increase in fluorescence at 443 mμ (ref. 15) and it was found that the increase in fluorescence which occurred when yeast alcohol dehydrogenase was added to a solution of NADH was unaffected by the prior incubation of the enzyme with 2-bromo-2-phenylacetaldehyde.

This compound would therefore appear to be a potent substrate-directed inhibitor of yeast alcohol dehydrogenase acting by reaction with a sulphydryl group at the active centre of the enzyme.

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Studies on the thyroidal UDPG pyrophosphorylase: Partial purification and some of its properties

The existence of a UDPG pyrophosphorylase (EC 2.7.7.9) in thyroids was recently established[1]. It appears that this and other enzymes involved in the metabolism of sugar donors contribute to the biosynthesis of the carbohydrate moiety of thyroglobulin. In the present communication, a procedure for the partial purification of the enzyme, extracted from calf thyroids, is described. Furthermore, observations of influences exerted by magnesium, pyrophosphate and hydrogen ions on the reaction catalyzed by the purified enzyme will be reported.

All reagents and auxiliary enzymes were purchased from commercial source. Only the UDPG pyrophosphorylase, used as assay control, was prepared in this

laboratory in a partially purified form (until Step 4 of the original method[2]) from calf liver. Methods applied for the determination of the enzyme are given in the figures.

Purification. The thyroidal UDPG pyrophosphorylase was purified about

TABLE I

PURIFICATION PROCEDURE OF UDPG PYROPHOSPHORYLASE EXTRACTED FROM CALF THYROIDAL GLANDS

Purification step	*Vol. (ml)*	*Total activity (units*)*	*Proteins** (mg)*	*Specific activity*	*Yield (%)*
I. Aqueous extract	525	414	42 000	0.01	100
II. Heat step, $(NH_4)_2SO_4$, 30% satn., 55°, 4 min	367	220	7 340	0.03	53.1
III. $(NH_4)_2SO_4$ fraction, 30–65% satn.	64	230	3 840	0.06	55.5
IV. DEAE-cellulose column	350	140	98	1.42	33.8
V. $(NH_4)_2SO_4$ fraction	2.5	135	70	1.92	32.6

* 1 unit corresponds to that amount of enzyme which catalyzed the transformation of 1 μmole of substrate per min under the conditions of the method described in Fig. 1.

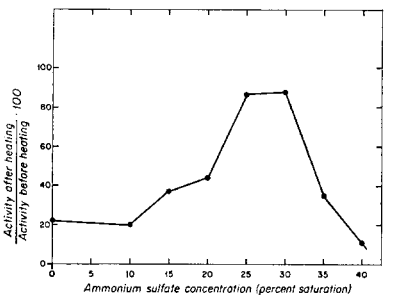
** Determined according to the method of LOWRY *et al.*[1].

200 times by a 5-step procedure (Table I). This was achieved mainly due to the relative heat stability of the enzyme in the presence of $(NH_4)_2SO_4$ (Fig. 1).

Calf thyroid glands collected at the slaughter house and immediately transported on ice to the laboratory were freed of connective tissue and fat. Stored at −15°, the material loses practically no enzymatic activity, at least not within five weeks.

The frozen tissue (220 g) was cut into small pieces and homogenized for 2 min in a Waring Blendor with 2 vol. of distilled water. The suspension was allowed to stand for 15 min and centrifuged at 13 200 × *g* for 20 min (Step I). Solid ammonium sulfate was added to the enzyme solution in order to obtain 30% satn. The resulting turbid solution was rapidly brought to a temperature of 55°, kept here during 4 min and then immediately cooled on ice (Step II). Enzyme activity was separated from the centrifuged solution by adding $(NH_4)_2SO_4$ to a concentration of 65% satn. The aquous solution of the precipitate was dialyzed for 2 h against 0.001 M phosphate buffer of pH 7.0 (Step III). Chromatography of the enzyme solution on a DEAE-cellulose column (3.5 cm × 37 cm), previously equilibrated with the same buffer, proved highly effective. The adsorbed enzyme was eluted by addition of NaCl to the buffer solution, at such a concentration that a linear gradient of 0.07–0.2 M was obtained. The peak enzymatic activity in the eluate (Fig. 2) was observed at a concentration of about 0.15 M NaCl (Step IV). The partially purified enzyme was concentrated by $(NH_4)_2SO_4$ precipitation (60% satn.) without loss of activity, but only little further purification was achieved during Step V.

Stability and interfering enzymes. The concentrated enzyme solution (28 mg/ml) kept at 4° proved to be stable, at least during 4 weeks of observation. Contrary to enzymes obtained from other sources, the thyroidal pyrophosphorylase had to be handled — during the extraction and purification steps — in neutral or slightly acidic solutions. Activity of UDPG 4-epimerase (EC 5.1.3.2), present in crude thyroidal



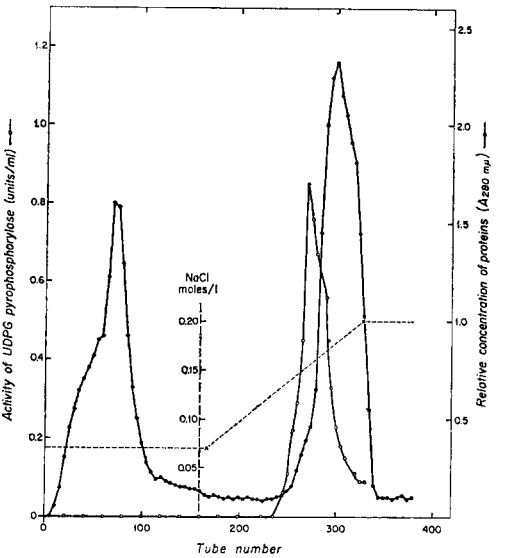


Fig. 1. Increased heat stability of thyroidal UDPG pyrophosphorylase in the presence of $(NH_4)_2SO_4$. After addition of $(NH_4)_2SO_4$ to the crude enzyme extract in the given amounts, the precipitate was separated by centrifugation. The supernatant solution was rapidly heated in a water bath to 55°, kept at this temperature during 4 min and then cooled on ice. Enzymatic activity was determined at 22° with aliquots of the enzyme solutions in a total volume of 1.0 ml solution containing 90 μmoles Tris–acetate (pH 7.8), 0.4 μmole of UDPG, 2.0 μmoles of $Na_4P_2O_7$, 1.0 μmole of magnesium acetate, 0.5 μmole of $NADP^+$, excess of phosphoglucomutase (20 μg) and glucose-6-phosphate dehydrogenase (7 μg) according to the method of MUNCH-PETERSEN[3].

Fig. 2. Column chromatography on DEAE-cellulose of partially purified UDPG pyrophosphorylase. Samples of 8 ml each at a flow rate of 1 ml/min were taken. Determination of enzymatic activity was performed according to the method described in Fig. 1.

extracts[1], was no longer detectable in the purified preparation. This enzyme appears to be eliminated by heat (Step II).

Concentration optimum for Mg^{2+} and $H_4P_2O_7$. Like other UDPG pyrophosphorylases[2,3,5–8], the thyroidal enzyme requires Mg^{2+} for its action. Maximum activity was observed in $4 \cdot 10^{-4}$ M Mg^{2+} concentrations. However, the thyroidal enzyme was not inhibited by higher concentrations of this ion as occurs with the pyrophosphorylases from yeast[3], peas[5], muscle[6], *Escherichia coli*[7] and mammary glands[8]. The study on the effect of increasing pyrophosphate concentrations on the enzymatic reaction taking place at pH 7.8 revealed that this substrate, in the range of $2 \cdot 10^{-3}$ to $1 \cdot 10^{-2}$ M, produced maximum activity. However, at higher pyrophosphate concentrations the reaction seems to be inhibited. Inhibition by excess of pyrophosphate had already been observed in studies on the pea enzyme[5].

Effect of pH. In the reaction which leads to UDPG formation, the thyroidal enzyme shows its maximum activity at about pH 8 (Fig. 3,b). The pH optimum for the formation of Glc-1-*P* observed in the reverse reaction (Fig. 3, a), seems to be shifted even more into the alkaline region. Also, it appears that the pH at which the onset of activity is observed differs, whether the reaction takes place in the forward or backward direction. Since the customary bell-shaped curve, generally obtained by plotting the enzyme-catalyzed reaction rate as a function of pH, is not observed

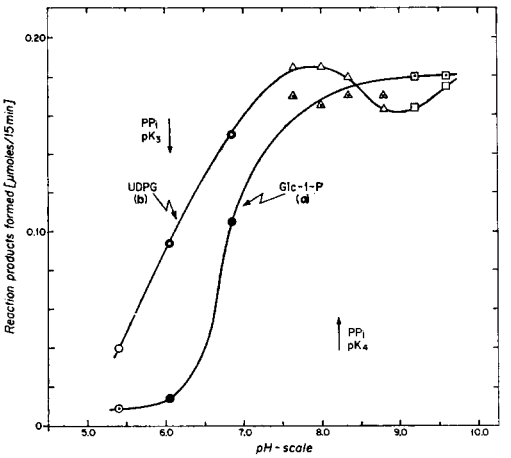


Fig. 3. Activity of the thyroidal UDPG pyrophosphorylase at different H^+ concentrations during the forward and back reactions. For the incubation experiments (37°, 15 min) buffer solutions (○—○, sodium acetate; ◎—◎, sodium maleate; △—△, Tris–acetate; □—□, glycine–NaOH) were used in amounts to obtain a final concn. of 0.09 M. (a) After incubation of UDPG (0.2 μmole) with $Na_4P_2O_7$ (0.5 μmole) in the presence of Mg^{2+} (0.5 μmole) and enzyme (22 μg or 0.037 unit) in a total volume of 0.5 ml, the Glc-1-*P* formed was determined by the method described in Fig. 1, using aliquots of the solutions obtained after arresting the enzymatic reaction by heating the samples in a boiling-water bath and then cooling on ice. (b) UTP (1.0 μmole) was incubated with Glc-1-*P* (0.5 μmole) under similar conditions. Determination of the UDPG formed was performed in 0.09 M glycine buffer (pH 8.65) measuring the reduction of added NAD^+ (1.0 μmole) catalyzed by UDPG dehydrogenase (0.1 mg).

in the present study, other effects besides those on prototropic groups at the active center of the enzyme protein must have been provoked by varying the hydrogen ion concentration. How the pyrophosphate ion, accepting different ionization stages according to the pH of the medium, may have affected the shape of the pH–activity curve needs to be studied more carefully.

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