.

Table 4. Specificity to cofactor of UDPG dehydrogenase of *B. licheniformis* IAM 11054.

Added cofactor	UDPGA formed (μmole/ml)
NAD	2.36
NADP	0.32
—	0.28

The concentrations of added cofactors were 8 mM and the reaction time was 3 hr.

5. *UDPG concentration* The effect of the concentration of UDPG toward formation of UDPGA is shown in Fig. 10. Although the maximal rate of conversion was shown to be around 8 to 12 mM, a more efficient concentration of the substrate seems to be 6 to 8 mM, because completion of the reaction at optimal concentration took a much longer

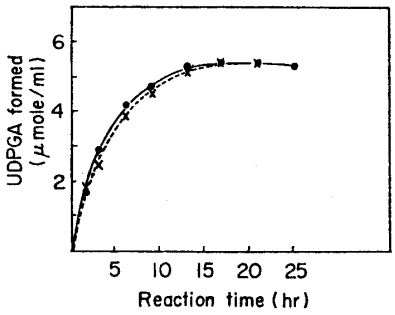

Fig. 6. Time course of reaction.
NAD concentration (mM): ×, 8; ●, 16.

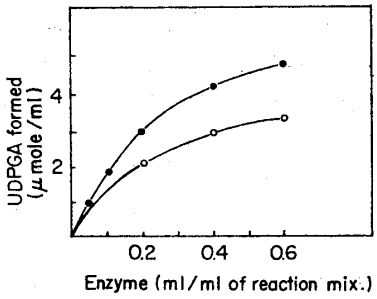

Fig. 7. Effect of enzyme concentration.
The concentration of NAD was 8 mM.
Reaction time (hr): ○, 3; ●, 5.

time than in the latter condition.

6. *NAD concentration* The cofactor of UDPG dehydrogenase was verified to be NAD and the oxidation of 1 mole of UDPG requires 2 mole of NAD theoretically according to the following equation. However,

$$\text{UDPG}+2\text{NAD}^{+}+\text{H}_2\text{O} \xrightarrow{\text{UDPG dehydrogenase}} \text{UDPGA}+2\text{NADH}+2\text{H}^{+}$$

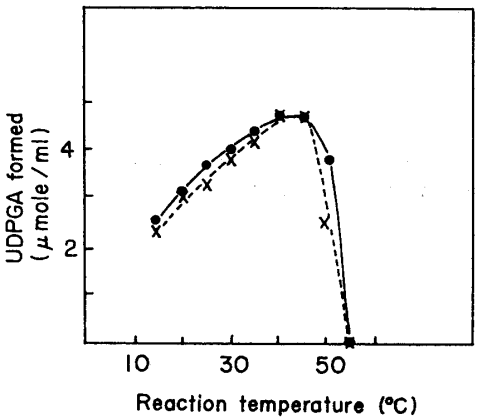

Fig. 8. Effect of reaction temperature.
Reaction time: 5 hr.
NAD concentration (mM): ×, 8; ●, 16.

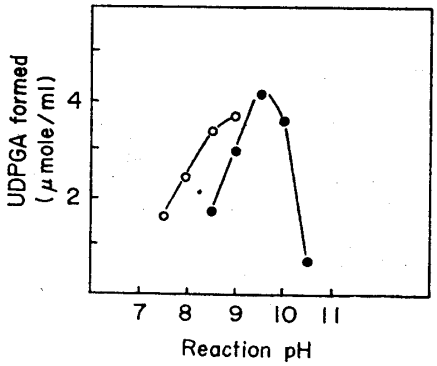

Fig. 9. Effect of reaction pH.
Reaction time: 5 hr.
Buffer: ●, Glycine buffer; ○, Tris buffer.

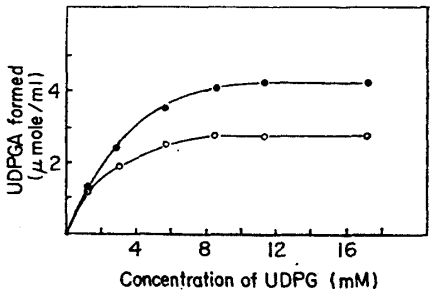

Fig. 10. Effect of concentration of UDPG.
The concentration of NAD was 8 mM.
Reaction time (hr): ○, 3; ●, 5.

using a crude enzyme preparation such as the sonicate, 0.5 mM of NAD which corresponds to one-twenty fourth of the concentration theoretically required was enough to dehydrogenate 6 mM of UDPG in 20 hr (Fig. 11). This result strongly suggested that the sonicate reoxidized NADH produced in the reaction mixture. Actually the uptake of oxygen was manometrically observed as shown in Fig. 12. NADH incubated with the sonicate of *B. licheniformis* IAM 11054 at pH 9.5 was stoichiometrically oxidized by atmospheric oxygen.

As the conclusion, the crude cell-free extract of *B. licheniformis* can be conveniently used for the production of UDPGA from UDPG since UDPG dehydrogenase in the extract is quite active and the oxidized form of the cofactor (NAD) is rapidly regenerated under aerobic conditions. It is also favorable for the completion of the reaction that both the substrate and the product are scarcely decomposed even on prolonged incubation.

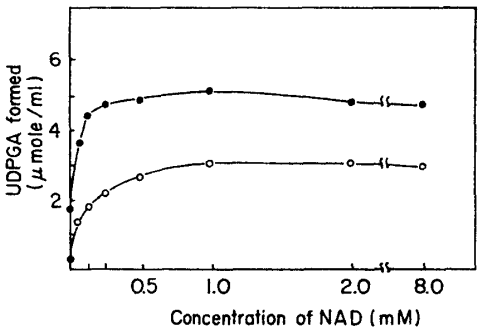

Fig. 11. Effect of concentration of NAD.
Reaction time (hr): ○, 3; ●, 20.

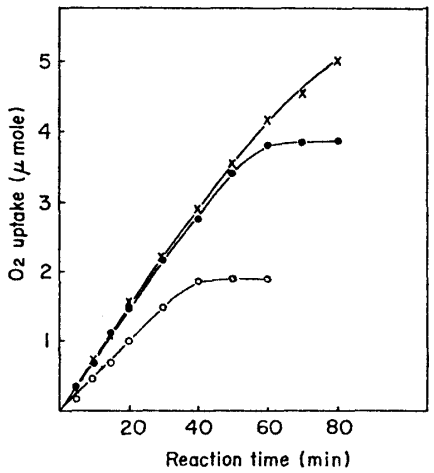

Fig. 12. Oxygen uptake resulting from oxidation of NADH.
The reaction mixture contained 250 μmole of glycine buffer (pH 9.5), 4~16 μmole of NADH, and 0.1 ml of enzyme solution in 2.4 ml. One-tenth ml of 20% KOH solution was placed in the center well of the vessel. The reaction was carried out at 30°C in a Warburg manometer.
NADH (μmole): ○, 4; ●, 8; ×, 16.

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