Aminotransferase Activity of *Hartmannella* (Culbertson Strain A-1) Grown Axenically

By M. M. HUSAIN AND V. K. MOHAN RAO

*Division of Biochemistry,
Central Drug Research Institute, Lucknow, India*

(Accepted for publication 24 February 1969)

SUMMARY

Cell-free extracts of trophozoites of *Hartmannella* (Culbertson strain A-1), grown axenically, catalysed transamination between 2-oxoglutarate and a number of amino acids. Aspartic acid, alanine and serine showed the maximum activity. The L-alanine-2-oxoglutarate aminotransferase (E.C. 2.6.1.2) was purified about 15-fold by adsorption with calcium phosphate gel and elution with 0.2 M-phosphate buffer. Stability during storage, pH optima in different buffers K_m values for alanine and 2-oxoglutarate, and the role of pyridoxal-5-phosphate are reported. *p*-Chloromercuribenzoate, cyanide and metal-chelating agents markedly inhibited the enzyme. Among dicarboxylic acids, maleic acid had the maximum inhibitory effect. Amoebicidal drugs and antibiotics had no marked inhibitory effect on the enzyme.

INTRODUCTION

Certain strains of the free-living amoebae belonging to the genus *Hartmannella* (Acanthamoeba) produce meningoencephalitis in mice and monkeys and are supposed to cause the same disease in man (Culbertson, Smith, Cohen & Minner, 1959; Patras & Andujar, 1966; Butt, 1966). The growth, nutritional requirements and metabolic activities (Adam, 1959, 1964; Band, 1959, 1961, 1962; Neff, 1957) of some of these strains have also been studied. The patterns of the proteolytic activities of *Hartmannella* (Ambrosioni, 1952; Bianchini, 1953*a, b*) and *Acanthamoeba* (Jarumilinta & Maegraith, 1961*a, b*) have been reported, using different substrates. In the intermediary metabolism of proteins and in the formation of pool amino-acids, transaminases play an important role. In view of this, the aminotransferase (E.C. 2.6.1.2) activity present in crude cell-free extract of trophozoites of *Hartmannella* has been partially purified and characterized. The action of certain amoebicidal drugs and antibiotics has also been studied and the results are presented in this communication.

METHODS

Source of enzyme. Pathogenic *Hartmannella* strain A-1, obtained from Dr C. G. Culbertson, was cultured axenically in medium containing Difco Proteose-peptone (1%, w/v), Oxoid tryptone (1%, w/v) and sodium chloride (0.5%, w/v), pH 6.8, in 1 l. Roux flasks. After 6 days at 37°, trophozoites were harvested and washed three times with glass-distilled water. The washed cells were suspended in water and

homogenized in a Potter-Elvehjem glass homogenizer at 4°. The homogenate was centrifuged at 3000 rev./min. in a MSE refrigerated centrifuge for 30 min. The resulting opalescent crude enzyme extract was purified over calcium phosphate gel.

Assay of enzyme activity

Colorimetric method. Glutamate-pyruvate transaminase (GPT) was estimated by the method of Reitman & Frankel (1957). In a typical assay system, 0.2 ml. of the enzyme was incubated for 60 min. with 2 μ mole 2-oxoglutarate, 200 μ mole DL-alanine and 100 μ mole Sørensen phosphate buffer (pH 7.4); total volume 1.2 ml. The reaction was stopped by addition of 1 ml. of 0.02% (w/v) 2,4-dinitrophenylhydrazine in 2 N-HCl. After 20 min. colour was developed by the addition of 10 ml. of 0.4 N-NaOH and the intensity of the colour was read at 505 m μ in a Beckman DU model spectrophotometer. Enzyme units were calculated from a standard curve for the extinction of dinitrophenyl-hydrazones of pyruvate in conditions similar to those of enzyme assay.

Chromatographic method. Wherever the above method could not be applied, paper chromatography was used for estimating the glutamate formed during transamination. The chromatogram was run for 16 hr in *n*-butanol + acetic acid + water (4 + 1 + 1 v/v) and the amino acid spots, after developing with ninhydrin, were quantitated according to the method of Giri, Radhakrishnan & Vaidyanathan (1952). In a typical assay, 0.5 ml. of reaction mixture contained 1 to 20 μ mole 2-oxoglutarate, 1 to 10 μ mole DL-alanine and 20 μ mole phosphate buffer, (pH 7.4). The reaction was run for 4 hr at 37°, stopped by heating 5 min. in a boiling water bath and the chromatograms were run using 0.02 ml. reaction mixture. Independent experiments (without enzyme) showed that under these conditions non-enzymic transamination did not occur due to heating.

Estimation of protein. Protein in the extract was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine plasma albumin as standard.

Preparation of calcium phosphate gel. Calcium phosphate gel was prepared according to the method of Keilin & Hartree (1938) and about 8-months old gel was used for adsorption purposes.

Chemicals and reagents. DL-alanine and 2-oxoglutarate (B.D.H.) and pyridoxal-5-phosphate (E. Merck) were used. All other reagents were of analytical grade.

Samples of paromomycin sulphate (Parke Davis (India) Ltd.), chlortetracycline hydrochloride (Aureomycin: Cyanamide (India) Ltd.), cycloheximide (Actidione: Upjohn Co., U.S.A.), 4-carbamidophenyl arsonic acid (Carbarsone: Burroughs Wellcome and Co. (India), Private Ltd.), chloroquine phosphate and emetine hydrochloride (Bengal Immunity Co. Ltd.), iodochlorhydroxyquin (Enterovioform: CIBA of India Ltd.) and chlorhexidine dihydrochloride (I.C.I.) were employed in these studies.

RESULTS

Preliminary screening of the crude extract of trophozoites of *Hartmannella* for aminotransferase activity, using 20 common amino acids as amino-group donors and the chromatography technique, revealed that 2-oxoglutarate was a more effective amino group acceptor than pyruvate or oxalacetate. Results with some of the active amino acids are given in Table 1.

Purification of the enzyme. Table 2 illustrates partial purification of the enzyme activity by calcium phosphate gel adsorption and elution by different molar concentrations of phosphate buffer. The most active enzyme protein was eluted by 0.2 M-phosphate buffer. Due to a paucity of material further purification was not attempted. In the following experiments about 15-fold purified enzyme was utilized for the estimation of GPT activity.

Table 1. *Aminotransferase activity of cell-free extracts of Hartmannella (Culbertson strain A-1) in 2-oxoglutarate system*

Donor amino acid	μ mole glutamic acid formed per mg. protein
L-Alanine	10
L-Aspartic acid	13
L-Cysteine	3
DL-Isoleucine	3
L-Methionine	0.7
L-Serine	8
L-Valine	6

Reaction conditions: 0.2 ml. of cell-free enzyme extract (43 μ g. protein), 10 μ mole 2-oxoglutarate, 10 μ mole L-amino acid (20 μ mole in the case of DL-amino acid), 20 μ mole Sørensen phosphate buffer (pH 7.4), total volume 0.5 ml; incubation 4 hr at 37°. 0.02 ml. of reaction mixture was used for the paper chromatographic estimation of glutamic acid formed.

Table 2. *Purification of alanine-2-oxoglutarate aminotransferase activity of Hartmannella (Culbertson strain A-1)*

Step	Vol. (ml.)	Protein (mg.)	Activity units* $\times 100$	Specific activity	Fold purification
Crude extract	7	18.9	126	666	1
Addition of calcium phosphate gel	7	—	—	—	—
Unadsorbed fraction	7.6	4.9	—	—	—
Elution with phosphate buffer pH 7.4					
0.05 M	5	2.6	—	—	—
0.05 M	5	1.6	—	—	—
0.1 M	5	1.2	42.5	3,541	5.5
0.1 M	5	0.8	30	3,750	5.7
0.2 M	5	1.1	95	8,636	13
0.2 M	5	0.5	55	11,000	16.5

* One unit of enzyme produces 1 μ mole pyruvate in 60 min. at 37°. Reaction conditions: 0.2 ml. enzyme, 2 μ mole 2-oxoglutarate, 200 μ mole DL-alanine, 100 μ mole Sørensen phosphate buffer (pH 7.4), total volume 1.2 ml; incubation 60 min. at 37°.

Properties of the enzyme

Stability. The partially purified enzyme solution was distributed into two tubes which were stored separately at 4° and -10° and assayed for GPT activity at intervals. Enzyme stored at 4° progressively lost more than half of its activity in 20 days, while freezing the enzyme extract at -10° for 2 days and thawing resulted in the loss of more than half of its activity.

Coenzyme requirement. Enzyme preparations, one dialysed overnight at 4° and

the other undialysed, were incubated with various concentrations of pyridoxal-5-phosphate for 15 min. and their activities determined. Figure 1 demonstrates that the coenzyme dissociated to some extent from the apoenzyme and higher concentrations of coenzyme inhibited the GPT activity.

Fig. 1. Cofactor requirement of alanine-2-oxoglutarate aminotransferase of *Hartmannella* (Culbertson strain A-1). The enzyme was preincubated with pyridoxal-5-phosphate (0 to 50 μ g.) for 15 min. Reaction conditions: 0.2 ml. enzyme (8 μ g. dialysed protein, 11 μ g. undialysed protein), 2 μ mole 2-oxoglutarate, 200 μ mole DL-alanine, 100 μ mole Sørensen phosphate buffer (pH 7.4), total volume 1.2 ml.; incubation 60 min. at 37°. ●—●, Enzyme dialysed; Δ--Δ, undialysed.

Fig. 2. Effect of pH on the alanine-2-oxoglutarate aminotransferase activity of *Hartmannella* (Culbertson strain A-1). Reaction conditions: 0.2 ml. enzyme (21 μ g. protein), 2 μ mole 2-oxoglutarate, 200 μ mole DL-alanine, 100 μ mole phosphate, tris-HCl, or boric acid-borate buffers, total volume 1.2 ml., incubation 60 min. at 37°. Enzyme preincubated 15 min. with 5 μ g. pyridoxal-5-phosphate.

Time-activity relationship. Thirty μ g. protein incubated for 15 min. with 5 μ g. pyridoxal phosphate (conditions otherwise as for Fig. 1) formed pyruvate linearly for 45 min. and reached a maximum by 60 min.

Enzyme concentration. In similar experimental conditions the relationship between the amount of pyruvate formed and enzyme protein was linear between 7.5 and 60 μg .

Effect of pH. The optimum pH in phosphate buffer was at 7.4, in tris-HCl buffer in the range 7.5–8.0 and in borate buffer at 7.9 (Fig. 2).

K_m values for substrates. Amounts of glutamic acid formed at different concentrations of alanine and 2-oxoglutarate were estimated. The K_m value for alanine was 2.22×10^{-3} M (Fig. 3) and for 2-oxoglutarate 7.1×10^{-3} M (Fig. 4).

Fig. 3. Determination of K_m value for alanine. Reaction conditions: 0.2 ml. enzyme (96 μg . protein), 10 μmole 2-oxoglutarate, 0 to 10 μmole DL-alanine, 20 μmole Sørensen phosphate buffer, total volume 0.5 ml.; incubation 4 hr at 37°. 0.02 ml. reaction mixture was used for paper chromatographic estimation of glutamic acid formed.

Fig. 4. Determination of K_m value for 2-oxoglutarate. Reaction conditions: 0.2 ml. enzyme (72 μg . protein), 10 μmole DL-alanine, 0 to 10 μmole 2-oxoglutarate, 20 μmole Sørensen phosphate buffer, total volume 0.5 ml.; incubation 4 hr at 37°. 0.02 ml. reaction mixture was used for paper chromatographic estimation of glutamic acid formed.

Effect of inhibitors. The actions of some common inhibitors on the enzyme activity are summarized in Table 3. Carbonyl agents such as hydrazine sulphate inhibited the enzyme by a quarter, while hydroxylamine hydrochloride inhibited the enzyme by about half. Metal chelating agents, *o*-phenanthroline, 8-hydroxyquinoline and α, α' -dipyridyl, markedly inhibited the enzyme, while EDTA had a slight stimulatory effect. Salts of heavy metals such as copper sulphate and mercuric chloride completely inactivated the enzyme. Structural analogues of pyridoxal-5-phosphate, namely isonicotinic acid hydrazide (INH) and pyridine-3-sulphonic acid at 5×10^{-3} M, inhibited the enzyme by only about one-fourth to one-fifth. Neither of these inhibitions could be annulled by 10 μg . of the cofactor. Most of the enzyme activity was inhibited by 10^{-3} M sodium cyanide. The sulphhydryl reagent *p*-chloromercuribenzoate (*p*CMB) at 10^{-4} M completely inhibited the enzyme, while 10^{-3} M-iodoacetate had no effect on the enzyme activity. The inhibitory action of *p*CMB could not be annulled by 5×10^{-5} M-cysteine. Cysteine at 10^{-2} M concentration, at neutral pH, inhibited the enzyme completely, but at 5×10^{-5} M it activated the enzyme by a quarter.

Effect of dicarboxylic acids. Fumarate, malate, succinate had a slight inhibitory

Table 3. *Effect of activators and inhibitors on alanine-2-oxoglutarate aminotransferase activity of Hartmannella (Culbertson strain A-1)*

Activator/Inhibitor	Concentration (M)	Inhibition (%)*
Hydroxylamine hydrochloride	1×10^{-4}	-54
Hydrazine sulphate	1×10^{-4}	-25
Sodium iodoacetate	1×10^{-4}	Nil
Sodium cyanide	1×10^{-3}	-95
Isonicotinic acid hydrazide (INH)	1×10^{-2}	-22
Pyridine-3-sulphonic acid	1×10^{-3}	-96
p-Chloromercuribenzoate (pCMB)	1×10^{-4}	-100
8-OH-quinoline	1×10^{-2}	-88
EDTA	1×10^{-2}	+8
α, α' -Dipyridyl	1×10^{-2}	-61
o-Phenanthroline	1×10^{-3}	-91
Sodium succinate	1×10^{-2}	-6
Sodium fumarate	1×10^{-2}	-15
Sodium malate	1×10^{-2}	-22
Sodium maleate	1×10^{-2}	-52
Sodium oxalate	1×10^{-2}	+10
Copper sulphate	3.6×10^{-4}	-100
Mercuric chloride	5×10^{-4}	-100
Cysteine hydrochloride	1×10^{-2}	-100
pCMB	5×10^{-5}	-100
Cysteine hydrochloride	5×10^{-5}	+26
pCMB + cysteine hydrochloride	5×10^{-5} each	-100
Pyridine-3-sulphonic acid	5×10^{-3}	-29
Pyridine-3-sulphonic acid + 10 μ g. pyridoxal-5-phosphate	5×10^{-3} each	-30
INH	5×10^{-3}	-17
INH + 10 μ g. pyridoxal-5-phosphate	5×10^{-3} each	-20

0.2 ml. inhibitor/activator or water (control) was incubated with 0.2 ml. enzyme for 15 min. and then 1 ml. of the buffered substrate (containing 2 μ mole of 2-oxoglutarate and 200 μ mole of DL-alanine and 100 μ mole Sørensen phosphate buffer at pH 7.4) was added. The reaction was then run for 60 min. at 37°.

* -, Inhibition; +, Activation.

Table 4. *Effect of amoebic drugs and antibiotics on alanine-2-oxoglutarate aminotransferase of Hartmannella (Culbertson strain A-1)*

Drugs	Inhibition (%)
Cycloheximide	3
Chlortetracycline hydrochloride	14
4-Carbamidophenyl arsonic acid	20
Chlorhexidine dihydrochloride	34
Chloroquine phosphate	11
Emetine hydrochloride	11
Iodochlorhydroxyquin	11
Paromomycin sulphate	5

0.2 ml. of enzyme (36 μ g. protein) was preincubated for 15 min. with 0.2 ml. of respective drug (1 mg./ml.) or water (control) and the reaction was run as described in Table 3.

effect on the enzyme, while oxalate had a slight stimulatory influence. Only maleate inhibited the enzyme by half.

Effect of antiamoebic drugs and antibiotics. The action of some antiamoebic drugs and antibiotics on the GPT of *Hartmannella* are presented in Table 4. Chlorhexidine dihydrochloride inhibited the enzyme by one-third. None of the antibiotics inhibited the enzyme markedly. Among the other potent amoebicidal drugs, 4-carbamidophenyl arsonic acid inhibited the enzyme to the maximum extent of one-fifth, while the remaining three drugs, namely emetine hydrochloride, chloroquine phosphate and iodochlorhydroxyquin, inhibited it by about one-tenth.

DISCUSSION

Just like in the aminotransferase system of *Entamoeba histolytica* (Mohan Rao & Dutta, 1966), 2-oxoglutarate was the most active amino-group acceptor compared with pyruvate and oxalacetate in the *Hartmannella* system. Valine, asparagine, leucine and aspartic acid have been reported to be the most active amino-group donors to 2-oxoglutarate in *E. histolytica* system, but in the *Hartmannella* aspartic acid, serine and alanine were most active. Serine was actively transaminated by *Hartmannella*, whereas *E. histolytica* failed to transaminate serine under similar experimental conditions. While *Hartmannella* poorly transaminated leucine, the same amino acid was actively transaminated by *E. histolytica* (Mohan Rao & Dutta, 1966). Amoebicidal drugs and antibiotics had almost the same type of effect either on *Hartmannella* or *E. histolytica* (Mohan Rao & Dutta, 1966) enzymes. In both these enzyme systems the maximum inhibition of about 30 % was effected by chlorhexidine dihydrochloride.

Higher concentrations of pyridoxal-5-phosphate inhibited either dialysed or undialysed enzyme extracts of *Hartmannella*. Similar findings were reported with the aminotransferase systems of *E. histolytica* (Mohan Rao & Dutta, 1966), animal tissues (Roberts & Bregoff, 1953) and human intestines (Ramaswamy & Radhakrishnan, 1964).

The inhibition caused by some dicarboxylic acids, especially by maleate, may be explained on the basis of an earlier observation (Jenkins, David & Irwin, 1959) that the enzyme forms an inactive complex with these acids.

The K_m value for alanine for the aminotransferase activity of *Hartmannella* compares favourably with those reported for cotton seeds (Turano, Bossa, Fasella & Fanelli, 1966) and equine erythrocytes (Balasarswathi & Krishna Murti, 1967).

The above discussion focuses some of the differences in the properties of aminotransferase activities of the two pathogenic amoebae, one belonging to free-living and the other to parasitic groups of organisms.

The authors are grateful to Dr M. L. Dhar, Director, for his keen interest in this work, and to Dr C. R. Krishna Murti and Dr B. N. Singh for helpful suggestions. The authors are very much obliged to Dr C. G. Culbertson, The Lilly Research Laboratories, Eli Lilly and Company, Indianapolis, U.S.A., for supplying the *Hartmannella* strain A-1. The authors are also indebted to the firms who supplied samples of drugs and antibiotics used in this investigation. The technical assistance of Mr S. K. Bose is gratefully acknowledged. One of the authors (M. M. H.) is grateful to the Council of Scientific & Industrial Research, New Delhi, for awarding a Junior

Research Fellowship. Communication no. 1329 from the Central Drug Institute, Lucknow.

REFERENCES

- ADAM, K. M. G. (1959). The growth of *Acanthamoeba sp.* in a chemically defined medium. *J. gen. Microbiol.* **21**, 519.
- ADAM, K. M. G. (1964). The amino acid requirements of *Acanthamoeba*. *J. Protozool.* **11**, 98.
- AMBROSIONI, P. (1952). Biology of *Hartmannella castellanii*. 1. Some biochemical properties of *Hartmannella castellanii* and of the proteolytic enzymes produced by it. *Riv. Patol. clin.* **7**, 90.
- BALASARSWATHI, K. & KRISHNA MURTI, C. R. (1967). Partial purification and properties of L-alanine 2-oxoglutarate aminotransferase of equine red blood cells. *Indian J. Biochem.* **4**, 22.
- BAND, R. N. (1959). Nutritional and related biological studies on the free-living soil amoeba, *Hartmannella rhyssodes*. *J. gen. Microbiol.* **21**, 80.
- BAND, R. N. (1961). Biotin, a growth requirement for four soil amoebae. *Nature, Lond.* **192**, 674.
- BAND, R. N. (1962). The amino acid requirements of the soil amoeba, *Hartmannella rhyssodes* Singh. *J. Protozool.* **9**, 377.
- BIANCHINI, P. (1953a). Proteolytic enzymes of *Hartmannella castellanii*. Suggestion for a new quantitative method to determine the activity of gelatinase. *Chem. Abstr.* **47**, 11269b.
- BIANCHINI, P. (1953b). A proteolytic enzyme excreted by *Hartmannella castellanii*. *Chem. Abstr.* **47**, 11319f.
- BUTT, C. G. (1966). Primary amoebic meningoencephalitis. *New Engl. J. Med.* **274**, 1473.
- CULBERTSON, C. G., SMITH, J. W., COHEN, H. K. & MINNER, J. R. (1959). Experimental infection of mice and monkeys by *Acanthamoeba*. *Am. J. Path.* **35**, 185.
- GIRI, K. V., RADHAKRISHNAN, A. N. & VAIDYANATHAN, C. S. (1952). Some factors influencing the quantitative determination of amino acids separated by circular paper chromatography. *Analyt. Chem.* **24**, 1677.
- JARUMILINTA, R. & MAEGRAITH, B. G. (1961a). The patterns of some proteolytic enzymes of *Entamoeba histolytica* and *Acanthamoeba sp.* I. The action of *E. histolytica* and *Acanthamoeba sp.* on protein substrates. *Ann. trop. Med. Parasit.* **55**, 505.
- JARUMILINTA, R. & MAEGRAITH, B. G. (1961b). The patterns of some proteolytic enzymes of *Entamoeba histolytica* and *Acanthamoeba sp.* II. Action of *E. histolytica* and *Acanthamoeba sp.* on various synthetic substrates. *Ann. trop. Med. Parasit.* **55**, 518.
- JENKINS, W. T., DAVID, A. Y. & IRWIN, W. S. (1959). Glutamic aspartic transaminase. I. Assay, purification and general properties. *J. biol. Chem.* **234**, 51.
- KEILIN, D. & HARTREE, E. F. (1938). On the mechanism of the decomposition of hydrogen peroxide by catalase. *Proc. R. Soc. B* **124**, 397.
- LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L. & RANDALL, R. J. (1951). Protein measurement with the Folin-phenol reagent. *J. biol. Chem.* **193**, 265.
- MOHAN RAO, V. K. & DUTTA, G. P. (1966). Transaminase activity of *Entamoeba histolytica*. *Indian J. Microbiol.* **6**, 63.
- NEFF, R. J. (1957). Purification, axenic cultivation and description of a soil amoeba, *Acanthamoeba sp.* *J. Protozool.* **4**, 176.
- PATRAS, D. & ANDUJAR, J. J. (1966). Meningoencephalitis due to *Hartmannella (Acanthamoeba)*. *Am. J. clin. Path.* **46**, 226.
- RAMASWAMY, K. & RADHAKRISHNAN, A. N. (1964). Studies on intestinal transaminases with special reference to human intestinal glutamic aspartic transaminase. *Indian J. Biochem.* **1**, 1.
- REITMAN, S. & FRANKEL, S. (1957). A colorimetric method for the determination of serum glutamic oxaloacetic and glutamic pyruvic transaminases. *Am. J. clin. Path.* **28**, 56.
- ROBERTS, E. & BREGOFF, H. M. (1953). Transamination of γ -amino-butyric acid and alanine in brain and liver. *J. biol. Chem.* **201**, 393.
- TURANO, C., BOSSA, F., FASELLA, P. & FANELLI, A. R. (1966). Purification and partial characterization of alanine oxoglutarate transaminase (AOT) from resting cotton seeds. *Enzymologia* **30**, 185.