STUDIES ON THE ACTIVATION AND INHIBITION OF THE ALKALINE PHOSPHATASE OF SALMONELLA TYPHOSA.

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Since the investigations of Paget and Vittu (1947) and Vittu (1947)on the inhibition and activation of the prosphatases of *Salmonella typhosa* by sodium cyanide and sulphonamide and by magnesium ions respectively, very little work has been done on the characterisation of this enzyme. In view of its important role in cellular metabolism and the variation in its properties depending upon the source, it was considered of importance to study its characters in *S. typhosa*. Earlier work on the effect of antibiotics (Singh et al., 1956) on this enzyme of the various antigenic strains of the typhoid bacilli showed activation with penicillin, streptomycin, chloromycetin and neomycin, whereas considerable inhibition was seen with achromycin and aureomycin. Studies have now been undertaken on the effect of a large number of inorganic and organic compounds on the alkaline phosphatase activity of *S. typhosa* and the results obtained are reported in the present communication.

MATERIALS AND METHODS

Culturing and harvesting of the cells were done as reported earlier (Singh et al., 1956) and a cell suspension having a transmission of 20 percent was used throughout these studies. Sodium-β-glycerophosphate was used as the substrate and the method of assay of the enzyme activity was essentially

the same as described in our previous communication (Singh et al., 1956). Orthophosphate was estimated in the presence of arsenate by first reducing the latter to arsenite, a non-interfering product (Pett, 1953). All the chemicals used were of reagent grade. The present studies were confined only to 0-901 strain as the other four antigentic strains viz. H-901, Vi1, Ty2 and Watson's V followed the same pattern with regards to their inhibition and activation.

RESULTS

The data presented in Table I show that most of the second group elements of the periodic table viz. Ca, Sr and Ba strongly activated this enzyme, whereas Zn, Cu, Cd, Ni, Co, Be and Mn salts inhibited it in the descending order. Decrease in the cationic concentration markedly reduced the inhibitory influence due to Co, Mn and Be ions, but no such effect was observed with Zn, Cu, Ni and Cd.

The effect of the period of contact of the enzyme with the various cations at 25°C (Table 2) showed that with the progressive increase of time, the inhibition was correspondingly more in the case of Cd, Cu, Zn, Co and Ni ions. Significantly, less increase was noted with Be and Mn ions.

The data presented in Table 3 on the influence of a peptide and a few amino acids on this enzyme showed that L-arginine, L-glutamic acid, L-glutathione, L-methionine and L-*a* and β-phenyl alanines did not have any action or affected the enzyme only slightly. Among the inhibiting amino acids, glycine and DL-serine had only a mild effect, whereas L-cysteine, L-alanine and DL-histidine were active inhibitors of this enzyme. On the other hand, DL-aspartic acid and L-asparagine had decidedly an activating influence.

Attempts made to reverse the inhibitory action of Zn ions by the addition of the activating amino acids showed that the inhibition could not be counteracted by the simultaneous addition of either aspartate, glutamate, glycine, alanine or glutathione and hence the results have not been presented in the table.

The significant activation of the enzyme by glucose and CNS is seen from the data present in Table 4. Sodium arsenate and arsenite, thioglycollic acid, fluoride, thiourea, nitrate and bromide had only slight positive or negative effect on the enzyme. Ammonium molybdate, cyanide, ascorbic acid, caffein and iodoacetate were strong inhibitors for this enzyme in the decreasing order. The inhibition obtained by ammonium sulphate was in between those obtained by ammonium chloride and sodium sulphate, which would presumably indicate the nonsynergistic action of NH_4 and SO_4 ions.

TABLE 1

Effect of certain cations on the alkaline phosphatase activity of *Salmonella typhosa* (0-901)*

Cations	Inhibition (—) or Activation (+) %			
	Concentration (Moles)			
	1×10^{-2}	5×10^{-3}	2.5×10^{-3}	5.6×10^{-4}
Zn^{++}	—100	—100	—96	—90
Ni^{++}	— 87	— 83	—77	—67
Cu^{++}	— 96	— 92	—84	—74
Mn^{++}	— 68	— 60	—45	—20
Cd^{++}	— 94	— 85	—79	—72
Co^{++}	— 86	— 76	—65	—15
Be^{++}	— 82	— 65	—25	— 8
Ca^{++}	—	—	—	+20
Sr^{++}	—	—	—	+14
Ba^{++}	—	—	—	+12

*The cell suspension was incubated with the cation solution for 2 hrs at 37°C and then the substrate-buffer mixture was added to start the reaction.

TABLE 2

Inhibition of the alkaline phosphatase activity of *Salmonella typhosa* (0-901) by certain cations as a function of the period of contact*

Cations	Inhibition (%)		
	Time of contact (hrs)		
	0	2	8
Zn^{++}	88	92	95
Ni^{++}	60	67	96
Cu^{++}	70	74	100
Mn^{++}	17	20	22
Cd^{++}	68	72	100
Co^{++}	12	16	56
Be^{++}	7	8	8

*The cationic concentration in the incubation mixture was 5.6×10^{-4} Moles.

TABLE 3

Effect of certain amino acids on the alkaline phosphatase activity of *Salmonella typhosa* (0-901)*

Amino acid	Activation (+) or Inhibition (—) %	Amino acid	Activation (+) or Inhibition (—) %
L-Arginine	0	L-Glutathione	+ 5
L-Alanine	—15	DL-Histidine	—18
L-Asparagine	+12	L-Methionine	+ 3
DL-Aspartic Acid	+ 9	L-*a*-Phenylalanine	— 5
L-Cysteine	—20	L-β-Phenylalanine	— 5
Glycine	— 9	DL-Serine	—10
L-Glutamic acid	+ 3	..	..

*The effective concentration of the amino acids in the incubation mixture was 2.6×10^{-3} Moles.

TABLE 4

Effect of certain reagents on the alkaline phosphatase activity of *Salmonella typhosa* (0-901)*.

Reagent	Activation (+) or Inhibition (—) %	Reagent	Activation (+ or Inhibition (—) %
Thiourea	+ 5	Sod. arsenite	+ 1
NaCn	—41	Sod. arsenate	+ 1
KF	— 5	caffeine Citrate	—21
NH_4Cl	—21	Ammo. molybdate	—65
$(NH_4)_2SO_4$	—16	Thioglycollic acid	+ 2
Na_2SO_4	—12	Ascorbic acid	—21
$NaNO_3$	+ 5	Iodoacetate	—10
NaBr	+ 5	Glucose	+23
NaCNS	+23	..	..

*The conditions of the experiment were the same as in Table I, but the concentration of NH_4Cl, $(NH_4)_2SO_4$ and Na_2SO_4 was $5x10^{-2}$ Moles, sodium arsenite and arsenate was $5x10^{-3}$ Moles and for the rest of the reagents it was $1x10^{-2}$ Moles.

TABLE 5

Effect of certain inhibitors and activators on the action of antibiotics on the alkaline phosphatase activity of *Salmonella typhosa* (0-901)*

Inhibitor or Activator	Increase or Decrease of activity %	Inhibitor or Activator	Increase or Decrease of activity %
Chloromycetin Control	+ 7	Terramycin Control	+13
+Glycerine	— 5	+Glycine	— 2
+Histidine	—15	+Histidine	— 5
+NaCN	—48	+NaCN	—67
+Achromycin	—68	+Achromycin	—73
+Aureomycin	—67	+Aureomycin	—75
Dihydrostreptomycin Control	+ 9	Achromycin Control	—78
+Glycine	+ 1	+Glucose	—94
+Histidine	+ 2	+Glutamate	—94
+NaCN	—65	+Aspartate	—94
+Achromycin	—75	+Ascorbic acid	—94
+Aureomycin	—75	+Ni++	—85
		+Mn++	—90
		+Co++	—77
Neomycin Control	+14	Aureomycin control	—82
+Glycine	0	+Glucose	—94
+Histidine	—10	+Glutamate	—92
+NaCN	—51	+Aspartate	—92
+Achromycin	—65	+Ascorbic acid	—95
+Aureomycin	—66	+Ni++	—92
		+Mn++	—86
		+CO++	—80

*The concentration of the antibiotics in the incubation mixture was 500µg/ml and that of the inhibitors and activators was $5x10^{3}$ Moles.

TABLE 6

Reversal of the cationic inhibition of alkaline phosphatase of *Salmonella typhosa* (0-901)* by Oxalate and Citrate.

Inhibitor	Inhibition %	Inhibitor	Inhibition %
Citrate[1] (alone)	4	Oxalate alone	9
Citrate[2] (alone)	9	Zn^{++} +Oxalate	91
Zn^{++} Control	91	Ni^{++} +Oxalate	55
Zn^{++} +Citrate[1]	90	Cu^{++} +Oxalate	74
Zn^{++} +Citrate[2]	72	Mn^{++} +Oxalate	22
Ni^{++} Control	67	Cd^{++} +Oxalate	65
Ni^{++} +Citrate[1]	65	Co^{++} +Oxalate	36
Ni^{++} +Citrate[2]	50	Co^{++} Control	16
Cu^{++} Control	74	Co^{++} +Citrate[2]	5
Cu^{++} +Citrate[2]	25	Cd^{++} Control	72
Mn^{++} Control	20	+Citrate[2]	78
Mn^{++} +Citrate[2]	13		

The concentration of citrate[2] was 2.5×10^{-3} Moles and the concentration of citrate[1] was 1/10 of citrate[2].

TABLE 7

Reversal of cationic inhibition of the alkaline phosphatase of *Salmonella typhosa* (0-901) by Chelating Agents*

Chelating agents	Inhibition %	Chelating agents	Inhibition %
EDTA (Versene)	12	Mn^{++} Control	20
0-phenanthroline	15	+ EDTA	15
8-Hydroxyquinoline	14	+ 0-phenanthroline	60
Zn^{++} Control	90	+ 8-Hydroxyquinoline	25
+ EDTA	67	Cd^{++} Control	72
+ 0-phenanthroline	82	+ EDTA	20
+ 8-Hydroxyquinoline	91	+ 0-phenanthroline	78
Ni^{++} Control	67	+ 8-Hydroxyquinoline	25
+ EDTA	45	Co^{++} Control	15
+ 0-phenanthroline	50	+ EDTA	10
+ 8-Hydroxyquinoline	60	+ 0-phenanthroline	32
Cu^{++} Control	74	+ 8-Hydroxyquinoline	32
+ EDTA	50		
+ 0-phenanthroline	74		
+ 8-Hydroxyquinoline	80		

*The metal binders and the cations were added simultaneously to the reaction mixture and the concentration of the cations and the metal binders was $5{\cdot}6 \times 10^{-4}$ and $2{\cdot}5 \times 10^{-3}$ Moles respectively.

The data representing the interaction of antibiotics with one another and a few other compounds (Table 5) showed that there was an enhancement of the inhibitory action of achromycin and aureomycin when each of them was used in combination with either glucose, glutamate, aspartate or ascorbic acid or in combination with the cations like Ni or Mn. Inhibition due to glycine was counteracted by dihydrostreptomycin and neomycin and only partially by chloromycetin and terramycin. Sodium cyanide was found to be invariably a strong inhibitor when used in combination with any of the non-inhibiting antibiotics; e.g., sodium cyanide by itself in a concentration of 5×10^{-3} moles gave an inhibition of only 38 per cent but when combined with chloromycetin, dihydrostreptomycin, neomycin and terramycin gave inhibition to the extent of 48, 65, 51 and 67 per cent respectively. The inhibitory action of achromycin and aureomycin was slightly less in the presence of any of the other four antibiotics.

Observations on the reversal of cationic inhibition (Table 6) showed that citrate was able to reactivate the enzyme to different proportions in the presence of Zn, Cu and Ni ions. This effect was only slight when Mn or Co ions were used as inhibitors. The results with Cd ions were slightly different, since in combination with citrate a little more inhibition was observed. The data with oxalate showed almost no action when Zn, Mn, Cu ions were used, since the per cent inhibition did not change appreciably. Significant reactivation with oxalate was seen when Ni ions were used as inhibitors. This effect was only slight with Cd ions. In contrast to the action of citrate the inhibitory effect due to Co ions was considerably increased due to the presence of oxalate.

The effect of chelating agents (Table 7) showed that EDTA was partially able to protect the enzyme against the inhibitory action of the various cations used, but this protective action was different for each element. o-Phenanthroline also showed a reversal of inhibition by Zn and Ni ions, though it did not affect the cationic inhibition by copper. It is interesting to note that o-Phenanthroline actully increased the inhibition caused by Mn, Cd and Co ions. 8-hydroxyquinoline again increased the inhibition when used in combination with Cu, Mn and Co ions, though it partially counteracted the inhibition due to Cd and Ni ions. However, it was without any influence when Zn ions were used as inhibitors.

DISCUSSION

The behaviour of alkaline phosphatase to various metal ions has been observed to vary extensively (Hove et al., 1940; Roche et al., 1947; Frohman et al., 1949; Khemperer et al., 1949; Roche et al., 1950; Varma and Srinivasan, 1954). The inhibitory action of Zn, Cu, Ni, Cd, Co, Mn and Be ions as indicated in the present studies rules out the possibility of any of these ions acting as cofactors for this enzyme system. The stimulatory effect of Ca, Sr and Ba ions, however, suggests the possibility of their participating as the coenzyme or its substitute for the alkaline phosphatase of *S. typhosa.*

With the increase in the time of contact of a few divalent inhibitory cations with the enzyme, there was a corresponding increase in the degree of the enzyme inhibition, indicating that the enzymic inhibitor complex formation is a slow time reaction process. The non-dependence of the enzyme inhibition on the time of contact in the case of a few other cations like Be and Mn suggests a spontaneous process in these cases.

There are numerous complex-forming compounds (Roche and Thoai, 1943; Roche, 1950) which have been known to inhibit the alkaline phosphatases. The alkaline phosphatase of *S. typhosa* is likewise inhibited though not very strongly by a few such compounds, like EDTA, o-Phenanthroline, 8-hydroxyquinoline, oxalate, and citrate. Cyanide also proved to be a strong inhibitor of this enzyme. Partial reversal of the enzyme inhibition against the adverse effect of the various cations was more satisfactory with EDTA and citrate and less with oxalate, o-phenanthroline and 8-hydroxyquinoline when added simultaneously with the inhibitors. The last three compounds on the contrary potentiate the inhibitory effect of some of these cations, the mechanism of which is not clear. It is possible that the metal complexes formed by these compounds are more toxic to the enzyme than the corresponding cations themselves.

Cloetens (1941) was able to distinguish the liver and kidney phosphatase on the basis of inhibition of the former with fluoride and the latter with cyanide and thiol compounds. The inhibition by cysteine and cyanide possibly suggests the alkaline phosphatase of *S. typhosa* to be more like the kidney phosphatase though very slight or no action has been obtained with some of the other thiol compounds like thioglycollic acid, methionine, glutathione and thiourea.

The inhibition of the alkaline phosphatase of *S. typhosa* by the ammonium and sulfate ions is in conformity with the results of London et al., (1954), on acid phosphatase of the prostate, even though no synergistic action was observed in this case.

The inhibition of the alkaline phosphatase of *S. typhosa* by caffeine is interesting in view of certain cytological and genetic changes induced by methylated oxypurines (Cheney and Lansing, 1955; Wihlman and Hansen, 1955) in microorganisms and plant roots besides affecting a large number of metabolically important enzymes.

The inhibitory influence of molybdate is in keeping with the results of previous workers (Bossard, 1947) whereas a neutral effect due to arsenate provides a situation which is contrary to that presented by other investigators (Zittle et al., 1947).

Amino acids have shown such a diverse behaviour towards the alkaline phosphatases, that no generalisation of their influence on this system are possible. They inhibit or have no effect or act as stimulators with respect to

alkaline phosphatases (Waldschmidt and Schaffner, 1935; Albers, 1935; Del-Regno, 1938; Bodansky, 1946, 1948, 1949; Fischer and Greep, 1948; Lora and Municio, 1951). All the three types of effects have been found on the *Salmonella* enzyme by the various amino acids tried in this study. The Co-joint effect of Zn ions with one peptide and any of the several amino acids attempted in this case, did not help at all in counteracting even partially the enzyme inhibition (Bodansky, 1946; Roche and Thoai, 1946). Aureomycin and achromycin inhibition increased in the presence of some of the enzyme activating compounds like glucose, glutamate, aspartate and an inhibiting compound ascorbic acid. Similarly the inhibition by sodium cyanide increased in the presence of any of the four antibiotics, which normally activate the enzyme. There is no acceptable explanation for this, though similar observations have been noted previously on different enzyme systems (Arora and Krishna Murti, 1955).

SUMMARY

1. The alkaline phosphatase of *S. typhosa* has been found to be inhibited by Zn, Ni, Cu, Mn. Cd, Co and Be, whereas it was activated by Ca, Sr and Ba ions.

2. Among the amino acids, L-cysteine, DL-histidine, L-alanine, DL-serine and glycine were able to inhibit the enzyme to an appreciable extent, while only L-asparagine and DL-aspartic acid proved to be the activators for this enzyme.

3. The inhibition by cysteine and cyanide possibly suggests the alkaline phosphatase of *S. typhosa* to be more like the kidney phosphatase, though very little or no action has been observed with other thiol compounds like thioglycollic acid, methionine, glutathione and thiourea.

4. The inhibition by achromycin increased when glucose, glutamic acid, aspartic acid and ascorbic acid were simultaneously added. Similarly the inhibition by sodium cyanide was enhanced by the presence of chloromycetin, dihydrostreptomycin, neomycin and terramycin, even though normally these antibiotics had an activating influence on the enzyme.

5. The alkaline phosphatase of *S. typhosa.* was also inhibited, though not strongly, by EDTA, o-phenanthroline, 8-hydroxyquinoline, oxalate and citrate individually, but EDTA, citrate and oxalate could partially protect the enzyme against the adverse effect of certain cations like Zn, Ni and Cu.

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