V. EXPERIMENTS ON S.FAECALIS RESPIRATION

CHAPTER V

EXPERIMENTS ON S.FAECALIS RESPIRATION

It has been demonstrated that methaqualone and SRC-820 inhibit the growth and acid production in S.faecalis at the early stages (see Chapter-IV, Growth Experiments). In the following experiments, the effect of SRC-820 and unsubstituted quinazolinone on the respiration of washed cell-suspensions of S.faecalis-R was studied using glucose and fructose as substrates.

Several substances including glucose and fructose were used as substrates. Among them, when pyruvate either alone or with citric acid cycle intermediates or with yeast extracts was employed as substrate, little oxygen consumption could be seen. The cell suspensions, it was found, were able to utilize L-cysteine as substrate when used at a concentration of 5×10^{-3}M (oxygen consumption 54 µl/ml cell suspension). Oxygen uptake by the cells was found to be more with glucose or fructose as substrate than that observed with L-cysteine. Oxygen consumption was 2 times more with fructose than that observed with glucose. As compared to glucose as substrates, 73 µl of oxygen uptake was seen with the same concentration of fructose under identical conditions. It was seen that in the case of

both these substrates, little oxygen consumption took place beyond 20 minutes.

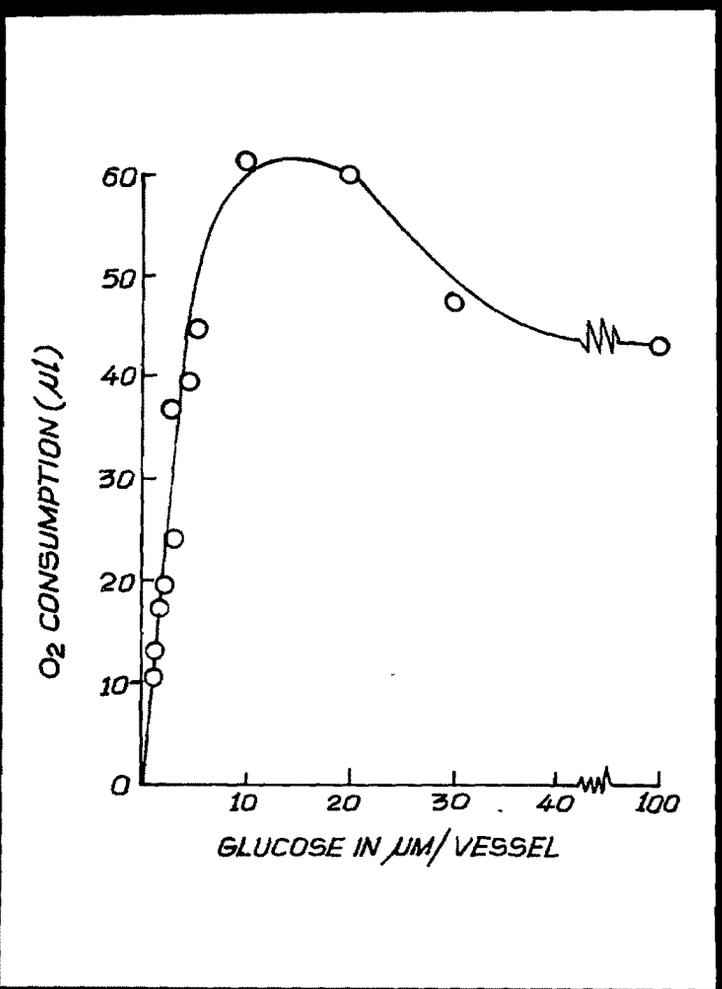
A.1 Effect of various concentrations of glucose and fructose on Cell Respiration:

In the next experiment, different concentrations of glucose ranging from 1 μM to 100 μM per reaction vessel was employed. Results are presented in Fig.6. As can be seen from Fig.6 a linear rate of oxygen uptake is observed upto a level of 10 μM of substrate per vessel. Beyond this substrate concentration, the oxygen uptake was not linear. At substrate concentrations higher than 10 μM, oxygen uptake was found to be low. Hence in subsequent experiments, the final concentration of glucose was kept at a level of 3.3 μM per reaction vessel to ensure a linear oxygen uptake, during the time of experiment.

A similar experiment was carried out using different concentrations of fructose. Concentrations of fructose ranged from 1 μM to 8 μM per reaction vessel, and the oxygen uptake is linear with increase in concentration of fructose upto 8 μM/vessel. At 3.3 μM/vessel of fructose, about 73 μl of total oxygen consumption was obtained. When the amount of fructose was doubled (6.6 μM/vessel) the oxygen uptake per hour was found to be about 137 μl. Unlike glucose, with fructose, no

Fig.6

Effect of various concentrations of glucose on S.faecalis respiration.



Assay System:

Reaction vessels contained in a total volume of 3 ml, Ca^{2+}-free Krebs Ringer phosphate, pH 6.6; concentrations of glucose as shown in the fig. and cell suspension, 0.5 ml.

inhibition in oxygen uptake due to increased substrate concentration was seen.

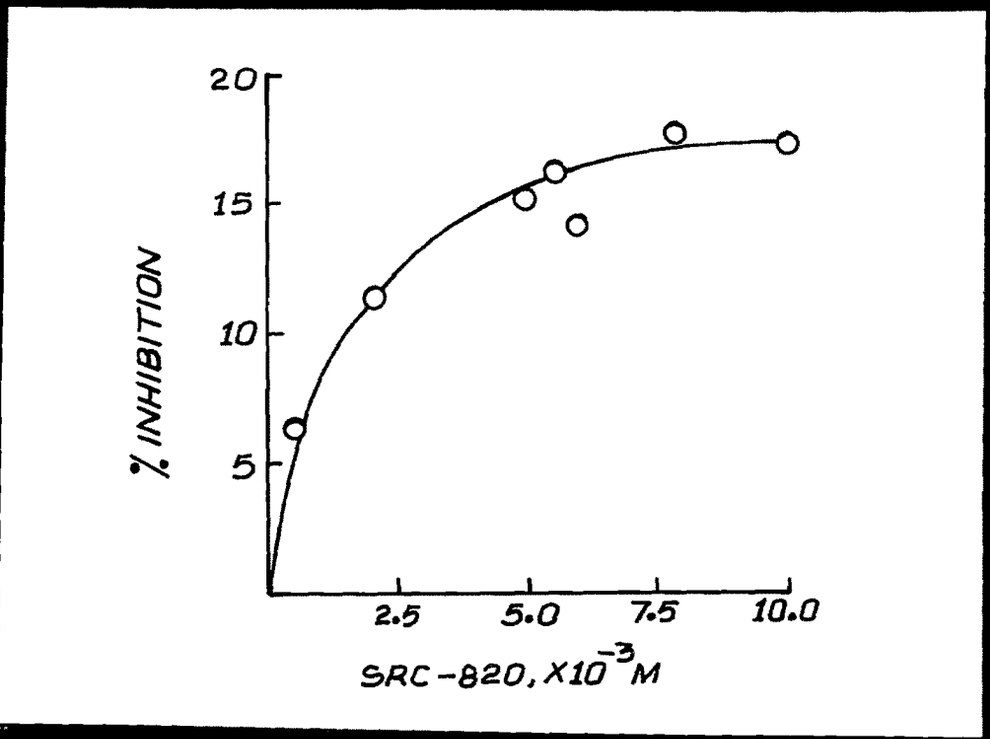
A.2 Effect of SRC-820 on Cell Respiration using glucose as substrate:

At ø the outset, the study of the effect of SRC-820 on cell-respiration was beset with difficulties due to its poor solubility in water and many inert solvents. Out of the various solvents tried (propylene-glycol, phosphate buffer, ethanol and glycerol), ethyl alcohol (10% v/v or 25% v/v when higher concentrations of SRC-820 were used) was found suitable. Ethyl alcohol at this level (10% v/v) inhibited cell-respiration by about 18% (Westhead and Malmstrom, 1957) and thus, this also proved to be unsatisfactory. Finally, the hydrochloride salt of SRC-820 was prepared and the solubility of this in water, though less than in ethanol, was satisfactory.

Different concentrations of SRC-820·HCl were used ranging from 0.55 X 10^{-3}M to 10 X 10^{-3}M, keeping glucose concentration at a final concentration of 1.1 X 10^{-3}M and the effect of SRC-820·HCl on S.faecalis cell-respiration was studied (Fig.7). As can be seen from Fig.7, inhibition was found to be about 17% even when SRC-820·HCl was used at nearly 10 times the substrate concentration.

Fig.7

Effect of different concentrations of SRC-820·HCl on the respirationn of S.faecalis using glucose as substrate.



Assay System:

All reaction vessels contained in a total volume of 3 ml: Ca^{2+}-free Krebs Ringer phosphate, pH 6.6, (tripple strength), 1 ml; glucose (side arm), 3.3 µmoles; cell-suspension, 0.5 ml and SRC-820·HCl as shown in fig.

Oxygen consumption without inhibitor was 30 µl at 20 min.

A.3 Effect of Iodoacetate on S.faecalis Respiration:

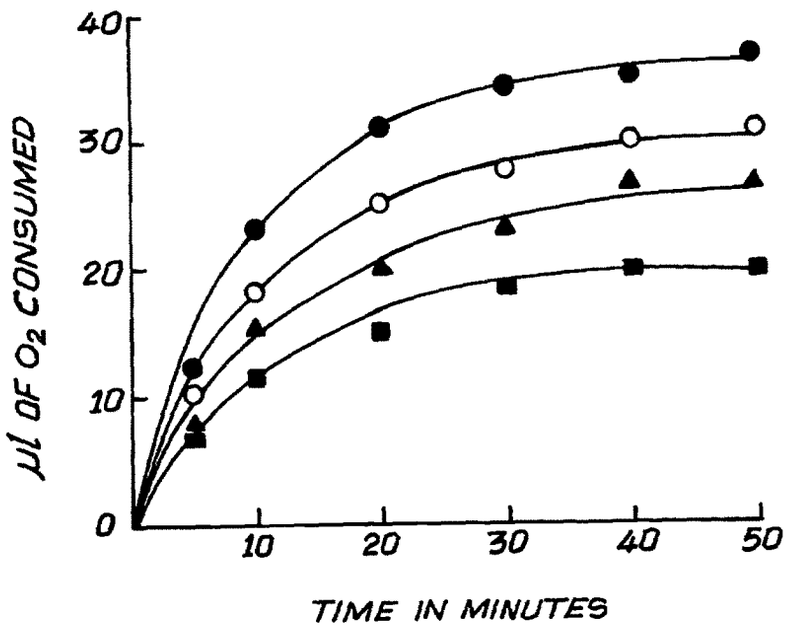
The next series of experiments were carried out to study the effect of iodoacetate on the respiration of S.faecalis. Iodoacetate at a level of 1.1×10^{-3}M completely inhibited the oxygen uptake. Various concentrations of iodoacetate ranging from 1.4×10^{-4}M to 2.3×10^{-4}M were used (Fig.8). As shown in Fig.8, inhibition in oxygen uptake increased with increase in concentration of iodoacetate. About 22% inhibition in oxygen uptake was seen with the lowest concentration of iodoacetate (1.4×10^{-4}M) and about 50% inhibition was seen at the highest iodoacetate concentration (2.3×10^{-4}M). In this experiment, iodoacetate was taken in the main compartment of the Warburg vessel along with the cells. Glucose was tipped in at 0 time.

A.4 Effect of SRC-820 and Iodoacetate on Respiration of S.faecalis.

i) Concentrations of iodoacetate and SRC-820 HCl were kept at 1.4×10^{-4}M and 1×10^{-2}M respectively. Cell-suspension, iodoacetate and/or SRC-820·HCl were taken in the main compartment with buffer. As can be seen from Fig.9 inhibition due to iodoacetate alone was about 37% which gets reduced to about 7% in presence of SRC-820·HCl while SRC-820·HCl alone showed about 15% inhibition.

Fig.8

Effect of Iodoacetate on the Respiration of S.faecalis.



Assay System:

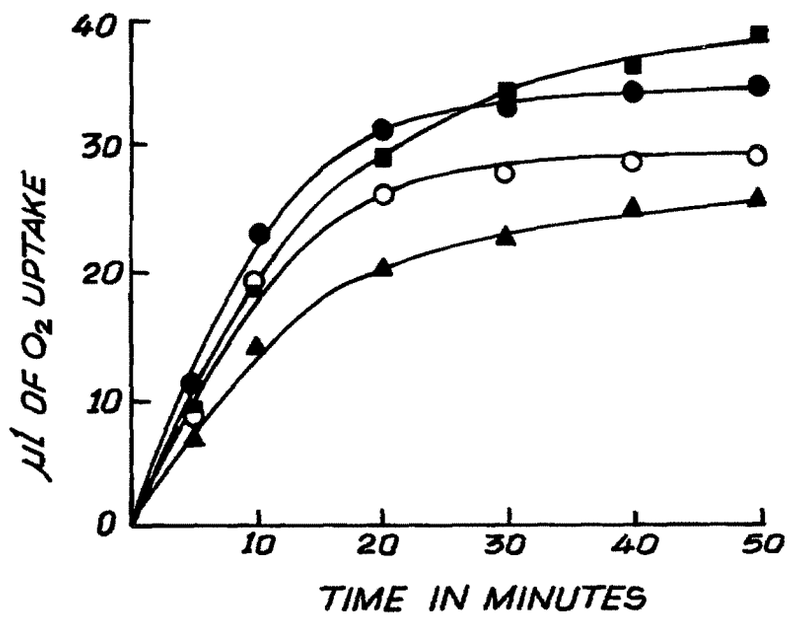
All reaction vessels contained in a total volume of 3 ml : Ca^{2+}-free Krebs Ringer phosphate, pH 6.6, (tripple strength), 1 ml; glucose, 3.3 µmoles (side arm) and cell-suspension, 0.5 ml.

Test vessels contained, in addition, different concentrations of iodoacetate (IAA).

●—● Control; ○—○ , ▲—▲ and ■—■ represent 1.4 X 10^{-4}M, 1.7 X 10^{-4}M and 2.3 X 10^{-4}M IAA respectively.

Fig. 9

Effect of SRC-820·HCl on Iodoacetate inhibition.



Assay System:

All reaction vessels contained in a total volume of 3 ml : Ca^{2+}-free Krebs Ringer phosphate, pH 6.6 (tripple strength), 1 ml; glucose, 3.3 µmoles (side arm) and cell-suspension, 0.5 ml.

Test vessels contained, in addition, iodoacetate, 0.43 µmole and SRC-820·HCl, 30 µmoles.

●—● Control (6); O—O With SRC-820 (6);
▲—▲ With IAA (3) and ■—■ With IAA + SRC-820 (5).

Figures in parentheses represent number of experiments.

ii) In the next experiment, effect of lower concentrations of SRC-820·HCl were studied, on inhibition due to iodoacetate. Concentrations of SRC-820·HCl ranged from 1.67 X 10^{-3}M to 6.6 X 10^{-3}M. It was seen that inhibition due to iodoacetate was found to be completely reversed, when SRC-820·HCl at a level of 6.6 X 10^{-3}M was also present. It must be noted that in the absence of iodoacetate, SRC-820·HCl did not exert any appreciable inhibition.

In the preceeding experiment, it was shown that the inhibition due to iodoacetate was reversed in presence of SRC-820·HCl. However, when iodoacetate was incubated with cells and SRC-820·HCl added later, no reversal in inhibition could be seen. The experimental conditions were kept the same as in the previous experiment (A.4-i).

iii) In another experiment, the concentration of SRC-820·HCl was reduced to the same level as that of iodoacetate i.e.; 1.4 X 10^{-4}M. No reversal of inhibition due to iodoacetate could be seen.

In the next experiment, less amount of iodoacetate (1 X 10^{-4}M) and the cells were incubated in the side arm and the effect of SRC-820·HCl at a concentration of 1 X 10^{-2}M was studied. It was seen, when iodoacetate alone was present (1 X 10^{-4}M), an inhibition of 55% was obtained.

This gets reduced to about 30% when both SRC-820·HCl and iodoacetate were present. Thus, a partial reversal of inhibition due to SRC-820 can be seen. This is discussed under "Discussion" (Chapter X).

In the next experiment, using the same experimental conditions (A.4-i) but with higher amounts of iodoacetate (now 0.7 μmole/3 ml of reaction mixture i.e., 2.3×10^{-4} M), similar results were obtained. However, with this high amount of iodoacetate, the extent of inhibition was also found to be high (about 42% inhibition was observed by iodoacetate alone which was reduced to about 17% in presence of SRC-820·HCl (1×10^{-2} M). Equimolar concentrations of SRC-820·HCl (2.3×10^{-4} M) and iodoacetate did not show such a reduction in inhibition due to iodoacetate.

Since there was a possibility that iodoacetate used in these experiments might have contained some free iodine and the phenomenon observed may be a combined effect exerted by these two substances, hence iodoacetate was treated with benzene (see "Methods and Materials") and the iodine-free iodoacetate, thus obtained, was used in the following experiments.

The effect of iodine-free iodoacetate either alone or with SRC-820·HCl on cell respiration was studied. The results are presented in Table-IV. As can be seen from Table-IV, treatment with benzene did not affect the

TABLE-IV

COMPARATIVE EFFECTS OF BENZENE-TREATED AND UNTREATED IODOACETATE ON S.FAECALIS RESPIRATION

Additions	µl. of O_2 consumed in 20min.	Inhibition (%)
1. None	26.8	-
2. + IAA	16.7	37
3. + IAA + SRC-820·HCl	22.1	18
4. + Benzene-treated IAA	16.9	37
5. + Benzene-treated IAA + SRC-820·HCl	24.0	10

Each Warburg vessel contained (in µmoles) in 3 ml, Krebs Ringer phosphate, pH 6.6; glucose, 3.3; benzene extracted or unextracted iodoacetate, 0.7; and SRC-820·HCl, 30, when present, and 0.5 ml cell-suspension in side arm.

Reaction started by tipping in cells at 0 min.

extent of inhibition or its reversal by SRC-820. The subsequent experiments were carried out using benzene extracted iodoacetate.

A5. Effect of Quinazoline-4-one·HCl and Iodoacetate on Respiration of S.faecalis:

In subsequent experiment, the effect of quinazoline-4-one·HCl (1 X 10^{-2}M) (QZ) on inhibition due to iodoacetate was studied. In this experiment, both the inhibitors (IAA and QZ) were added at the same time. Results are presented in Table-V. As can be seen from Table-V, QZ alone at 1 X 10^{-2}M concentration exerted about 18% inhibition similar to the action of SRC-820·HCl but did not reverse the inhibition due to iodoacetate.

When cells were first treated with QZ and then iodoacetate and glucose were tipped in at 0 time, about 15% reversal in inhibition was seen (Table-VI). In another experiment, when cells were first treated with iodoacetate (2.3 X 10^{-3}M) in a small volume and then tipped in the main compartment containing QZ, almost no reversal in inhibition occured. Under these conditions, iodoacetate alone exerted a strong inhibition (97%) (not shown in the Table).

TABLE-V

EFFECT OF QUINAZOLINE-4-ONE·HCl AND IODOACETATE ON THE RESPIRATION OF S.FAECALIS

Additions	µl of O_2 consumed in 20 min.	Inhibition (%)
1. None (3)	27.2	-
2. + Iodoacetate (4)	20.9	23.2
3. + QZ (3)	23.7	13.0
4. + Iodoacetate + QZ (6)	20.8	23.5

Figures in parentheses represent number of experiments.

Each Warburg vessel contained (in µmoles) 3 ml of Krebs Ringer phosphate, pH 6.6; glucose 3.3; iodoacetate, 0.7; Quinazoline-4-one·HCl, 30 and 0.5 ml of cell-suspension in side arm.

Reaction started by tipping in the cells at 0 minute.

TABLE-VI

EFFECT OF QUINAZOLINE-4-ONE AND IODOACETATE ON *S. FAECALIS* RESPIRATION (CELLS TREATED WITH QZ.HCl)

Time (min)	µl of O_2 consumed			
	Control glucose (1.1×10^{-3} M)	Glucose + IAA	Glucose + QZ·HCl	Glucose + IAA + QZ·HCl*
5	14.5	10.1	14.0	13.0
10	22.3	15.8	18.6	19.0
20	33.5	21.4	25.6	26.6
30	36.8	24.8	26.8	30.2
40	37.9	25.9	27.9	32.6
50	40.1	28.2	29.1	34.9

* Mean of 2 experiments.

Each Warburg vessel contained (in µmoles) 3 ml of Krebs Ringer phosphate, pH 6.6; glucose 3.3; Iodoacetate, 0.7 (both in side arm), Quinazoline-4-one·HCl, 30 and 0.5 ml of cell suspension in the main compartment.

Reaction started by tipping in glucose + IAA at 0 min.

A.6 Effect of β-picoline and Iodoacetate on Respiration of S.faecalis cells.

Since SRC-820 also contains a β-picoline ring, the effect of β-picoline (1×10^{-2} M) was studied on the inhibition due to iodoacetate. Results are presented in Table-VII. As can be seen from Table-VII, β-picoline does not reverse the inhibition exerted by iodoacetate and also did not inhibit the oxygen uptake.

A.7 Effect of Sodium Fluoride on S.faecalis Respiration:

A few more experiments on the respiration of S.faecalis cells were carried out using the well known inhibitor, sodium fluoride. Sodium fluoride upto a concentration of 1.67×10^{-3} M exerted no inhibition on oxygen uptake. At higher concentrations ranging from 3.3×10^{-3} M to 1.67×10^{-2} M, it was observed, that a concentration of sodium fluoride equal to 13.3×10^{-3} M was required to give about 50% inhibition (Table-VIII). As can be seen from Table-VIII, in presence of sodium fluoride, very little oxygen uptake could be seen upto the first 10 minutes, beyond this time, the rate of oxygen uptake is faster and the total oxygen uptake reached nearer to the control value. There is, thus, a time lag till first 10 minutes. If percentage inhibition was calculated at 20 minutes, a high extent of inhibition (about 50%) could be seen.

TABLE-VII

EFFECT OF β-PICOLINE AND IODOACETATE ON S.FAECALIS RESPIRATION

Additions	µl of O_2 consumed in 20 min.	% Change from control
1. None (3)	29.0	-
2. + Iodoacetate (2)	19.7	-32.1
3. + β-picoline (3)	31.0	+ 6.9
4. + Iodoacetate + β-picoline (3)	20.4	-29.7

Figures in parentheses represent number of experiments.

Each Warburg vessel contained (in µmoles) 3 ml of Krebs Ringer phosphate, pH 6.6 : glucose, 3.3 and 0.5 ml cell-suspension in side arm. Iodoacetate, 0.7 and β-picoline, 30, when present.

Reaction started by tipping in cells at 0 min.

TABLE-VIII

EFFECT OF SODIUM FLUORIDE ON THE RESPIRATION OF S.FAECALIS

Time (min)	µl of O_2 consumed			
	Concentration of NaF X 10^{-3}M			
	0	3.3	8.3	13.3
5	4.5	1.1	+1.3	+2.2
10	16.7	14.7	2.5	1.1
20	26.8	28.2	22.8	13.3
30	31.2	28.2	27.8	27.8
40	33.5	30.3	29.1	31.1
50	34.6	31.5	31.6	31.1

Each Warburg vessel contained (in µmoles) 3 ml of Krebs Ringer phosphate, pH 6.6 : glucose, 3.3 and 0.5 ml of cell suspension in side arm, sodium fluoride, as shown in the Table.

Reaction started by tipping in cells at 0 min.

A8. Effect of SRC-820·HCl and Sodium Fluoride on Respiration of S.faecalis cells.

In the next experiment, the effect of sodium fluoride was studied in presence of SRC-820·HCl. For this the concentration of NaF and SRC-820·HCl taken were 13.3 X 10^{-3}M and 1 X 10^{-2}M respectively and cells were taken in the side arm. Concentration of glucose was taken at a level of 1.1 X 10^{-3}M. Results are presented in Fig.10. As can be seen from Fig.10, sodium fluoride alone inhibited the oxygen uptake by about 47%. In presence of SRC-820, inhibition was found to be aboutn80%. The values shown in Fig.10 are mean of 5bexperiments carried out on different days.

When cells were allowed to remain in contact with sodium fluoride and SRC-820·HCl and then glucose was tipped in, very little oxygen uptake could be seen upto 20 minutes. By the end of 50 minutes, however, oxygen consumption in presence of both these inhibitors was found to be about 60% of the control.

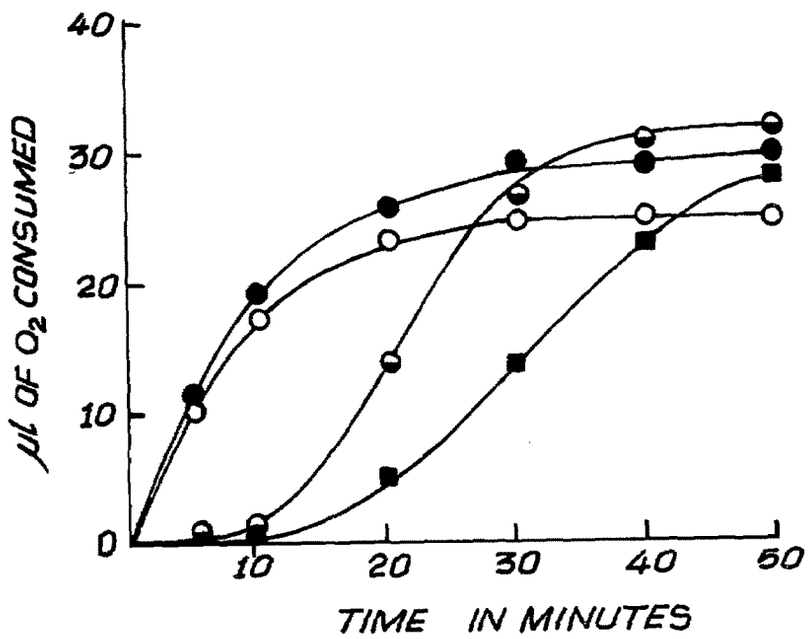
When the concentration of glucose was increased three times (3.3 X 10^{-3}M), the added effect of SRC-820·HCl was not seen (Table-IX).

B.Experiments using Fructose as Substrate:

1. Subsequent experiments on S.faecalis respiration were carried out using fructose (1.1 X 10^{-3}M) as

Fig.10

Effect of SRC-820·HCl and sodium fluoride on respiration of S.faecalis.



Assay System:

All reaction vessels contained in a total volume of 3 ml : Ca^{2+}-free Krebs Ringer phosphate, pH 6.6 (tripple strength), 1 ml; glucose, 3.3 µmoles and cell-suspension, 0.5 ml (side arm).

Test vessels contained, in addition, sodium fluoride, 40 µmoles and SRC-820·HCl, 30 umoles.

●—● Control(5); O—O With SRC-820·HCl (10); ⊖—⊖ With sodium fluoride (9) and ■—■ With SRC-820·HCl + sodium fluoride (10).

Figures in parentheses represent number of experiments.

TABLE-IX

EFFECT OF SRC-820·HCl AND SODIUM FLUORIDE ON RESPIRATION OF S.FAECALIS

Time (min)	µl of O_2 consumed			
	Control glucose (3.3×10^{-3}M)	Glucose + NaF (1.0×10^{-2}M)	Glucose + SRC-820·HCl (1.0×10^{-2}M)	Glucose + NaF (1.0×10^{-2}M) + SRC-820·HCl (1.0×10^{-2}M)
5	5.1	+3.4	3.5	+5.6
10	20.2	+1.1	15.1	+5.6
20	44.2	5.6	39.5	3.4
30	-	15.6	47.7	13.4
40	54.4	27.8	52.3	25.7
50	58.1	35.6	54.7	39.0

Each Warburg vessel contained (in µmoles) 3 ml of Krebs Ringer phosphate, pH 6.6 : glucose, 10; sodium fluoride and SRC-820·HCl, when present, 30 each and 0.5 ml of cell suspension in side arm.

Reaction started by tipping in cells at 0 min.

substrate. SRC-820·HCl at 1 X 10^{-2}M inhibited oxygen uptake by about 11%. With higher amounts of fructose (2.2 X 10^{-3}M), no inhibition was seen a with SRC-820.

2. Effect of Iodoacetate:

In this experiment, concentration of fructose was kept at a level of 1.1 X 10^{-3}M and the effect of iodoacetate was studied on the respiration of S.faecalis. Iodoacetate at a concentration of 2.3 X 10^{-4}M inhibited the oxygen consumption by about 66% as compared to 42% observed with glucose as substrate.

3. Effect of SRC-820·HCl and Iodoacetate:

With fructose as substrate, the effect of SRC-820·HCl on inhibition due to iodoacetate was studied just as in the case of glucose. Concentrations of iodoacetate, SRC-820 and fructose were kept at 2.3 X 10^{-4}M, 1 X 10^{-2}M and 1.1 X 10^{-3}M respectively. Fructose was tipped in at 0 time. SRC-820·HCl alone does not inhibit and also does not reverse the inhibition due to iodoacetate. These results are quite different from those obtained with glucose as substrate. (Fig.9).

4. Effect of Quinazoline-4-one(QZ), β-picoline and Iodoacetate on Respiration of S.faecalis.

Just as in the case of glucose as substrate, the effect of quinazoline-4-one and β-picoline were

studied individually on inhibition due to iodoacetate. The concentrations of both of these inhibitors were kept at a level of 1 X 10^{-2} M and iodoacetate at 2.3 X 10^{-4} M. Results are presented in Table-X.

As can be seen from Table-X that iodoacetate alone showed about 59% inhibition which is not reversed either by unsubstituted quinazoline-4-one or β-picoline. Quinazoline-4-one alone showed almost no effect whereas β-picoline showed about 15-20% inhibition in oxygen uptake.

TABLE-X

EFFECT OF QUINAZOLINE-4-ONE, β-PICOLINE AND IODOACETATE ON THE RESPIRATION OF S.FAECALIS (FRUCTOSE AS SUBSTRATE)

Time (min)	µl of O_2 consumed					
	Control + fructose (1.1×10^{-3} M) n = 5	Fructose + IAA (2.3×10^{-4} M) n = 7	Fructose + QZ (1.0×10^{-2} M) n = 1	Fructose + β-picoline (1.0×10^{-2} M) n = 3	Fructose + IAA (2.3×10^{-4} M) + QZ (1.0×10^{-2} M) n = 3	Fructose + IAA (2.3×10^{-4} M) + β-picoline (1.0×10^{-2} M) n = 2
5	6.0	3.7	4.5	4.6	5.3	3.7
10	16.5	11.0	15.8	14.0	10.9	10.4
20	46.0	19.1	44.0	36.7	20.0	19.4
30	66.3	24.0	59.7	56.3	25.3	25.5
40	70.5	26.1	63.1	61.6	27.9	27.4
50	71.9	28.1	63.1	63.1	29.0	26.9

n = Number of experiments.

Each Warburg vessel contained (in µmoles) 3 ml of Krebs Ringer phosphate, pH 6.6: fructose, 3.3; and 0.5 ml of cell suspension in side arm. Iodoacetate (IAA), Quinazoline-4-one·HCl (QZ) and β-picoline when present, 0.7, 30 and 30 respectively.

Reaction started by tipping in the cells at 0 min.

S U M M A R Y

1. With glucose, fructose or L-cysteine as substrates, respiration in washed cell-suspension could be demonstrated.

2. Oxygen uptake by the cells is higher with glucose or fructose as substrates than with L-cysteine. Oxygen uptake with fructose is almost twice that obtained with glucose as substrate.

3. Substrate inhibition of oxygen uptake is shown with glucose whereas no such effect occurs with fructose.

4. SRC-820 (10^{-2} M) inhibits the oxygen uptake by about 17% (substrate:glucose). With fructose, the inhibition is slightly less (11%).

5. With glucose as substrate, iodoacetate (2.3×10^{-4} M) inhibits (about 42%) the respiration of *S. faecalis* while with fructose as substrate, about 66% inhibition is seen.

6. Inhibition of cell-respiration due to iodoacetate (1.4×10^{-4} M) can be overcome by SRC-820 (1×10^{-2} M or 6.6×10^{-3} M). With fructose (1.1×10^{-3} M) as the substrate, such reversal is not seen.

7. If iodoacetate (1.4×10^{-4} M) is incubated first with cells and SRC-820·HCl (1×10^{-2} M) added later, no reversal in inhibition can be seen but a partial reversal (about 15%) in inhibition can be seen under the identical conditions if a less amount of IAA (1×10^{-4} M) is employed.

8. Quinazoline-4-one·HCl (QZ) at 10^{-2} M inhibits (about 18%) the respiration of S.faecalis cells with glucose (1.1×10^{-3} M) as substrate but does not reverse the inhibition due to IAA. However, a partial reversal in inhibition (about 15%) can be seen if cells are first incubated with QZ.HCl (10^{-2} M).

9. β-picoline (10^{-2} M) neither inhibits nor reverses the inhibition due to iodoacetate.

10. Respiration of S.faecalis (substrate:fructose) is inhibited (15-20%) by β-picoline (10^{-2} M) but not by QZ.HCl (10^{-2} M). Under these conditions, inhibition due to IAA is reversed by either QZ.HCl or β-picoline (10^{-2} M).

11. Sodium fluoride (NaF) (1.3×10^{-2} M) inhibits the respiration of S.faecalis (substrate:glucose 1.1×10^{-3} M). With this inhibitor, there is a time lag of 10 minutes during which practically

no oxygen uptake is seen and then the rate of oxygen uptake is resumed at a faster rate.

12. SRC-820 (10^{-2} M) augments the effect of NaF. The added inhibitory effect of SRC-820 is abolished by increasing the concentration of glucose (3.3×10^{-3} M).