X. DISCUSSION

CHAPTER-X

D I S C U S S I O N

The present study was undertaken to find out what stages or steps of metabolism are affected by SRC-820, 2-methyl-3-(3'-methyl-2'-pyridyl)4(3H) quinazolinone, and compounds of similar structure. SRC-820 was synthesized and shown by another group of workers[1] to possess analgesic and tranquilizing properties. In the case of some other quinazoline-4-one derivatives, it was shown that they possess bacteriostatic properties (Baker et al, 1958; Hutchison, 1968; Muraveva et al, 1971 and Varma, 1975). It was felt that bacterial system will prove to be a simpler tool for finding out the exact step influenced by SRC-820, the compound under investigation.

Because of its having a pyrimidine ring substituted with alkyl and aryl radicals, it is possible that, like many other substituted pyrimidines - methotrexate, aminopterine (Seeger et al, 1949; Hutchings and Burchall, 1965), SRC-820 may have the potential to act as a folic acid antagonist (Albrecht and Hutchison,1970).

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Thus the folate requiring organism Streptococcus faecalis-R (ATCC-8043) was chosen for this study.

Before proceeding into metabolic studies, it was felt that a colourimetric method simpler than the available methods for estimating substituted quinazolinones such as methaqualone, should be devised. A method based on acid hydrolysis followed by diazotization and coupling with beta-naphthol was devised (Jani and Srinivasan, 1972). The method is described in detail in Chapter-III and is suitable for estimation of this compound in the range of 10 to 200 µg. Also, it was found that the increase in optical density (at 300 nm) subsequent to acid hydrolysis can be utilized for estimating smaller amounts of SRC-820 in the range of 2-16 µg. Such an increase in optical density at 300 nm after acid hydrolysis was not observed in the case of methaqualone.

After having successfully devised a method, the effect of SRC-820 and methaqualone on the growth of S. faecalis was investigated. The results (Chapter-IV) clearly indicated that both methaqualone and SRC-820 at a concentration of 5×10^{-3} M inhibited the early stages of growth of this organism (Jani and Srinivasan, 1977). Sometime after these results were published,

Seshavataram and Rao (1977) also reported, using an empirical method, the inhibition of bacterial growth by quinazolone derivatives. The growth inhibition study presented in this thesis not only takes into account of the amount of cells but also their metabolic activity in the form of lactic acid production. Both were found to be inhibited. The fact that methaqualone is more potent in inhibiting the growth and acid production than SRC-820 can be explained by the greater lability of SRC-820 in acid than methaqualone (Chapter-III).

An alternate explanation can be that the organism degrades SRC-820 but not methaqualone. In this context, it can be mentioned that some microorganisms such as Pseudomonas aeruginosa, Sarcina lutia and flavobacterium metabolize L-tryptophan through o-aminoacetophenone and subsequently to 4-methyl-2-alkyl quinazoline which can be further degraded by dioxygenase system present in these organisms. (Mann, 1967; see also Doelle, 1975). The conversion of 4-hydroxyquinazoline to 2,4-dihydroxyquinazoline is also reported to occur in some bacterial species as part of a degradative pathways (Grant and Al Najjar, 1975). It is therefore, not unlikely that S. faecalis also may possess the ability to breakdown or convert quinazolinone derivatives to simpler compounds. In animals, it was reported, 2,3-disubstituted quinazolinones are converted to N-↑ oxide derivatives and

excreted (Murata and Yamamoto, 1970). When unsubstituted quinazoline was employed as substrate for milk and rabbit liver xanthine oxidase in *in vitro* experiments, 4-hydroxyquinazolone was found to be one of the products (McCormack, 1978; Cf.Krenitsky *et al*, 1972).

It is possible, therefore, that the organism degrades SRC-820 but not methaqualone, as seen by gradual reduction in the extent of inhibition at later stages of growth. Apparently, the substitution of quinazolinone ring with beta-methylpyridine ring renders the compound susceptible to breakdown by acids as well as by cellular enzymes.

Inhibition of growth of *S.faecalis* by SRC-820 can be reversed by addition of liver extract (source of folic acid) and shows the potential of this compound to act as a folic acid antagonist. Several disubstituted quinazolinone derivatives other than SRC-820 were tested by a group of workers against *S.faecalis* and were found to act as folate antagonist (Lespagnol *et al*, 1973), but these compounds were much less active as compared to methotrexate. The search for more effective quinazoline-antifolates is a continuing one and a vast literature is available (Montgmomery and Wood, 1964; Hutchison, 1968 and Elslager *et al*, 1972). However, it will be necessary to carry out further studies with SRC-820 to confirm its antifolate action.

In the next stage of investigation, washed cell suspensions were used to study the effect of SRC-820 on S. faecalis respiration. The results obtained by using glucose as substrate (Chapter-V), show that at concentrations higher than 3.3×10^{-3} M respiration is inhibited (Barker et al, 1964). With fructose as substrate, a similar effect could not be shown. This may be due to higher Km for fructose for phosphorylation by hexokinase (Axelrod, 1967). It should be pointed out that, beyond a concentration of 2.67×10^{-3} M, higher concentrations of fructose were not tested.

With either of the substrates, SRC-820 was shown to inhibit respiration although the inhibition of fructose respiration was slightly less than that found in the case of glucose.

Inhibitory effect of iodoacetate and sodium fluoride was also demonstrated using cell suspension. Inhibitory effect of iodoacetate is reduced when SRC-820 was also present in the medium. Such reduction in inhibition in presence of SRC-820 could not be demonstrated when fructose was the substrate. Although no definite explanation can be given for this, it is well known that the entry of fructose into the cell is mediated by a phosphoenolpyruvate : D-fructose-1-

phosphotransferase system (Fraenkel, 1968; Hansen and Anderson, 1968; see also Thompson, 1978) yielding fructose-1-phosphate. The latter substance is converted to fructose-1, 6-diphosphate by another inducible enzyme D-fructose-1-phosphatekinase (Hanson and Anderson, 1966). The lack of reversal of inhibition due to iodoacetate by SRC-820 may be due to its inhibitory effect on the above two reactions. This will result in less FDP formation and in turn less formation of GAP. In the light of the results obtained with acetone powder extracts in latter experiments, this explanation appears to be more probable (Chapter-VI).

The cause for reduction in inhibition due to iodoacetate in presence of SRC-820 is perhaps due to the possibility of the latter substance undergoing alkylation, thus effectively reducing the amount of iodoacetate reacting with the -SH groups of the enzyme proteins. Of additional interest is the findings of Cho and Pitman (1974) and Pitman and Morris (1979) that the unsubstituted quinazoline can react with sulfhydral groups. SRC-820 may also compete with iodo-acetate for the -SH groups. This explanation appears all the more probable in the light of the results obtained by incubating the cells with iodoacetate at first and later adding SRC-820 when no reduction in

inhibition takes place (Chapter-V, Section A.4). It was also shown in this study that the parent compound (Quinazoline-4-one·HCl) inhibits glucose respiration to the same extent as that of SRC-820 while β-picoline, the aryl substituent, does not. The inhibitory action of SRC-820 is due to the quinazoline nucleus rather than to the substituents. The reverse is true in the case of fructose respiration viz. quinazoline-4-one·HCl does not inhibit whereas β-picoline does. Both these compounds do not lessen the iodoacetate inhibition of both glucose and fructose respiration. This may point to the existence of a different pathway for utilization of fructose in this organism.

Whereas SRC-820 reduces the iodoacetate effect on glucose respiration, in the case of fluoride, it augments the inhibitory effect. This can be explained on the basis of the chelating property of quinazolinone and its derivatives (Dave et al, 1969; Sanghavi and Pai, 1974; Pellizzi and Pellizzi, 1975; Battaglia et al, 1976). Like fluoride which acts at the enolase step probably forming a complex with magnesium and phosphate (Warburg and Christian, 1941; Cf. Malmström, 1961), SRC-820 may also form a complex with magnesium thus adding to the influence of fluoride. Such perhaps is

the case is shown in a latter study (Chapter-VII) where the inhibitory effect of SRC-820 is reversed by higher concentrations of magnesium.

Results obtained with acetone powder extracts of S. faecalis-R confirm the inferences arrived at in the respiration studies (Chapter-V). It is shown in this study that aldolase of S. faecalis-R belongs to Class-II, requiring metal ions for activity. Although, a definite requirement for Fe^{2+} or Co^{2+} could not be demonstrated, the fact that the enzyme is inhibited by EDTA shows a definite requirement for metal cations. The metal, in this case, is perhaps covalently bound to the enzyme and hence no stimulation due to externally added ions could be shown (Horecker et al, 1972). In this connection, it can be mentioned that even amongst the same group of organisms, FDP-aldolase of Lactobacillus casei, was shown to belong to Class-I (London, 1974). The occurrence of a Class-II aldolase in a related strain of S. faecalis was reported by the same investigator (London, 1974). In the present study, it was also shown by the use of different buffers, that aldolase of S. faecalis-R resembles serum aldolase (Beck, 1957) in having an optimum pH, near to 7.4.

Inconclusive results as to the effect of SRC-820 on aldolase can be explained as due to its interference in the method of estimation of phosphate or its lability in acids or alkalies. However, when the influence of this compound on GAP-dehydrogenase with fructose-1,6-diphosphate (FDP) as substrate was studied, a definite stimulatory effect was seen. (In a few experiments, a stimulatory effect of SRC-820 on aldolase was also seen). It looks as though, that SRC-820 may act as a stimulatory of aldolase rather than of GAP-dehydrogenase. It was also observed in aldolase experiments that fluoride acts as a stimulator of activity. Probably it brings about this action by removing some of the inhibitory ions present in the acetone powder extracts. EDTA, being a more powerful metal complexing agent, removes the favourable cations also, thus inhibiting the enzyme activity. A similar explanation can be offered, in the case of SRC-820 also, based on its complexing property (Cf. Dave et al, 1969; Malesani et al, 1972; Pelizzi and Pelizzi, 1975; Battaglia et al, 1976).

The well known inhibitory effect of iodoacetate on GAP-dehydrogenase is also shown in this study. It can be inferred that the active site of the enzyme in

S. faecalis-R contains sulfhydral group (Boyer and Segal, 1954). Results, identical to those obtained in respiration experiments, namely that the inhibition due to iodoacetate is reduced in presence of SRC-820, were also obtained with acetone powder extracts and a similar explanation is also applicable here. The observed stimulation in GAP-dehydrogenase activity by sodium fluoride can arise from its effect on aldolase, since the reaction (GAP-DH) was studied using FDP as substrate. But in the case of SRC-820, its stimulatory effect cannot be said to be confined to a single enzyme, either aldolase or GAP-dehydrogenase. It is more probable, however, that, because of its metal chelating property similar to that of fluoride, its action lies at the level of aldolase than at GAP-DH.

The inhibitory effects of sodium fluoride and SRC-820 on respiration are confirmed in the study on enolase in acetone powder extracts. Enolase of yeast, as well as 3-PGA mutase, requires Mg^{2+} for its activity (Grisolia, 1962). Enzymes of S. faecalis-R also do not differ from them. As expected, EDTA is found to be a powerful inhibitor of the reaction. Inhibition due to SRC-820, it is found, can be overcome by increasing the concentration of Mg^{2+} (Chapter-VII, Table-XII). The total abolition of respiration in the combined presence

of sodium fluoride and SRC-820 finds confirmation in the results of the enolase experiment, where the effect of both these compounds is observed to be additive. The reversal of inhibition due to SRC-820 by added Mg^{2+} indicates that similar to fluoride, SRC-820 also may form a complex with magnesium and phosphate.

The effect of SRC-820 at the level of GAP-dehydrogenase and enolase shows that its effect on carbohydrate metabolism is brought about due to its metal complexing property which may result in either the removal of inhibitory ions or the activating ones. Due to this property, stimulation of aldolase activity and inhibition of enolase activity result. In the case of aldolase, the action is one of removal of inhibitory ions and in the case of enolase, it is of reacting with ions required for activity. It can be speculated that the inhibition of glycolysis by the major Embden-Meyerhof Pathway results in the organism resorting to alternate pathways of utilization. That, such a possibility exists in S.faecalis, can be inferred from the gradual weakening of the inhibitory effect of SRC-820 on growth with time (Chapter-IV). Several reports (Gunsalus et al, 1955; Sokatch et al, 1956; Sokatch and Gunsalus, 1957; Brown and Wittenberger, 1971)

indicate that one of the alternate pathways, the Hexose Monophosphate (HMP) pathway for glucose utilization is operative in S. faecalis and the possibility that the effect of SRC-820 may be absent on this pathway was next explored.

A few characteristics of Glucose-6-phosphate dehydrogenase of S. faecalis-R were also brought to light in this study. These are: (1) the enzyme is specific for NADP and shows little or no activity with NAD^+ even when ten times the amount of $NADP^+$ was used (ii) Mg^{2+} is required for activity and (iii) chloride ions stimulate its activity (Cf. Mangiarotti and Garre, 1965). This Mg-requiring enzyme is also inhibited by SRC-820. With the use of artificial electron acceptor, such as 2,6-dichlorophenolindophenol (DCIP), a similar inhibitory effect of SRC-820 was seen. In this reaction, the inhibitory effect of SRC-820 can be explained again on the basis of its chelating property. Unlike, in the case of enolase, reversal of inhibition by higher concentrations of magnesium, could not be studied. Thus, it can be inferred that both glycolysis and HMP pathway - are affected by SRC-820.

It can be safely assumed that blocking of both these pathways will result in complete abolition of respiration with the attendant loss of energy for the

organism to survive or maintain itself. The continued uptake of O_2 in presence of SRC-820 can be explained on the basis of breakdown of this compound by cellular enzymes or its lack of ability to completely inhibit both glycolysis or direct oxidation of glucose. It should also be borne in mind that this organism is versatile in its metabolic ability to employ many alternate routes for acquiring energy. Thus, the oxidation of reduced coenzymes and transport of hydrogen to oxygen should play an important role whatever may be the alternate routes resorted to by this organism.

The oxidation of NADH and NADPH by extracts of S.faecalis cells was thus investigated and the results presented in Chapter-IX. The general characteristics of these reactions are in agreement with those reported by Dolin (1955, 1961) with other organisms such as Clostridium and other strains of S.faecalis (Cf.Weber and Kaplan, 1954).

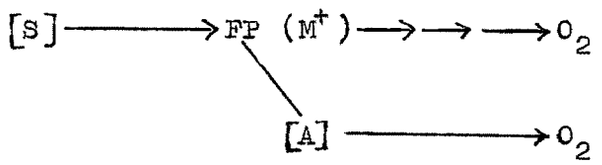
It is known that lactic acid bacteria have a rudimentary respiratory system (Wittenburry, 1960). They may contain cytochromes but depend entirely on flavoproteins (Dolin, 1961) for their respiration. However, some lactic acid bacteria including S.faecalis can synthesize a functional membrane bound cytochrome system when they are grown in media containing a haematin-like

compound (Bryan-Jones and Wittenburry, 1969; Ritchey and Seeley, 1974). It was observed by Pritchard and Wimpenny (1978) that S.faecalis variety zymogens strain TR, showed low level of membrane bound NADH-oxidase activity and this was possibly due to the traces of haem compounds present in yeast extract used in the medium. In the present investigation also, S.faecalis-R was grown in a medium containing 0.4 gm % of yeast extract (see Chapter-II, Materials and Methods) and the crude cell extracts of S.faecalis showed good amount of "NADH-oxidase" activity.

S.faecalis-R cells possess active enzymes such as NADH-oxidase or diaphorase (Dolin, 1960). The peroxidase activity, however, could not be demonstrated. NADH-oxidase activity was found to share many of the properties reported by Hoskins et al (1962), Walker and Kilgour (1965) in S.faecalis 10C1 and L.casei, respectively in its instability to storage and dialysis. This loss in activity cannot be prevented by addition of albumin to increase the concentration of protein.

SRC-820 inhibits, perhaps competitively, the activity of NADH-oxidase (Chapter-IX). The enzyme is also inhibited by pCMB and iodoacetate showing that -SH groups are necessary for its activity (Cf. Hoskins et al, 1962). Unlike what was observed in the case of

iodoacetate, with GAP-dehydrogenase, presence of SRC-820 increases the inhibitory effect of these compounds on this enzyme. It has been shown by several workers (Mahler and Elowe, 1954; Bray et al, 1961) that metal ions such as Fe play a significant role in this reaction. The additive effect of SRC-820 in this case can be due to its forming a more stable complex with Fe than with magnesium as was seen in the case of enolase. SRC-820 does not seem to exert its action on -SH groups of this protein. The compound 8-hydroxyquinoline (Albert et al, 1947; Albert, 1970), another well known metal chelator is also found to inhibit NADH-oxidase activity. Thus, it can be inferred that, in this strain of S.faecalis-R also, oxidation of NADH is brought about by a metalloflavoprotein as in many other organisms, as given below:



where [S] stands for substrate, FP for flavoprotein, [A] for artificial electron acceptor and (M^{+}) for metal.

Similar results were obtained when oxidation of NADH was followed using DCIP as electron acceptor. It is assumed that by this method, the activity estimated is that of what was once known as 'diaphorase' (Lipoamide dehydrogenase, NAD: lipoamide oxidoreductase EC.1.6.4.3; Massey, 1960). By this method, oxidation of both NADH and NADPH by S.faecalis extracts was demonstrated (Chapter-IX). The activity was higher in tris buffer than in phosphate buffer. Neither SRC-820 nor the free quinazolinone-4-one inhibit the oxidation of NADH, when DCIP was used as the electron acceptor.

The use of artificial electron acceptors may eliminate the participation of the natural acceptors such as metal, cytochromes etc (Mahler and Elowe, 1953). Peterson and Peterson, as early as 1945, have shown that various lactic acid bacteria and E.coli contain compounds with vitamin K activity, participating in electron transport. Baum and Dolin (1963) have isolated a membrane bound, borohydride reducible compound, (2-solanesyl-1, 4-naphthoquinone), from the lipid extract of S.faecalis.

S.faecalis diaphorase functions preferentially with such quinones as electron acceptors (Dolin and Wood, 1960), from flavoproteins through metal ions.

The lack of inhibitory effect of SRC-820 can be attributed to the by-passing of the metal cofactor requirements as outlined earlier.

The above results taken together lead to the conclusion that the effect of SRC-820 on carbohydrate metabolism in brought about by chelating the metal cations needed for enzyme activity. The effect is more prominent at the level of conversion of 3-PGA to PEP and reoxidation of NADH. The inhibition of respiration of S.faecalis cells by SRC-820 is more due to its effect on NADH-oxidation and the inhibition of glycolysis is more due to its effect on enolase. The gradual reduction in inhibition of growth and respiration with time points to the possible existence of alternate pathway of utilization of carbohydrate in S.faecalis. It also indicates that SRC-820 does not compete with NAD as reported by some workers (Seth et al, 1964; Parmar and Seth, 1965) in the case of methaqualone in animal tissues but only inhibits the oxidation reduced NAD by complexing with metal ions.

Also, the study has revealed that SRC-820 may act as an antifolate with repercussions on the metabolism of other substances, besides carbohydrates. But further studies will be needed to explore this fully.

In conclusion, it can be said that the quinazolinone derivative, SRC-820, at millimolar concentrations inhibits glycolysis and HMP pathway and reoxidation of NADH by NADH-oxidase. The inhibition is brought about by its forming complexes with essential cations needed for enzyme activity.