VI. ALDOLASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN ACETONE-POWDER EXTRACTS OF *S. FAECALIS*

CHAPTER-VI

ALDOLASE AND GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE IN ACETONE-POWDER EXTRACTS OF S.FAECALIS.

A. Fructose-1, 6-Diphosphate Aldolase:

Introduction:

Fructose-1, 6-diphosphate aldolase (fructose-1, 6-diphosphate: D-glyceraldehyde-3-phosphate-lyase, EC.4.1.2.13) catalyzes the reversible cleavage of fructose-1, 6-diphosphate (FDP) to form glyceraldehyde-3-phosphate (GAP) and dihydroxy-acetone phosphate (DHAP). This enzyme is known to occur in all plants and animal tissues and in most microorganisms. It is reported to be absent in heterofermentative bacteria such as L. mesenteroids and in some aerobic organisms such as A. suboxidans (Horecker et al, 1972).

FDP-Aldolases are divided into two distinct classes - (i) Class-I and (ii) Class-II on the basis of their metal ion requirements and other properties (Rutter, 1964). Class-I aldolase requires no metal ions and occurs in animal and higher plants and is inactivated by reduction with $NaBH_4$; it is not inhibited by ethylenediamine-tetraacetate (EDTA). Class-II aldolases

require metal ions and are found in bacteria including S.faecalis and molds; they are inhibited by EDTA but not $NaBH_4$. Certain blue-green algae and bacteria such as Euglina gracilis, Chlamydomonas mundana and E.coli (Crookes' strain) contain both types of aldolases depending on growth conditions (Stribling and Perham, 1973).

Results:

Due to the lack of facilities for the preparation of large size culture, isolation and purification of aldolase from cell-extracts could not be carried out. Activities of aldolase and glyceraldehyde-3-phosphate dehydrogenase were studied in crude and dialysed acetone powder extracts.

Activity of FDP-aldolase was estimated by the modified method of Sibley and Lehninger (1949; Cf.Beck, 1957) as well as by estimating the released inorganic phosphate (Fiske and SubbaRow, 1925) after alkaline hydrolysis (Meyerhof and Lohman, 1934; Herbert et al, 1940; see also Beck, 1957).

In a preliminary experiment, it was observed that undialysed cell-extract was found to contain a good amount of aldolase activity. The activity in absence of Tris-HCl buffer (pH 8.0) was higher than that

observed in its presence. Activities obtained with undialysed extracts were found to vary considerably from day to day experiments. Beck (1957) had also observed that the choice of buffer may influence the result of aldolase activity. At pH 8.6, activity was reported to be higher in Tris-buffer than in collidine buffer, the reverse being the case at pH 7.4. On the other hand, in serum, aldolase activity was reported to be higher at pH 7.4 in Tris-buffer than in collidine buffer (Cook and Dounce, 1954).

S.faecalis extracts also, like serum, showed higher aldolase activity at pH 7.4 in Tris-buffer than at pH 8.6. Hence subsequent experiments were carried out at pH 7.4 using Tris-HCl buffer.

1. Effect of SRC-820·HCl on Aldolase Activity:

Different concentrations of SRC-820·HCl ranging from 6.7×10^{-4} M to 1×10^{-2} M were used. When aldolase assay was carried out by determining alkali labile inorganic phosphate by Fiske SubbaRow's method, it was found that SRC-820 interfered in the final colour development and hence, recourse to another method (Simmonsen et al, 1947) of estimation of inorganic phosphate, though less sensitive and more time-consuming, was taken. However, it should be pointed out that

SRC-820·HCl did not interfere in the dinitrophenyl (DNP) hydrazone method of Sibley and Lehninger (1949), which was also employed in this study.

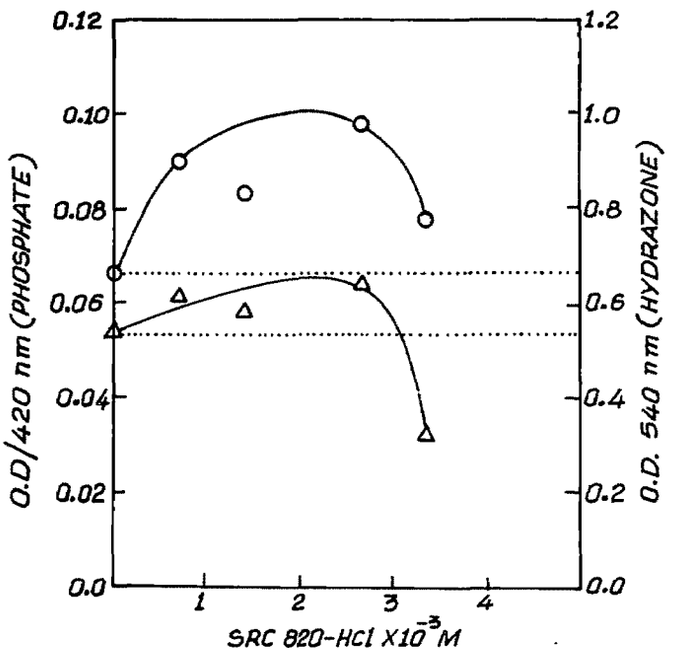
Estimations of both hydrazone and inorganic phosphate were carried out in aliquots of reaction mixtures. It was found that by inorganic phosphate estimation, SRC-820·HCl exerted a stimulatory effect on aldolase activity upto a level of 2.67×10^{-3} M (by the method of Simmonsen et al, 1947). Beyond this concentration it showed either less stimulation (phosphate estimation) or inhibition at the concentration of 3.3×10^{-3} M (hydrazone estimation) (Fig.11). However, it should be mentioned that with the hydrazone method, the effect of SRC-820 (at lower concentration) on this enzyme activity varied widely from day to day ranging from no stimulation to inhibition. Because of this, no definite conclusions as to the influence of SRC-820 could be reached.

2. Effect of Sodium Fluoride:

The effect of sodium fluoride on aldolase activity was also studied. It was found that NaF at a concentration of 1×10^{-2} M exerts a stimulatory effect to the extent of 35% (Fig.12).

Fig. 11

Effect of SRC-820 on Aldolase activity



Assay System:

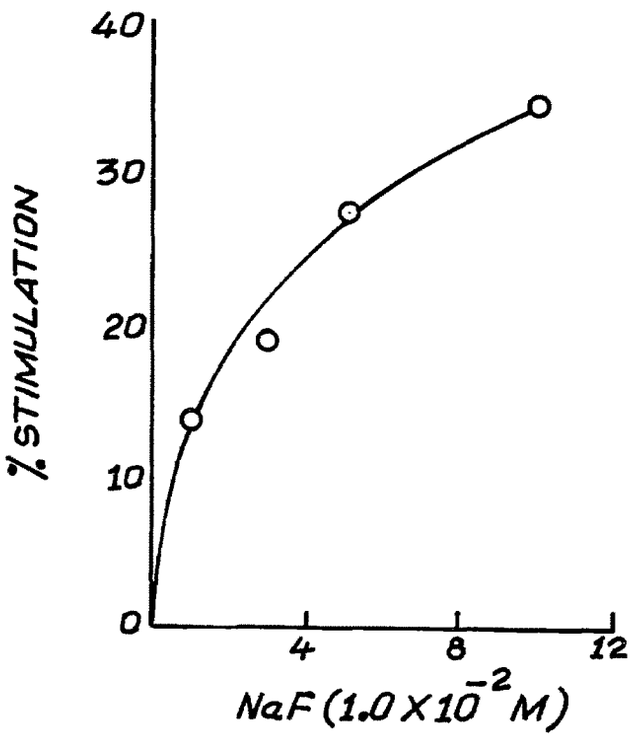
Reaction mixture contained in a total volume of 3 ml (in µmoles) : Tris buffer, pH 7.4, 100; Hydrazine-SO_4, pH 7.4; 55; FDP, 12.5; 24 hr. dialysed extract (sup.), 0.2 ml equivalent to 2 mg of acetone powder and SRC-820·HCl as shown in figure.

Reaction carried out at 37°C for 15 min and terminated by heat, 100°C for 3 min. 1 ml protein-free filtrate processed for phosphate estimation after alkaline hydrolysis (O——O) and also for DNP Hydrazone estimation (Δ——Δ).

FDP was added to the blanks after terminating the reaction.

Fig.12

Effect of sodium fluoride on Aldolase activity



Assay System:

Reaction mixture contained in a total volume of 3 ml (in μmoles) : Hydrazine-SO_4, pH 7.4, 55; FDP, 12.5, 24 hr. dialysed extracts (sup.), 0.2 ml equivalent to 2 mg of acetone powder and sodium fluoride as shown in figure.

Reaction (37°C for 15 min) started by addition of enzyme and terminated by addition of 2 ml of cold TCA (10% w/v). 1 ml of protein-free filtrate processed for DNP-Hydrazone estimation.

FDP was added to the blanks after terminating the reaction.

3. Effect of Divalent Metal Ions and EDTA:

Aldolase in S.faecalis was not found to be dependent on added divalent metal ions such as Fe^{2+} and Co^{2+} (effect of Co^{2+} not given in Table) but was found to be inhibited by EDTA. EDTA at a level of 2×10^{-5} M exerted about 30% inhibition (Table-XI).

B. Glyceraldehyde-3-Phosphate-Dehydrogenase:

Introduction:

In the previous chapter (Chapter-V "Experiments on S.faecalis Respiration"), it was shown that the inhibition of cell-respiration due to iodoacetate was reversed in presence of SRC-820. It was, therefore, thought of interest to study this effect in more detail with cell-free extracts. Iodoacetate is known to act at the level of Glyceraldehyde-3-phosphate dehydrogenase (GAP-DH), (D-glyceraldehyde-3-phosphate: NAD^+ oxido-reductase (phosphorylating), EC.1.2.1.12).

GAP-DH catalyzes the oxidation of glyceraldehyde-3-phosphate in presence of phosphate and NAD and a high-energy phosphate compound 1,3-diphosphoglyceric acid is formed.

Results:

1. Activity of GAP-Dehydrogenase in acetone powder extracts of S.faecalis cells was followed using

TABLE-XI

EFFECT OF Fe^{2+} AND EDTA ON ALDOLASE ACTIVITY

Exp. No.	Additions	O.D. at 540 nm (Hydrazone Method)	Activation / Inhibition (%)
1.	Control	0.467[b]	-
2.	Control + Fe^{2+} (2 X 10^{-5} M)	0.493[a]	+ 5.6
3.	Control + EDTA(2 X 10^{-5} M)	0.315	- 32.5
4.	Control + EDTA(1 X 10^{-3} M)	0.063	- 86.5
5.	Control + EDTA(1 X 10^{-5} M)+ Fe^{2+} (2 X 10^{-5} M)	0.345	- 26.1
6.	Control + EDTA(2 X 10^{-5} M) + Fe^{2+} (2 X 10^{-5} M)	0.319	- 31.7
7.	Control + EDTA (4 X 10^{-5} M) + Fe^{2+} (2 X 10^{-5} M)	0.297	- 36.4

a - Mean of two experiments

b - Mean of five experiments

Reaction mixture in a total volume of 3 ml contained (in µmoles): Tris-HCl, pH 7.4, 100; Hydrazine-sulphate, 55; FDP, 12.5; dialysed acetone powder extracts (supernatant), 0.2 ml, equivalent to 2 mg of acetone powder. Fe and EDTA as shown in the table. Reaction stopped by cold 10% (w/v) TCA.

FDP was added to the blanks after terminating the reaction.

0.5 ml protein-free filtrate out of 5 ml taken for hydrozine estimation.

Fructose-1, 6-diphosphate (FDP) (to generate glyceraldehyde-3-phosphate) and by estimating the increase in absorption at 340 nm due to reduced NAD (Krebs, 1955).

(i) Requirement for L-cysteine:

GAP-Dehydrogenase activity of S.faecalis cell-extracts was also found to require L-cysteine (Cf.Krebs, 1955). Almost no activity could be seen in its absence. With 1.2 X 10^{-3} M L-cysteine, very high activity could be observed. At concentrations of L-cysteine higher than this, no further increase in activity could be seen.

(ii) Influence of Deoxycholate treatment of the Acetone-Powder extract.

When the acetone powder was extracted with buffer containing sodium-deoxycholate (0.1 % w/v, final concentration) there was a reduction in the O.D. of blanks. It was also observed that if the extraction with deoxycholate is carried out prior to dialysis, activity was completely lost.

(iii) Need for Preliminary Incubation:

As shown by Krebs (1955), in the case of yeast, in the present case also, preliminary incubation with NAD^+ and L-cysteine was found to be necessary for

maximum activity. Hence, a preliminary incubation of enzyme extracts with L-cysteine and NAD was carried out for 7 minutes.

Using different amounts of acetone powder extracts, it could also be shown that the activity increases with increase in the amount of extract. When 1 mg equivalent of acetone powder was present in extracts an increase of 0.8 in O.D. at 340 nm occurred by the end of 2 min.

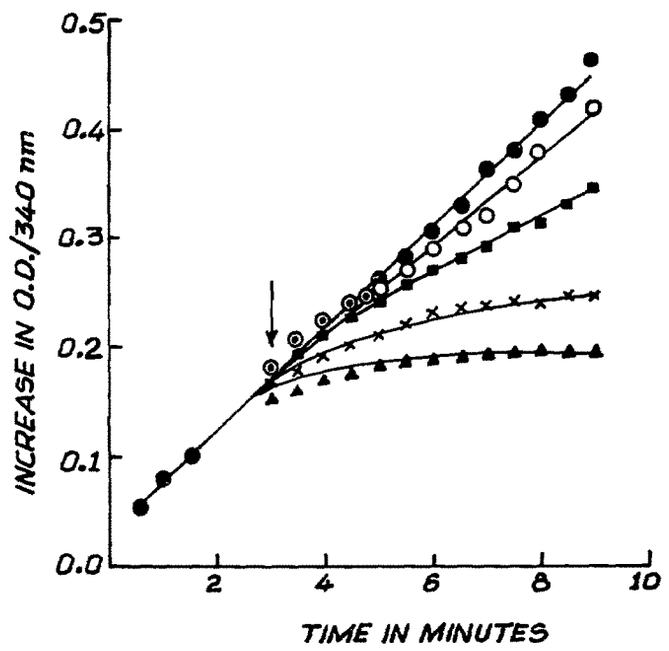
2. Effect of Iodoacetate:

In a preliminary experiment, it was observed that iodoacetate at a concentration of 3.3×10^{-4} M completely inhibited GAP-Dehydrogenase activity. Hence, the effect of lower concentrations of iodoacetate ranging from 8.3×10^{-6} M to 5×10^{-5} M was studied (Fig.13). Reaction was allowed to proceed without the inhibitor for 3 min and then iodoacetate was added. Increase in O.D. at 340 nm was followed every 30 seconds for a period of further 6 minutes.

As can be seen from Fig.13, the rate of increase in O.D. in control is linear upto 9 minutes showing an average rise of about 0.048 in O.D./min. In presence of the lowest concentration of iodoacetate used (8.3×10^{-6} M) the rate of activity was reduced to 0.032 O.D./min

Fig.13

Effect of Iodoacetate on Glyceraldehyde-3-phosphate dehydrogenase activity.



Assay System:

Reaction mixture contained in a total volume of 3 ml (in μmoles): Tris-buffer, pH 7.4, 20; disodium-arsenate, 17; NAD, 3; L-cysteine, 3.6; FDP, 12.5; 4 hr. dialysed and deoxycholate treated extract (sup.) in 0.002 M Tris-buffer, pH 7.4, 0.2 ml equivalent to 0.25 mg of acetone powder and the concentrations of iodoacetate as following:

●—● Control; O—O 0.83×10^{-5} M;
■—■ 1.67×10^{-5} M; X—X 3.3×10^{-5} M and
▲—▲ 5×10^{-5} M.

Cell_extract was pre-incubated with NAD and L-cysteine at 37°C for 7 min. Reaction started by addition of FDP.
↓ indicates time at which IAA added to the reaction.

(about 33% inhibition). Thus, inhibition increased with the amount of iodoacetate and reached 58% at a concentration of 5 X 10^{-5}M iodoacetate.

3. Effect of SRC-820·HCl:

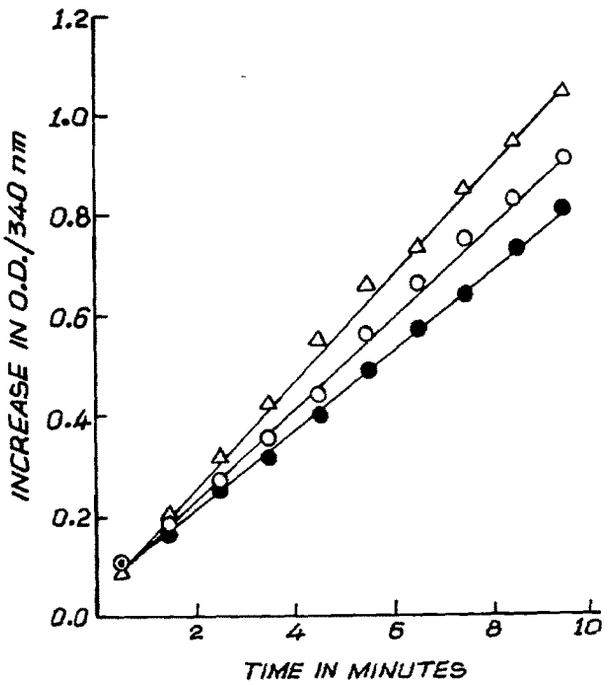
Effect of two different concentrations 6.6 X 10^{-3}M and 10 X 10^{-3}M of SRC-820·HCl on GAP-Dehydrogenase activity was studied. As can be seen from Fig.14, in presence of SRC-820·HCl, there is a stimulation in activity by about 10 to 30%. The extent of stimulation due to SRC-820·HCl was found to vary from day to day.

4. Effect of SRC-820·HCl and Iodoacetate on GAP-Dehydrogenase:

Next series of experiments were carried out to see the combined effect of SRC-820·HCl and iodoacetate on GAP-dehydrogenase activity. Final concentrations of iodoacetate and SRC-820·HCl were 1.25 X 10^{-5}M and 1 X 10^{-2}M, respectively. As can be seen from Fig.15, inhibition due to iodoacetate was found to be reversed in presence of SRC-820·HCl. It should be pointed out that the enzyme concentration in these experiments was so adjusted as to give an increase in O.D. of 0.06 at 6 min.

Fig. 14

Effect of SRC-820·HCl on Glyceraldehyde-3-phosphate dehydrogenase activity.



Assay System:

Reaction mixture contained in a total volume of 3 ml (in μmoles): Tris-buffer, pH 7.4, 20; disodium arsenate, 17; NAD, 3; L-cysteine, 3.6; FDP, 12.5; 4 hr. dialysed and deoxycholate treated extract (sup.) in 0.002 M Tris-buffer, pH 7.4, 0.2 ml equivalent to 0.5 mg of acetone powder and the concentrations of SRC-820·HCl as following:

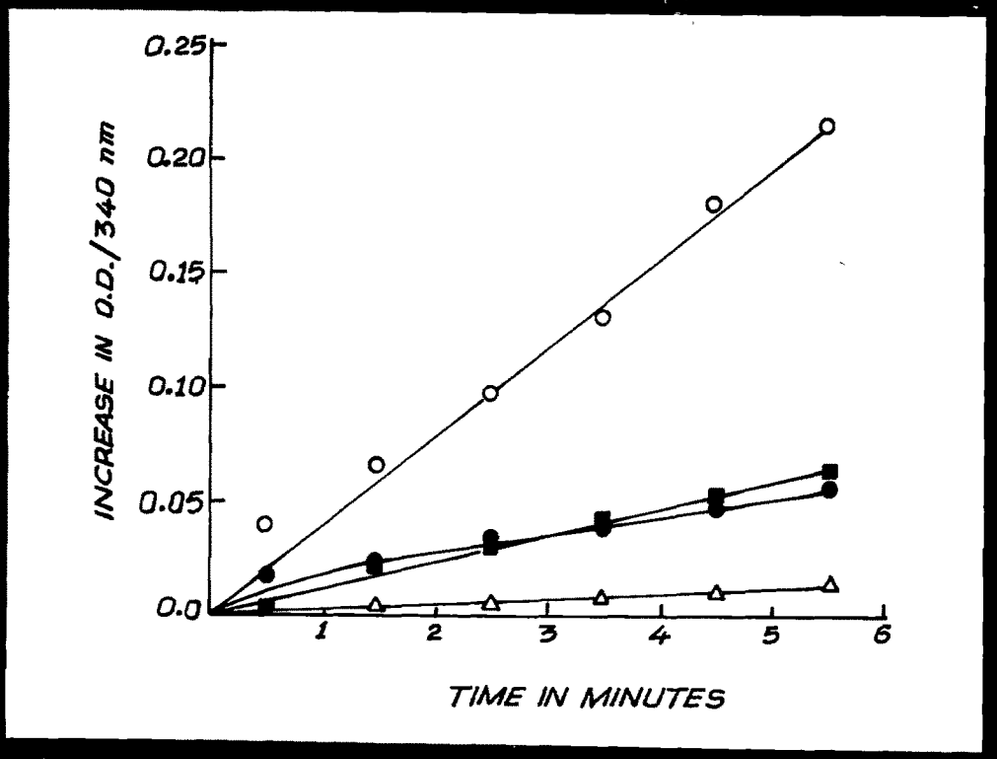
●—● Control; O—O 6.6×10^{-3} M and
△—△ 10×10^{-3} M SRC-820·HCl.

Cell extract was pre-incubated with NAD and L-cysteine at 37°C for 7 min.

Reaction started by addition of FDP.

Fig. 15

Effect of Iodoacetate and SRC-820·HCl on Glyceraldehyde-3-phosphate dehydrogenase.



Assay System:

Reaction mixture contained in a total volume of 3 ml (in µmoles): Tris-buffer, pH 7.4, 20; disodium arsenate, 17; NAD, 3; L-cysteine, 3.6; FDP, 12.5; 4 hr. dialysed and deoxycholate treated extract (sup.) in 0.002 M Tris-buffer, pH 7.4; 0.2 ml equivalent to 0.25 mg of acetone powder. Iodoacetate and SRC-820·HCl, when present, 0.0375 µmole and 30 µmoles, respectively.

Cell extract was pre-incubated with NAD and L-cysteine at 37°C for 7 min.

Reaction started by addition of FDP.

●—● Control; Δ—Δ With IAA; O—O With SRC-820·HCl and ■—■ With IAA + SRC-820·HCl

Similar experiments carried out on 3 different days using a slightly higher concentration of iodoacetate (1.67×10^{-5} M) showed the same effect.

5. Effect of Sodium Fluoride:

Sodium fluoride (1×10^{-2} M) was found to exert a stimulatory effect (about 40%) on the activity of GAP-dehydrogenase.

Stimulation of enzyme activity seen with SRC-820 and NaF may indicate that the effect may primarily be on aldolase activity, where the stimulatory effect of SRC-820 could not be conclusively demonstrated. The possibility that this effect may also occur at the level of GAP-dehydrogenase however, cannot be ruled out.

SUMMARY

1. Aldolase and Glyceraldehyde-3-phosphate (GAP) Dehydrogenase activities were demonstrated in acetone powder extracts of S. faecalis cells.
2. Sodium fluoride (10^{-2}M) stimulates aldolase activity by about 35%.
3. Inconclusive results as to the effect (inhibitory or stimulatory) of SRC-820 on aldolase activity, were obtained.
4. Aldolase of S. faecalis is not dependent on added divalent metal ions such as Fe^{2+} and Co^{2+} for its activity.
5. EDTA (2×10^{-5}M) inhibits aldolase activity by about 32%.
6. Prior incubation of cell-extract with L-cysteine protects GAP-Dehydrogenase activity from inhibition by iodoacetate.
7. Iodoacetate (5×10^{-5}M) inhibits GAP-Dehydrogenase activity by about 58%.
8. SRC-820·HCl (10^{-2}M) stimulates GAP-Dehydrogenase activity by about 30%.
9. Sodium fluoride (10^{-2}M) also stimulates GAP-Dehydrogenase activity by about 40%.
10. Inhibition of GAP-Dehydrogenase activity by iodoacetate can be reversed by SRC-820.