Table II. In vivo inhibition of MAO from cattle tick larvae after treatment with chlorodimeform and N-desmethylchlorodimeform

Time (h)	Treatment (% inhibition)			
	Chlorodimeform	Chlorodimeform and piperonyl butoxide	N-desmethylchlorodimeform	N-desmethylchlorodimeform and piperonyl butoxide
0	0	0	0	0
5	74	65	60	54
24	81	81	68	65
48	82	81	75	74
Mortality (%)				
5 h	0	0	0	0
24 h	100	0	100	100

Results are the mean of up to 5 separate experiments.

in vitro nor was there any detectable mortality using such compounds. This can probably be attributed to their lack of penetration of the cattle tick larvae.

These results suggest that inhibition of MAO cannot be the primary mode of action of chlorodimeform in cattle ticks. Chlorodimeform and its metabolite N-desmethylchlorodimeform are equally good inhibitors of MAO in vivo, and yet ticks survive with low MAO activities when metabolism of chlorodimeform is inhibited by the presence of piperonyl butoxide. Our results also support the view that in the tick N-desmethylchlorodimeform rather than chlorodimeform may be the actual toxicant[1].

The mode of action of chlorodimeform could still involve an interference with neuroactive amines, either by inhibition of other regulatory enzyme systems or by interference with the uptake processes for monoamines. We are continuing our investigations along these lines.

Summary. The action of the acaricide, chlorodimeform and its metabolite, N-desmethylchlorodimeform, on the activity monoamine oxidase (MAO) from the cattle tick, *Boophilus microplus*, were studied, Both compounds were found to be potent in vitro and in vivo inhibitors of the enzyme. However the inhibition of MAO does not seem to be related to the toxic action of the acaricide.

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α^5 Pyridoxalacetic Acid and α^5 Pyridoxyl-L-Phenylalanine Acetic Acid: Their Action on some B_6-Dependent Enzymes

The interaction between pyridoxal-P and apoenzymes pyridoxal-P dependent has been studied using a variety of analogues of pyridoxal-P in an attempt to investigate the role of functional groups of the coenzyme in binding and in catalysis[1].

The results of Groman et al.[2] on coenzymatic activity of 6 analogues with the position 5'-modified on 3 bacterial enzymes tryptofanase, D-serine dehydratase, arginine decarboxylase, support the view that 5'-phosphate group, in addition to contributing importantly to binding, is also important for precise positioning of the coenzyme on the enzyme surface and that the requirement for achieving this precise positioning also must vary from one enzyme to another.

In the present paper we describe the interaction between 4 apoenzymes pyridoxal-P dependent, tyrosine transaminase from rat liver (TAT), tyrosine decarboxylase from *Streptococcus faecalis* (TDC), aspartate transaminase from pig heart and aspartate transaminase from wheat germ (AAT) and an analogue of pyridoxal-P, the α^5-pyridoxalacetic acid, and a pyridoxalacetic acid derivative, the α^5-pyridoxyl-L-phenylalanine acetic acid, compound with structure similar to that generally postulated for the initial substrate-coenzyme complex formed during the enzymic reaction catalyzed by B_6-dependent enzymes[3].

Materials and methods. The purification of TAT[4,5], AAT from wheat germ[6,7] and from pig heart[8,9] and TDC[10], their conversion to their respective apoproteins, and assay of their enzymatic activities were performed as described in the cited references. The pyridoxal-P analogue which contains the substituent CH_2CH_2COOH in the 5'-position has been synthesized according to Iwata and Metzler[11]: further purification was achieved

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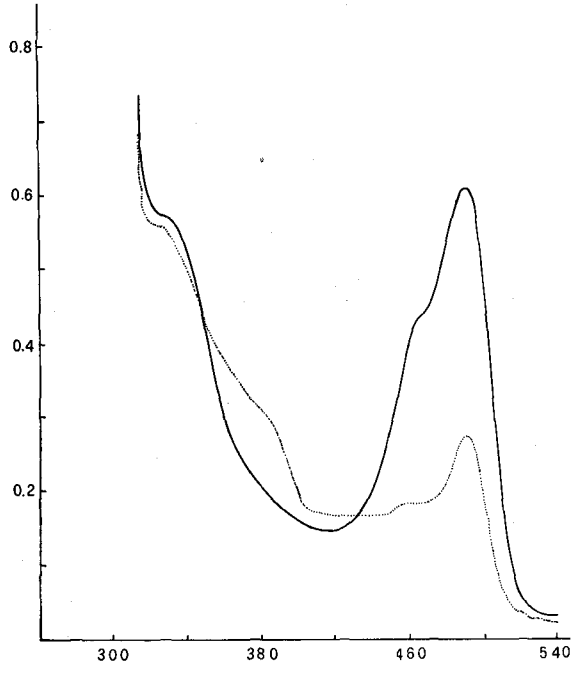
Table I.

Enzymes	Catalysis	$[I]_{50}$ (M)
1. Apotyrosine transaminase from rat liver	Inactive	1×10^{-6}
2. Apoaspartate transaminase from pig heart	Inactive	5.5×10^{-6}
3. Apoaspartate transaminase from wheat germ	Inactive	3.8×10^{-6}
4. Apotyrosine decarboxylase from *Streptococcus faecalis*	Inactive	$\gg 1 \times 10^{-3}$

1. Pyridoxal-P concentration in the assay = 1.5×10^{-8} M; 2. = 1×10^{-7} M; 3. = 1×10^{-7} M; 4. = 1.5×10^{-8} M.



Absorption spectra of the pyridoxal-P apoaspartate transaminase complex (—) and of the α^5-pyridoxalacetic acid aspartate transaminase complex (. . . . .) from pig heart in presence of 0.02 M β-erythrohydroxyaspartate.

Table II.

Enzymes	α^5-Pyridoxyl-L-phenylalanine acetic acid $[I]_{50}$ (M)	P-pyridoxyl-L-phenylalanine $[I]_{50}$ (M)
1. Apotyrosine transaminase from rat liver	2.25×10^{-5}	3.8×10^{-7}
2. Apotyrosine decarboxylase from *Streptococcus faecalis*	$\gg 1 \times 10^{-4}$	3×10^{-6}

1. Pyridoxal-P concentration in the assay = 1.5×10^{-8} M; 2. = 1.5×10^{-8} M.

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by means of column chromatography through an Amberlite CG-50 (130×1.5) (H^+) first in water and then in acetic acid 5% with a flow rate of 8 ml/h. Fractions which contain the product were collected and liophylized. The UV and IR spectral properties and the Rf value on thin layer chromatography were in accordance with those of IWATA and METZLER[11].

The synthesis of the α^5-pyridoxyl-L-phenylalanine acetic acid was carried out as follows: 0.05 mmoles each of α^5-pyridoxalacetic acid and L-phenylalanine were added to 10 ml methanol and brought into solution with 0.15 mmoles of 50% KOH. $NaBH_4$ was added to the clear yellow solution until the latter was turned colorless.

The solution was filtered off and the filtrate concentrated in vacuo to dryness. The residue dissolved in 1 ml of water was chromatographed on Amberlite CG-50 (13×1) (H^+). The column was developed with water and 2 ml fractions were collected. Those fractions that showed a maximum at 325 nm, which caracterizes B_6 derivatives lacking a double bond at 4′ C, were collected and liophylized.

Results. The α^5-pyridoxalacetic acid does not replace pyridoxal-P as coenzyme for any of the apoenzymes tested when the catalytic activity was measured at low enzyme concentration.

The analogue behaves as a competitive inhibitor with respect to pyridoxal-P for TAT at 37 °C and 30 °C, but gives a non-competitive inhibition at 20 °C for TAT and also for both the AAT tested at 37 °C. The dissociation constants for TAT at 37 °C and 30 °C are 6.27×10^{-7} and 3.4×10^{-7} M, respectively. No inhibition has been observed in TDC at a concentration of 1×10^{-3} M of this analogue, which is the higher solubility in our experimental conditions.

For all the transaminases tested the concentration of the analogue which causes a 50% inhibition of the assayed reaction is about 10^{-6} M, at least 1000 times lower than for tyrosine decarboxylase, as shown in Table I. Some spectral studies have been carried out on AAT from pig heart to characterize the functional properties of the α^5-pyridoxalacetic acid-apoenzyme complex. The reaction of the bound analogue with DL-alanine was observed spectrophotometrically measuring the absorption decrease at 360 nm; in presence of 0.0166 M DL-alanine at pH 7.8 the native holoenzyme transaminates with a $t^1/_2$ = 9.4 min, while the α^5-pyridoxalacetic acid-apoenzyme with a $t^1/_2$ = 300 min.

We have also tested the effect of erytro β-hydroxyaspartate on the complex α^5-pyridoxalacetic acid apoAAT from pig heart. The Figure shows that the complex α^5-pyridoxalacetic acid-apoenzyme in presence of 0.02 M β-erythrohydroxyaspartate has a peak at 490 nm significantly lower than that of the corresponding holoenzyme.

The interaction of α^5-pyridoxyl-L-phenylalanine acetic acid, which is analogue of P-pyridoxyl-L-phenylalanine (pyridoxyl-5′-phosphate L-phenylalanine), has been investigated for TAT and for TDC: the inhibition is of the non-competitive type for TAT at 37 °C, and no inhibition is observed for TDC at a concentration 1×10^{-4} M (the limit of its solubility).

The $[I]_{50}$ of the α^5-pyridoxyl-L-phenylalanine acetic acid is about 100 times lower than of P-pyridoxyl-L-phenylalanine for both the apoenzymes, as shown in Table II.

Discussion. The α^5-pyridoxalacetic acid binds to apoTAT at 37 °C and 30 °C with a K about 100 times higher than for the real coenzyme[12].

The non-competitive inhibition observed in TAT at 20 °C and in both apoAAT at 37 °C is unexpected and diffi-

cult to explain because the α^5-pyridoxalacetic acid has a chemical structure very similar to that of pyridoxal-P and also shows a high affinity for the apotransaminases tested. These data suggest that the analogue binds at the pyridoxal-P binding site.

FURBISH et al.[13] have observed that the α^5-pyridoxalacetic acid-apoAAT from pig heart complex undergoes reversible transamination by L-glutamate: this observation is consistent with the above suggestion.

The α^5-pyridoxalacetic acid-apoAAT from pig heart complex shows an efficient but reduced catalytic activity, evidenced as using, not the glutamate which has an extremely high rate of transamination[14], but a more suitable substrate, the DL-alanine, as well as the possibility to form the intermediate complex enzyme-substrate, proposed by JENKINS[15] in the transamination reaction.

Furthermore, if we compare the ratio between $[I]_{50}$ of the α^5-pyridoxalacetic acid and its pyridoxyl derivative and the ratio between the dissociation constants for pyridoxal-P and P-pyridoxyl-L-phenylalanine[12] we found a similar value. This observation suggests that the substitution of the phosphate with a carboxyl group does not hinder the formation of the enzyme-substrate complex, but affects the catalytic activity.

It is possible that the binding of the α^5-pyridoxalacetic acid to the active site has some peculiar characteristics which could result in an imperfect fit at the active center so as not to allow an efficent interaction and catalysis to take place. The extent of the binding is much more lower in apoTDC, as compared to apotransaminases, according to the evidence reported by GROMAN et al.[2].

Riassunto. I risultati ottenuti dall'interazione dell'α^5-piridossal acido acetico e dall'α^5-piridossil-L-fenilalanina acido acetico su alcuni enzimi B_6 dipendenti suggeriscono che la sostituzione del fosfato con un gruppo carbossilico nel piridossal-5'-fosfato non impedisce la formazione del complesso enzima-substrato, ma influenza l'attività catalitica.

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Molecular Heterogeneity of Human Placental Aminopeptidase Isozymes

An aminopeptidase (AP) that hydrolyzes L-leucyl-β-naphthylamide (leucine aminopeptidase, LAP) or L-cystine-di-β-naphthylamide (cystine aminopeptidase, CAP or oxytocinase) appears in maternal sera during pregnancy[1–3]. GOEBELSMANN and BELLER[4] successfully used chromatography on Sephadex for separating this pregnancy serum AP from an LAP which is present in all human sera. In an earlier report we presented evidence for the existence of 2 AP isozymes in human placenta: the lysosomal and microsomal isozymes, which were distinct from normal serum LAP[5]. In this paper we present new data on the molecular heterogeneity of placental AP isozymes obtained from a gel filtration study.

Lysosomal and microsomal extracts were prepared from homogenates of human placentae as previously prepared[5]. Sepharose 6B column (*2.5 × 100 cm*) was prepared and equilibrated with 0.1 *M* sodium phosphate buffer (pH 7.0). Elution was carried out with the same buffer and the effluent was collected in 4-ml-fractions. LAP and CAP activities were determined by the method of TAKENAKA[6] with slight modifications. Techniques for disc electrophoresis and the enzyme staining were also described previously[5].

Figure 1 represents a typical elution pattern of AP isozymes by chromatography on Sepharose 6B column. Non-pregnancy sera and fetal sera exhibited only 1 peak of LAP activity. Pregnancy sera at term showed in addition another tall LAP peak (Peak I) which was separated from the LAP peak in non-pregnancy sera (Peak II). Corresponding to Peak I, a peak of CAP activity was found in pregnancy sera, whereas non-pregnancy sera and fetal sera had no CAP activity in any fraction. Lysosomal LAP and CAP were eluted in the same position as Peak I; microsomal LAP and CAP as Peak II.

Since only pregnancy sera exhibited biphasic LAP peaks, these 2 separated peaks were subjected to disc electrophoresis. As is presented in Figure 1, Peak I showed 2 CAP bands (CAP_1 and CAP_2)[7] as the lysosomal enzyme did; Peak II one LAP band.

These results support our view that the origin of pregnancy serum AP is the lysosomes of placenta, from which the enzyme is released into maternal circulation during pregnancy[5,8]. Furthermore, it was shown that the pregnancy serum AP of lysosomal origin displaying 2 CAP bands can be separated from the LAP in all human sera by gel filtration. This is in agreement with the results of GOEBELSMANN and BELLER[4].

As is shown in Figure 2, according to the method of ANDREWS[9], the molecular weight of the lysosomal enzyme was estimated to be approximately 320,000; that of the microsomal enzyme and normal serum LAP to be approximately 145,000. The former is nearly equal to the molecular weight of retroplacental CAP as estimated to be 325,000 by YMAN and SJÖHOLM[10]. From the present gel filtration experiment, the lysosomal and microsomal isozymes are apparently multiple molecular forms differing in their molecular sizes.

Absence of Peak I (2 CAP bands) in fetal serum suggests that this enzyme does not leak into fetal circulation. It is likely that the high molecular weight of pregnancy serum AP is also responsible for its failure to pass from the blood of pregnant women through the placental barrier into fetal circulation.

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