DISCUSSION
DISCUSSION

The existence of low molecular weight antithiamine factors (not structural analogues) in different food-stuffs throws new light on the dietary source and availability of thiamine. The natural antithiamines isolated till date from different sources have diverse structures although their mode of action may not so much different towards thiamine. All these antithiamines so far isolated from different food-stuffs viz. caffeic acid from fern (97), chlorogenic acid and pyrochatechins from coffee (163), fraction A from rice bran (95), methyl sinapate from mustard seed (102) and 3,5 dimethoxy salicylic acid (103) are of phenolic nature having one or more than one phenolic hydroxyl groups. The number and position of the hydroxyl groups are of primary importance for the antithiamine activity. Molecules with ortho- hydroxyl groups showed (99) a marked antithiamine effect, those with hydroxyl in para- position a medium one and diphenols with hydroxyl groups in meta- position are inactive. Further, the antithiamine activity does not depend upon the side chain of the phenolic compounds and a significant amount of antithiamine activity is reduced when phenolic hydroxyl group is substituted (99). Thus, compound 'G' characterized as 3,4 dihydroxy cinnamic acid, as isolated from Phaseolus radiatus, with two hydroxyl groups in the orthoposition and a side chain possesses a marked antithiamine activity.

Preliminary extraction of the antithiamine factor from Phaseolus radiatus with 10% chloroform-water mixture indicated that the compound was likely to be polar. Before acid hydrolysis the dry mass of the centrifuged chloroform- water extract of Phaseolus radiatus was not soluble in pure methanol indicating the polar nature of the active compound in the impure state and suggesting that it might exist in a bound form. On acid hydrolysis the active principle could be extracted with isobutanol. In the process of purification in silica gel G column chromatography, the active principle was obtained only by eluting with 50% methanol-chloroform mixture.
The active antithiamine factor isolated from *Phaseolus radiatus* was found to be a pure compound as tested on thin layer chromatography using three different solvent systems in which this compound gave a single spot. Various colour reactions of this isolated factor indicated the presence of phenolic hydroxyl group(s). Acidic property of this antithiamine factor was supported by its high solubility in alkaline solution and its reaction towards litmus paper.

That the isolated active component was 3,4 dihydroxy cinnamic acid was first suspected from the similarity of its antithiamine activity with that of known 3,4 dihydroxy cinnamic acid. This was first confirmed by its various physico-chemical properties viz. colour, texture, solubility, stability, dialysis, different chemical reactions, detection of elements, micro analysis and melting point. Further, the isolated compound gave the identical spots when tested on thin layer chromatography using three different solvent systems, with the corresponding R_\text{f} values which were identical to that of commercial 3,4 dihydroxy cinnamic acid (Figures - 6,7,8). Lastly, conclusive proof about the identity of the isolated compound as 3,4 dihydroxy cinnamic acid was obtained from spectral studies. The ultraviolet absorption maxima of the isolated compound was similar to that of standard 3,4 dihydroxy cinnamic acid. Moreover, infrared absorption studies of the isolated compound gave a superimposeable curve with that of known 3,4 dihydroxy cinnamic acid (Figure - 9). Thus, the antithiamine compound isolated from *Phaseolus radiatus* was 3, 4 dihydroxy cinnamic acid as represented below.

\[
\begin{align*}
\text{CH} &= \text{CHCOOH} \\
\text{HO} &= \text{OH}
\end{align*}
\]

3, 4-dihydroxy cinnamic acid.

The antithiamine activity of the phenolic compounds was generally determined on the weight basis by thiochrome method (102). Accordingly, 1 mg of 3,4 dihydroxy cinnamic acid (isolated antithiamine factor) inactivated 135.0|\mu g of thiamine hydrochloride, whereas fraction A (95), methyl sinapate (102), 3,5 dimethoxy salicylic acid (103) and caffeic acid (102) inactivated 26.5|\mu g, 45.0|\mu g, 20.5|\mu g and 135.0|\mu g of thiamine hydrochloride respectively.

As far as could be ascertained from the available literature, no study had yet been conducted to evaluate the nutritional, enzymological and microbiological (using *S. aureus*) status of 3,4 dihydroxy cinnamic acid as an antithiamine compound although several studies had already been conducted to explore different properties of 3,4 dihydroxy cinnamic acid, (164-176). The in vivo effect of 3,4 dihydroxy cinnamic acid (antithiamine factor as isolated from *Phaseolus radiatus*) on growth of rats was thus studied. It was observed that on administration of preincubated mixture of thiamine hydrochloride and 3,4 dihydroxy cinnamic acid to the rats caused acute thiamine deficiency such as anorexia, loss in weight, fall of hair etc. as in case of thiamine deprived rats. Thiamine deficient symptoms
of 3,4 dihydroxy cinnamic acid treated rats disappeared within 2-3 days after administration of thiamine hydrochloride.

The plasma cholesterol (free) level of the thiamine deficient rats was reduced to about half in comparison to that of normal rats. It is therefore presumed that the isolated antithiamine compound i.e. 3,4 dihydroxy cinnamic acid had an inhibitory effect on pyruvate oxidase (thiamine pyrophosphate requiring enzyme) system as a result of which formation of acetyl Co A, the precursor for cholesterol biosynthesis was considerably inhibited. The accumulation of high level of pyruvate in blood also supported this fact that there was a block in the oxidative removal of pyruvate.

Inactivation study between thiamine hydrochloride and the isolated antithiamine factor (3,4 dihydroxy cinnamic acid) was extended at the enzymic level. A number of workers (145) studied the effect of structural analogues of thiamine which caused the change in thiamine pyrophosphate dependend enzyme activity. But no extensive studies on the enzyme activity had yet been made by administering the natural antithiamines to the animal system. The in vivo studies on the inactivation of transketolase enzyme by natural antithiamines were carried out by Chaudhuri and his associates (96, 104, 105, 106). It is known that transketolase enzyme catalyses the conversion of ribose-5-phosphate to sedoheptulose-7-phosphate in pentose phosphate pathway requiring thiamine pyrophosphate as co-enzyme. It was found that on administration (by injection) of 3,4 dihydroxy cinnamic acid (a preincubated mixture of thiamine hydrochloride and 3,4 dihydroxy cinnamic acid) the transketolase activity of hemolysate and intestinal mucosa was markedly decreased as shown by increase in TPP effect in comparison to that of normal rats. This depressed enzyme activity was found to be restored on further administration of thiamine hydrochloride (by injection).

The loss of enzyme activity was probably due to a non-competitive inhibition, because only the structural analogues can compete with thiamine pyrophosphate to combine with the apo-transketolase protein. The restoration of the enzyme activity with the administration of thiamine hydrochloride led to the conclusion that 3,4 dihydroxy cinnamic acid had no inhibitory effect on transketolase enzyme but only on the thiamine pyrophosphate.

The TPP effect of brain was not much elevated in the case of 3,4 dihydroxy cinnamic acid (the isolated antithiamine factor) treated rats as compared with that of normal rats indicating that transketolase activity of brain was least affected in the case of thiamine deprived rats as well as 3,4 dihydroxy cinnamic acid treated rats during the forty-five days of experiment. It might be of interest to add that Salcedo and co-workers (177) showed that brain could retain its thiamine for about three weeks in thiamine deficient rats.

The inhibitory study of thiamine hydrochloride in presence of the isolated antithiamine factor from Phaseolus radiatus i.e. 3,4 dihydroxy cinnamic acid as determined by thiochrome method as well as on thiamine pyrophosphate-transketolase (TPP-TK) system was further extended to the microbiological level. The growth of Staphylococcus aureus, a thiamine dependent strain was retarded when 3,4 dihydroxy cinnamic acid was added to the medium.
supplemented with thiamine hydrochloride. In presence of 3,4 dihydroxy cinnamic acid thiamine was inactivated resulting in the block of carbohydrate metabolism which might ultimately be responsible for the inhibition of growth of the organism. The growth of *S. aureus* in the medium containing 3,4 dihydroxy cinnamic acid was restored to the normal level with the further addition of thiamine hydrochloride to the medium. This evidence also supported the fact that 3,4 dihydroxy cinnamic acid had only inhibitory effect on thiamine. When thiamine hydrochloride was added to the medium of 3,4 dihydroxy cinnamic acid treated *S. aureus* cell, the growth of the organism became normal after one hour, suggesting that 3,4 dihydroxy cinnamic acid had no toxic effect on *S. aureus*.

In conclusion, it may be stated that the antithiamine factor isolated from *Phaseolus radiatus* was found to be chemically analogous to 3,4 dihydroxy cinnamic acid. It possesses marked antithiamine activity. Its antithiamine property was initially estimated by thiochrome method and finally confirmed by nutritional, enzymological and microbiological studies.

Lastly, it can be added that in some preliminary chromatographic and absorption studies, it was observed that the interaction between thiamine and 3, 4 dihydroxy cinnamic acid (antithiamine) was probably a reversible process involving the formation of a complex. Further study on the exact mechanism involved in this interaction is now in progress.