Comparison of Specific and Relative Alanine and Aspartate Aminotransferases of *Trypanosoma brucei* Subgroup Trypanosomes [1]

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Abstract

Using Biochemica Test Kits (Boehringer GmbH, Mannheim) a significant difference in alanine aminotransferase : aspartate aminotransferase (ALAT : ASAT) ratios was found between *Trypanosoma brucei brucei* (4 strains; mean $\pm$ S.E.; 4.204 ± 0.342) and *T. b. rhodesiense* (4 strains; mean $\pm$ S.E.: 2.973 ± 0.174) bloodstream stage crude soluble enzyme extracts.

This difference is a stable characteristic, unaffected by the strains tested, level of parasitaemia, and number of subpassages in rats. There was also a significant difference between *T. b. brucei* and *T. b. rhodesiense* specific ALAT activities.

ALAT : ASAT ratios and specific ALAT activities in bloodstream and culture stages of *T. b. brucei* were significantly different.

Control tests elucidated the value of the test assay used for crude soluble enzyme preparations of both *T. brucei* subspecies.

Within either subspecies there was a positive significant correlation between the degree of pleomorphism (percentage of slender forms) and ALAT : ASAT ratios.

Introduction

Alanine aminotransferase, E.C. 2.6.1.2 (ALAT), and aspartate aminotransferase, E.C. 2.6.1.1. (ASAT), have been found in numerous microorganisms, higher plants and animals.

Isoenzymes of ALAT and/or ASAT have been used for species characterization in lower vertebrates (e.g. Filosofova-Lyzlova 1972), for phenotyping of the bay mussel (Johnson & Utter 1973), and as genetic markers in man (e.g. Chen & Giblett 1971).

The relative activities (ratios) of ALAT : ASAT (or ASAT : ALAT) have been extensively studied in human serum because of their importance in the diagnosis of cardiac and liver diseases, and drug reactions. Recently, it has been found that it may be possible to differentiate between bloodstream stages of the parasitic protozoan family, Trypanosomatidae, on the basis of their ALAT : ASAT ratio in

soluble crude cell extracts (GODFREY & KILGOUR 1973), or on the basis of electrophoretic mobility of ALAT and ASAT (KILGOUR & GODFREY 1973).

There have been no reports on the use of this method to differentiate between the two very closely related *Trypanosoma brucei* subgroup subspecies, *T. b. brucei* and *T. b. rhodesiense*. It is difficult to differentiate between these two subspecies; the Blood Infectivity Incubation Test (BIIT) (RICKMAN & ROBSON 1970 a, b) is still open to discussion and human volunteer tests are the only reliable technique for identification.

It, therefore, seemed useful to determine whether ALAT:ASAT ratios of *T. b. brucei* and *T. b. rhodesiense* were significantly different since this could be the basis of a test for species identification and strain characterization.

A study was undertaken to compare specific and relative ALAT and ASAT activities in well known strains of *T. b. brucei* and man-tested *T. b. rhodesiense* by assaying for soluble aminotransferases from crude extracts of bloodstream and culture stages of the two subspecies. GPT (ALAT) and GOT (ASAT) "Biochemica Test Combination Kits" (Boehringer GmbH, Mannheim) were used to simplify the assays as much as possible.

A preliminary report of some of the findings was given at the IVth International Congress of Protozoology (STEIGER et al. 1973).

Material and Methods

The strains of *T. b. brucei* used for transaminase assays came from the following stabilates:

1. EATRO 1961 – a primary cyclically passaged pleomorphic strain from a mouse on which an infected *Glossina morsitans* was permitted to feed. The parent strain was EATRO 1093, isolated from *Hippotragus niger* (GEIGY et al. 1967).

2. EATRO 1529 – a pleomorphic strain originating from frozen, infected *G. pallidipes* salivary glands injected into a mouse and subsequently syringe-passaged 7 times in mice (GOEDBLOED et al. 1971).

3. EATRO 1532 – a pleomorphic strain originating from frozen, infected *G. pallidipes* salivary glands injected into a mouse and subsequently syringe-passaged 4 times in mice (GOEDBLOED et al. 1971).

4. STIB 335 – a nearly monomorphic, cloned strain derived from an old laboratory strain, Lister 427, obtained in 1972 from the Molteno Institute, Cambridge University.

5. S 42 – a culture strain derived from a pleomorphic *T. b. brucei* infection (see p. 204).

The strains of *T. b. rhodesiense* used for transaminase assays came from the following stabilates:

1. EATRO 1873 – a man-tested, BIIT-positive (GEIGY et al. 1973), pleomorphic strain isolated from *Alcelaphus buselaphus* in the Serengeti National Park in 1970 by mouse inoculation (GEIGY et al. 1971).

2. EATRO 1132 – a pleomorphic, BIIT-positive strain (GEIGY et al. in preparation) isolated in 1967 from a Sleeping Sickness patient in Tororo, Uganda.

3. STIB 338 – a pleomorphic strain derived from the Dumelo strain isolated in 1960 from a Sleeping Sickness patient in Botswana (ORMEROD & VENKATESAN 1971), obtained from Dr. W. F. Ormerod, London School of Tropical Medicine, in frozen ampoules and injected into rats from which stabilates were made.

4. STIB 339 – a pleomorphic, cloned strain derived from STIB 338.

Blood and culture techniques

Bloodstream trypomastigotes were maintained in RA 25 Ivanovas white male rats (200–300 g) by syringe passage (up to 7 passages for strain EATRO 1961) with transfers made between 7–20 days post inoculation. Parasites used for transaminase assays were harvested (7–20 days post inoculation) by exsanguinating rats with heart punctures. Syringe needles were pre-coated with a small amount of heparin to prevent clotting. Infected blood was immediately mixed 1 : 1 with phosphate-saline-glucose (PSG) 4 : 6, pH 8.0 (LANHAM & GODFREY 1970) and kept in ice. Blood smears were also made to determine the degree of pleomorphism (Giemsa stain).

Single donor rats were selected when their infection reached a preselected arbitrary figure based on the number of trypomastigotes/red blood cells in a fresh wet smear of tail blood; hosts with a rating of at least +++ (a net of 10^8 trypanosomes or more) were used for all tests.
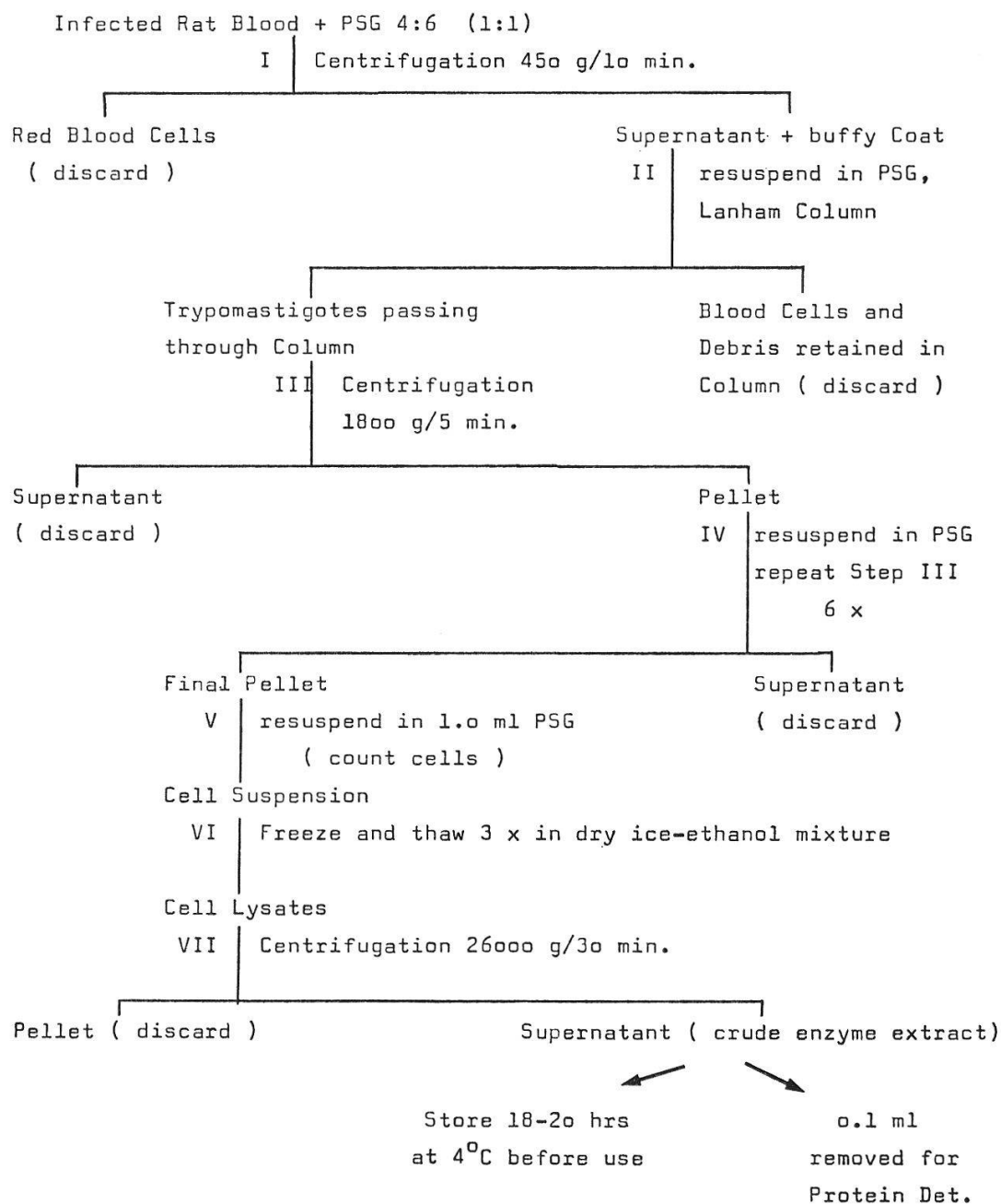
Culture stage trypomastigotes were grown in Cross' HX 12 Semi-Defined Medium (CROSS & MANNING 1973). It was somewhat difficult to adapt bloodstream cells to HX 12. It was not possible to culture bloodstream cells directly in this monophasic medium; the cells required an adaptive phase in diphasic medium followed by growth in monophasic media containing blood derivatives before continuous growth in semi-defined medium was possible. The following procedure was designed to obtain yields sufficient for enzyme assays: 0.5 ml infected rat blood (with a small amount of ESG buffer [EDTA-saline-glucose] to prevent coagulation) was added to a slant of modified Tobie's Medium (TAYLOR & BAKER 1968) lacking an overlay. 0.5 ml of HX 12 was then added and the cells permitted to adapt for 5 days. After 5 days, the cell suspension was transferred into 30 ml Falcon disposable screwcap Tissue Culture flasks containing 1.0 ml of modified Pittam's Medium (DIXON & WILLIAMSON 1970), in which fresh horse blood was substituted for human blood. Four days later 2.0 ml of Pittam's medium was added to the flasks giving a total of 4.0 ml of medium. After 4 days, the cell suspension was divided into two equal parts of 2.0 ml in Tissue Culture flasks to which 2.0 ml HX 12 was added and the cells allowed to adapt for another 4 days. 1.0 ml of this suspension was added to flasks containing 4.0 ml HX 12 and the remaining subcultures carried out in HX 12. Using this procedure we have been able to adapt every strain of *T. b. brucei* thus far tested.

In addition to the above strains, we also tested S 42, an old culture strain derived from a pleomorphic *T. b. brucei* infection (obtained from G. Cross, The Molteno Institute, Cambridge University). This strain was already adapted to HX 12.

All cultures were maintained at 25–26 °C with transfers made every fourth day. It was necessary to have at least 10^6 cells in the inoculum to ensure a sufficiently high yield of organisms on day 4 (approximately 5.0×10^7 cells/5.0 ml HX 12).

Enzyme measurements

Enzyme extracts were prepared as in Fig. 1, with bloodstream material starting at step I and culture material starting at step IV. Phosphate-saline-glucose (PSG) 4 : 6, pH 8.0 for bloodstream forms and pH 7.4 for culture forms, was prepared as directed by LANHAM & GODFREY (1970). DEAE-cellulose (Whatman DE 52) column separation was carried out according to the method described by the above authors, with four steps carried out at 4 °C to retard cell deterioration. There was no change in the % of pleomorphism after column separation. Cell counts

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Infected Rat Blood + PSG 4:6  (1:1)
         I │ Centrifugation 45o g/1o min.
    ┌──────┴────────────────────────────────┐
Red Blood Cells                      Supernatant + buffy Coat
( discard )                          II │ resuspend in PSG,
                                         Lanham Column
                          ┌──────────────┴────────────┐
              Trypomastigotes passing         Blood Cells and
                 through Column               Debris retained in
                    III │ Centrifugation      Column ( discard )
                          18oo g/5 min.
        ┌─────────────────┴──────────────────┐
    Supernatant                           Pellet
    ( discard )                           IV │resuspend in PSG
                                              repeat Step III
                                                   6 x
            ┌─────────────────────────────────────┴───────┐
        Final Pellet                              Supernatant
            V │ resuspend in 1.o ml PSG          ( discard )
                   ( count cells )
        Cell Suspension
           VI │ Freeze and thaw 3 x in dry ice-ethanol mixture
        Cell Lysates
          VII │ Centrifugation 26ooo g/3o min.
    ┌─────────┴────────────────────────────────────────┐
Pellet ( discard )                    Supernatant ( crude enzyme extract)
                                          ↙           ↘
                              Store 18-2o hrs        o.1 ml
                              at 4°C before use      removed for
                                                     Protein Det.
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N.B. Steps II,III,IV,VII were carried out in the cold.

Fig. 1. Flow diagram of preparation procedure for crude enzyme extracts from *T. b. brucei* and *T. b. rhodesiense* bloodstream and culture stages. Bloodstream material starts with step I, whereas culture cells start with step IV.

were done with a haemocytometer and total protein content was estimated by the Folin Phenol method (Lowry et al. 1951).

We found that cells lysed with distilled water before freezing and thawing did not give ALAT : ASAT ratios that were different from those obtained by our usual method. Crude enzyme extracts obtained by sonication (MSE, 100 Watt) rather than by freezing and thawing had a slightly lower transaminase ratio. In

some tests high speed centrifugation of the lysate was done at 100,000 g/40 min; enzyme extracts in these gave an ALAT:ASAT ratio that was slightly lower (less than 10 %). Occasionally enzyme extracts were kept for longer than 20 hours; in these cases 1 mM dithiothreitol was added as a stabilizing agent; otherwise there was about a 10 % daily loss of transaminase activity.

ALAT and ASAT activity was measured spectrophotometrically with Biochemica Test Combination GOT (ASAT) and GPT (ALAT) Kits according to the manufacturer's specifications at 25 °C. These indicator (coupled enzyme) tests were read at 366 nm in an Eppendorf spectrophotometer. Indicator enzymes are present in excess to obviate the effect of physical change. The reaction mixture contained 3,000 μl 0.1 M phosphate buffer, pH 7.4 (with 80 mM DL-alanine for ALAT or 40 mM L-aspartate for ASAT), 50 μl NADH$_2$ (12 mM), 50 μl indicator enzyme (0.25 mg/ml LDH for ALAT or 0.25 mg/ml LDH and 0.25 mg/ml MDH for ASAT), 500 μl test solution, and 100 μl α-oxoglutarate (0.25 M).

Only the initial, linear reactions (first 3–5 minutes) were followed. ALAT and ASAT values were calculated according to test kit directions and are given in both mU/ml or U/mg protein. In order to determine if the test kits' substrate concentration or pH were limiting transaminase activity, apparent Km (α-oxoglutarate, double reciprocal plots) and pH optima values for ALAT and ASAT in the crude extracts were studied as described by BERGMEYER & BERNT (1970) with the methods adapted for microcuvette analysis. In these tests linearity in O.D. decrease of NADH was noted for at least two minutes. Controls were performed with bloodforms (EATRO 1971) and culture forms (S 42) to determine what changes in activity were due to the test enzyme and which were the result of ancillary factors such as NADH$_2$ recycling or other competing reactions. The procedures for kinetic and pH optima studies were essentially those of FAIR & KRASSNER (1971).

In several experiments transaminase activity was measured after cells had been subjected to the BIIT or to ESG buffer controls. The method employed was that of RICKMAN & ROBSON (1970 a, b).

Statistical analyses

Most of the statistical tests used to compare specific and relative aminotransferase activities are listed in the results. Specific and relative aminotransferase activities in crude extracts from bloodstream trypomastigotes did not fall into a normal distribution (χ^2 test and David test, SACHS 1969), so that non-parametric techniques were necessary for the analyses of these forms (DOCUMENTA GEIGY, Scientific Tables; SIEGAL 1956). Despite this fact means and standard errors of the enzyme values are given for convenience. Culture stage specific and relative aminotransferase activities were normally distributed so that statistical tests based on normality were used for culture forms except when they were compared with bloodstream trypanosomes. All values of strains (cloned and non-cloned) were tested with the H-test (SACHS 1969) to see if all the strains within a species formed a statistically single population; this turned out to be a proper assumption (P < 0.05). Within each species the activities of the strains were therefore pooled.

Occasionally we found that cells used for enzyme tests appeared abnormal (e.g. they moved abnormally or were cytologically in poor condition). In these cases, the specific and relative transaminase activities were tested with the outlying range method (MOSHMAN & ATLA) to see if the values should be excluded from the sample. Two values were discarded as a result of this test and were not included for analyses.

Table 1. Specific and relative activities of the soluble alanine and aspartate amino-transferases (ALAT and ASAT) of *Trypanosoma brucei* subgroup Trypanosomes (means ± S.E.)

Subspecies	Strains	Stage [a]	Specific [b] activity ALAT	Specific [b] activity ASAT	Relative [c] activity ALAT:ASAT
T. b. brucei (15) [d]	EATRO 1961	B	0.534 ± 0.108	0.179 ± 0.057	4.096 ± 0.418
T. b. brucei (6)	EATRO 1532	B	0.287 ± 0.064	0.077 ± 0.016	4.383 ± 0.796
T. b. brucei (25) [e]	All strains	B	0.422 ± 0.068	0.130 ± 0.032	4.204 ± 0.342
T. b. brucei (26)	S 42	C	0.506 ± 0.059	0.107 ± 0.010	4.870 ± 0.267
T. b. brucei (8)	EATRO 1529, 1532, 1961	C	0.215 ± 0.032	0.083 ± 0.014	3.500 ± 0.488
T. b. rhodes. (15)	EATRO 1873	B	0.373 ± 0.083	0.098 ± 0.016	3.117 ± 0.235
T. b. rhodes. (5)	EATRO 1132	B	0.376 ± 0.072	0.138 ± 0.027	2.740 ± 0.358
T. b. rhodes. (25) [f]	All strains	B	0.374 ± 0.046	0.122 ± 0.038	2.973 ± 0.174

[a] B = bloodstream stage, C = culture stage.
[b] Units enzyme (1 micromole substrate/min)/mg Protein N at 25 °C.
[c] ASAT = 1.0.
[d] ( ) = number of determinations.
[e] Including values from Stabilates EATRO 1529 and STIB 335.
[f] Including values from Stabilates STIB 338/339.

Results

Specific and relative transaminase activity

The specific and relative activities of the soluble alanine and aspartate aminotransferases of *T. b. brucei* and *T. b. rhodesiense* (bloodstream and culture stages) are shown in Table 1. The standard errors of enzyme values are greater for blood stream forms than those found in culture stages. The *T. b. brucei* (all blood strains) mean relative ALAT:ASAT value was 4.204 ± 0.342 while the mean ALAT:ASAT value for *T. b. rhodesiense* (all blood strains) was 2.973 ± 0.174.

In *T. b. brucei* culture forms, a relative activity of 4.87 ± 0.267 was found for strain S 42 whereas 3.50 ± 0.488 was found for the other strains.

We obtained, however, overlapping ranges for the ALAT:ASAT ratios in bloodforms of both subspecies (*T. b. brucei:* 1.95–7.48; *T. b. rhodesiense:* 1.36–5.90).

Comparative analyses

Comparative analyses of specific and relative transaminase activity between the different subspecies, strains and stages are listed in Table 2 (Group I vs. Group II).

Table 2. Comparison of specific and relative activities of the soluble alanine and aspartate aminotransferases (ALAT and ASAT) of *Trypanosoma brucei* subgroup Trypanosomes

Group I			Group II			Activity	Stat.	P	Signif.
Ssp.	Strains	Stage	Ssp.	Strains	Stage	compared	tests		diff.
brucei	1961	B	*brucei*	1532	B	ALAT : ASAT	I *	> 0.05	no
brucei	S 42	C	*brucei*	1961, 1529, 1532	C	ALAT : ASAT	II	0.01	yes
brucei	1961, 1529, 1532	B	*brucei*	1961, 1529, 1532	C	ALAT : ASAT	III	0.05	yes
							IV	0.01	
brucei	S 42	C	*brucei*	1961, 1529, 1532	C	ALAT	II	0.02	yes
brucei	S 42	C	*brucei*	1961, 1529, 1532	C	ASAT	II	0.30	no
brucei	1961, 1529, 1532	B	*brucei*	1961, 1529, 1532	C	ALAT	III	0.01	yes
							IV	0.01	
brucei	1961, 1529, 1532	B	*brucei*	1961, 1529, 1532	C	ASAT	III	> 0.05	no
							IV	> 0.05	
rhodes.	1873	B	*rhodes.*	1132	B	ALAT : ASAT	I	> 0.05	no
rhodes.	All	B	*brucei*	All	B	ALAT : ASAT	V	0.02	yes
							VI	0.05	
							VII	0.05	
rhodes.	All	B	*brucei*	All	B	ALAT	V	0.05	yes
							VI	0.01	
							VII	0.05	
rhodes.	All	B	*brucei*	All	B	ASAT	V	0.10	no
							VI	0.10	
							VII	0.20	

* I = Fisher Exact Probability Test; II = t-Test; III = Sign-Test; IV = Wilcoxon Matched-Pairs Signed-Ranks Test; V = U-Test; VI = Wald and Wolfowitz Test; VII = Median Test.

There was no difference in relative activity between any of the bloodstream intra-subspecies strains. All the values measured for *T. b. brucei* bloodstream forms fell into a single population and the same was true for *T. b. rhodesiense* bloodstream forms.

A significant difference in the relative ALAT : ASAT activity was found between *T. b. brucei* bloodstream and culture stages. Ratio values and ALAT values between S 42, an old laboratory culture strain, and the other, recently culture-adapted strains, were significantly different; for this reason values obtained for S 42 were not pooled with the other culture strains in the comparative analyses.

The most significant findings were that the mean ALAT : ASAT ratio and ALAT specific activity of *T. b. brucei* and *T. b. rhodesiense* blood stream forms were significantly different.

Table 3. Factors having no significant effect on specific and relative activities of the soluble alanine and aspartate aminotransferases (ALAT and ASAT) of T. brucei subgroup trypanosomes

Factor	Subspecies	Stage	Activity	Statistical tests	P >
Pleomorphism	T. brucei *	B	ALAT ASAT	II ***	0.10 0.05
	T. rhodesiense *	B	ALAT ASAT	II	0.10 0.10
Parasitaemia (number of parasites)	T. brucei or T. rhodesiense	B	ALAT : ASAT ALAT ASAT	II	0.10 0.10 0.10
BIIT incubation or ESG control incubation	T. brucei ** or T. rhodesiense	B	ALAT : ASAT	III	0.10
Number of passages in culture	T. brucei S 42	C	ALAT : ASAT ALAT ASAT	IV	0.10 0.10 0.10
Number of passages in rats	T. brucei	B	ALAT : ASAT	II	0.10

* The average pleomorphic proportions do not differ between T. brucei and T. rhodesiense tested. However, within each subspecies there was a positive significant correlation between enzyme ratio and pleomorphism; T. brucei, all strains – P = 0.001; T. rhodesiense, all strains – P = 0.02 (Spearman's Rank Correlation Test).

** T. brucei does not survive BIIT but does survive ESG Control Incubation.

*** I = Median Test; II = Spearman's Rank Correlation (R); III = Wald and Wolfowitz Test; IV = Correlation Test (r).

We found a positive significant correlation between enzyme ratios and the degree of pleomorphism (percentage slender forms) in both T. b. brucei and T. b. rhodesiense (Fig. 2). Although we did not subject the data to statistical analysis, the values shown in Fig. 2 suggest that the correlations between these two parameters in both subspecies is the same.

The level of parasitaemia had no effect on specific or relative activities of bloodstream forms (Table 3). BIIT incubation and/or ESG control incubation had no detectable effect on specific or relative aminotransferase activities (Table 3). BIIT incubation inhibits T. b. brucei so that this was not tested.

The number of subpassages in culture was not related to specific and relative enzyme values found in T. b. brucei, S 42 (Table 3). It has
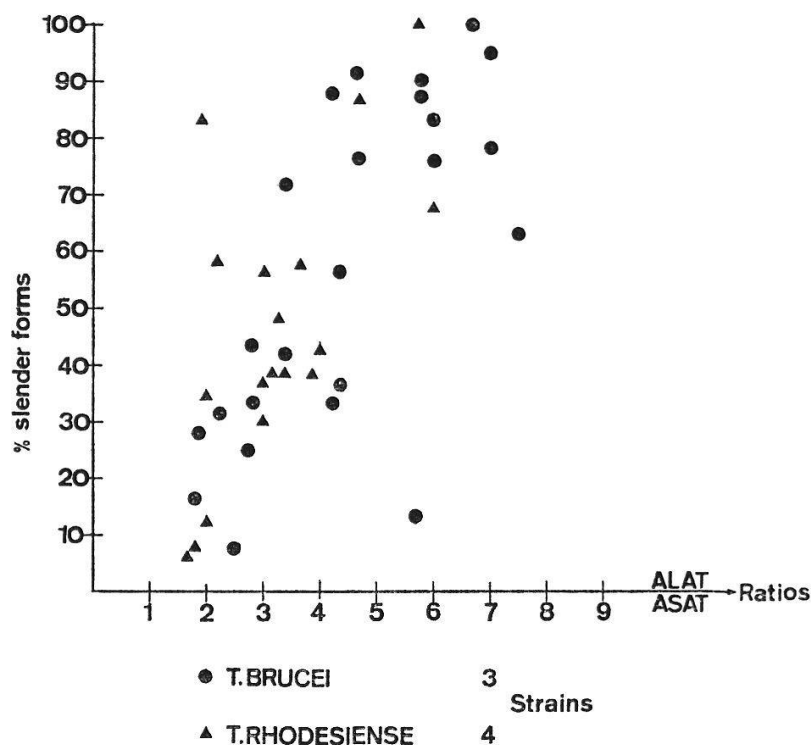
Fig. 2. The effect of pleomorphism (% slender forms) in *T. b. brucei* and *T. b. rhodesiense* rat infections on ALAT:ASAT ratios of soluble enzyme extracts.

not been possible to obtain forms infective for mice or rats in any of the culture forms grown in HX12; this suggests that only midgut-like trypomastigotes prevail in the culture flasks, a view supported by observations of Giemsa stained smear preparations. The number of subpassages in rats (up to 7) also had no influence on *T. b. brucei* bloodstream ALAT:ASAT values (Table 3).

Kinetic analyses

Preliminary apparent Km and pH optima tests on *T. b. brucei* S 42 culture forms and EATRO 1961 bloodstream stages indicate that the buffer used in the test kits was probably not optimal. Maximal activity in crude extracts of *T. b. brucei* bloodforms was obtained at pH 8.0 with TRIS buffer for both ALAT and ASAT (Table 4).

The test kit phosphate buffer (pH 7.4) permitted 50% of the maximal activity for ALAT and 90% for ASAT.

In crude extracts of *T. b. brucei* culture forms the activities read at the test kit pH represented 90% for ALAT and 70% for ASAT of the maximal activities obtained at pH 6.0 for ASAT (phosphate buffer), and 6.0 (phosphate buffer) and 7.2 (TRIS) for ALAT.

This demonstrates quite clearly that the values for specific and

Table 4. Kinetic analyses of specific activities of the soluble alanine and aspartate aminotransferases (ALAT and ASAT) of *T. brucei brucei*

Test	Strain	Stage	Enzyme	
			ALAT	ASAT
Apparent * Km	1961 S 42	B C	6.4×10^{-4} M 5.1×10^{-4} M	2.5×10^{-4} M 4.0×10^{-4} M
pH * Optima	1961 S 42	B C	$\backsim$ 8.0 $\backsim$ 6.0 and 7.2 **	$\backsim$ 8.0 $\backsim$ 6.0

    * Reaction mixture given in Material and Methods.
** pH Optima appeared as two plateaus.

Table 5. Endogenous rates of control reactions

Stage	Test	No. of Det.	Substrate omitted	Average percentage of normal assay activity
Blood	ALAT	4	α-oxoglutarate	2.8
		4	alanine	0
		4	LDH	0
		2	$NADH_2$	0
		4	test enzyme	0
	ASAT	4	α-oxoglutarate	0
		4	aspartate	0
		4	MDH	77
		2	$NADH_2$	0
		4	test enzyme	0
Culture	ALAT	4	α-oxoglutarate	7.7
		4	alanine	21.0
		6	LDH	0
		2	$NADH_2$	0
		4	test enzyme	0
	ASAT	4	α-oxoglutarate	21.0
		4	aspartate	74.0
		6	MDH	83.0
		2	$NADH_2$	0
		4	test enzyme	0

relative aminotransferase activities under test kit conditions do not represent true, optimal enzyme activities.

The control tests performed revealed endogenous reaction rates (Table 5). $NADH_2$ recycling was not observed in either bloodforms or culture forms.

Discussion

Transaminases seem to play an important role in amino acid metabolism of haemoflagellates (FAIR & KRASSNER 1971; CHAPPELL et al. 1972), especially in proline oxidation (KRASSNER 1969; FORD & BOWMAN 1973).

GODFREY & KILGOUR (1973), however, were the first to suggest the use of ALAT : ASAT ratios as an aid in differentiation between the bloodstream forms of haemoflagellate species. These workers presented specific and relative aminotransferase values for *T. b. brucei*, but made no attempt to differentiate it from the morphologically indistinguishable closely related subspecies *T. b. rhodesiense*. They did show, though, that ALAT : ASAT ratios were stable species characteristics. In addition, the same authors show subgeneric differences in the electrophoretic mobility of ALAT between two strains of *T. b. brucei* (KILGOUR & GODFREY 1973). At the same time, by using the ratio values, they could delineate trypanosome phylogenetic relationships (GODFREY & KILGOUR 1973); bloodstream trypanosomes of the archaic *T. lewisi* group show the lowest ratios (mean: 0.3) and those of the more evolved *T. brucei* subgroup the highest ones (mean: 5.9). Specific ASAT activity and electrophoretic patterns of ASAT have been used for determining evolutionary ranks from lower to higher vertebrates (SEREBRENIKOVA & FILOSOFOVA 1969; SERBAN & COTARIU 1970).

In the present study, specific and relative ALAT and ASAT activities in soluble crude cell extracts of *T. b. brucei* and *T. b. rhodesiense* bloodforms were found to be subspecies characteristics, at least with our strains tested and the mean values concerned. The number of subcultures and the number of subpassages or level of parasitaemia (number of parasites) in rats had no effect on transaminase values. A problem in our investigation was that the *T. b. brucei* strain used for studying the effect of subculturing on enzyme activity, S 42, is an old adapted culture strain, which differs significantly from the other freshly adapted culture strains. A previous study showed that S 42 bloodforms differed from another bloodform *T. b. brucei* strain on the basis of ALAT electrophoretic behaviour (KILGOUR & GODFREY 1973). It would be valuable to study possible enzyme changes in freshly adapted strains during and after the adaptation procedure. In addition, tests should be carried out on *T. b. rhodesiense* culture forms since they have not yet been studied.

Our *T. b. brucei* specific and relative aminotransferase values and their S.E. are almost identical to those reported by GODFREY & KILGOUR (1973, and personal communication). Since they used different strains from ours, this strongly supports their conclusion that aminotransferase activity is a stable species (and/or subspecies) characteristic.

The variation (S.E.) in specific enzyme activity was less in culture

forms than in bloodstream forms (Table 1). This interesting result and the nonnormal distribution of bloodstream enzyme values may be due to the facts that:

1) harvesting of culture forms is simpler than that of bloodstream trypomastigotes,
2) bloodstream form crude enzyme extracts may sometimes contain a slight host ALAT and ASAT contamination, in spite of thorough washing, and
3) population heterogeneity, such as pleopmorphism, in blood stream parasites affects transaminase activity levels.

However, crude extract contamination by endogenous substrates and/or competing enzymes was more obvious in control tests of culture forms than in those of bloodstream forms.

It is known that defined *in vitro* conditions, such as glucose concentration in *Tetrahymena* (PORTER & BLUM 1973), and ion concentration in *T. b. brucei* (GODFREY & TAYLOR 1972) and in mitochondrial preparations from vertebrate cells (CHENG et al. 1971; RENDON & WAKSMAN 1971) can affect the activities of ASAT. Moreover, casein-hydrolysate, which is one of the constituents in Cross' HX 12 Medium, increased the hepatic level of ALAT, but not ASAT, in protein-depleted rats (PESTANA 1969).

Similarly, different buffers at the same pH have an influence on transaminase levels; this was shown before for ALAT in rat liver (SEGAL et al. 1962) and by SMIRNOV et al. (1970) for ALAT and ASAT in swine duodenum. We found a clear buffer effect in specific ALAT activity in *T. b. brucei* bloodforms, and in specific ASAT activity in *T. b. brucei* culture stages, S 42 (TRIS > phosphate). The pH optima and apparent Km values of ALAT and ASAT found during the present study (Table 4) are similar to those found in culture promastigotes of *Leishmania tarentolae* (FAIR & KRASSNER 1971), even though our data are derived from crude extracts only.

It is well known that, apart from extract impurity, modifications of cell preparation affect transaminase activity levels: e.g. ultrasonic rupture of the cells stimulates ALAT, but inhibits ASAT in blood serum of white rats (CHIRKIN & KOZIN 1970). The anticoagulant applied also has an influence on aminotransferase activity; heparin, which was used in the present study, is the most suitable anticoagulant for determing serum ALAT activities (KOUDELA et al. 1969). Freezing and thawing is superior to other cell disruption methods. This is due to higher activities of mitochondrial ASAT isoenzyme released from the more thoroughly disrupted mitochondria (ZELEZINSKAYA 1969).

The physiological basis for the positive significant correlation between pleomorphism and enzyme ratio in bloodforms of *T. b. brucei* and *T. b. rhodesiense* is not yet fully understood. Our supposition that

the correlation between these two parameters was the same for both subspecies is not disproved by our data (Fig. 2). Thus enzyme activity differences between the two subspecies are probably not due to a significantly different pleomorphic "force".

The positive significant correlation found indicates quite clearly that an increasing proportion of stumpy bloodforms parallels a decrease in enzyme ratio. This effect is caused by a decrease of ALAT. ASAT may play a relatively more important role than ALAT in the respiration of the short stumpy forms. It was shown earlier that stumpy forms oxidize α-oxoglutarate (BOWMAN et al. 1972; FLYNN & BOWMAN 1973). Hence, ASAT could regulate the interconversion between the latter substrate and glutamate or oxaloacetate. Glutamate can be derived from the oxidation of proline, which is a major substrate for respiration in culture haemoflagellates (FAIR & KRASSNER 1971; BOWMAN et al. 1972; KRASSNER & FLORY 1972; SYLVESTER et al. in press), but can be metabolized, in part, also by the stumpy bloodforms of *T. b. brucei* (BOWMAN et al. 1972; FLYNN & BOWMAN 1973).

Biochemically and ultrastructurally, stumpy forms can be regarded as precursors of the established culture forms. Oxidative metabolism of the former is only partly sensitive, whereas that of the latter is 100 % sensitive to KCN. This indicates the presence of an active cytochrome chain in culture forms.

These facts possibly explain the even lower ALAT : ASAT ratio in culture forms of *T. b. brucei*.

ALAT, on the other hand, may be more important for slender bloodforms, an idea supported by the positive correlation between enzyme ratio and percentage of slender forms (Fig. 2). Alanine aminotransferase has a high level of activity in monomorphic *T. b. gambiense* (CHAPPELL et al. 1972).

Probably our most important finding was the significant difference in bloodform ALAT : ASAT and specific ALAT activity between the two subspecies. However, as mentioned before, there was an overlap in these activities. Many more, well characterized strains should be tested, especially field isolates. Possible transitions between *T. b. brucei* and *T. b. rhodesiense* cannot be ruled out.

The significant activity differences between *T. b. brucei* bloodstream and culture stages is fascinating, but it is not possible to state whether these differences reflect true underlying metabolic properties of either stage because of the crude nature of the extracts. The use of crude enzyme extracts brings along disadvantages, such as endogenous reaction rates caused by effects of competing enzymes and/or hidden substrate pools (Table 5). This is especially the case in culture forms where the active mitochondrial enzyme systems may somehow interact with the two reaction steps of our test kit assay. In addition, the non-

optimal conditions in the present assay may also affect the enzyme values. True specific and relative enzyme activities and kinetic analyses should be determined on purified material to ascertain if the enzyme differences found between subspecies, strains or stages are not due to the present assay conditions. In general, we can assume that the enzyme activities found in the crude extracts were affected by the test kit assay conditions.

Studies should likewise be done to determine if isoenzymes are present, and where these are located (mitochondrion, cytoplasm, peroxisome).

In conclusion, we feel that, in conjunction with other methods such as the BIIT, use of ALAT : ASAT relative activities as an aid in differentiation between closely related haemoflagellate species is a promising field that should be further explored. Transaminase studies could be a start for determination of enzyme patterns in these trypanosomes, as has been done in other protozoans (e.g. HOFER et al. 1972)

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Zusammenfassung

Bei Verwendung von «Biochemica Test Kombinationen» (Boehringer GmbH, Mannheim) wurde ein signifikanter Unterschied im Alanin Aminotransferase : Aspartat Aminotransferase-(ALAT : ASAT)-Quotienten zwischen Blutformen von *Trypanosoma brucei brucei* (4 Stämme, Mittelwert ± S.E.: 4,204 ± 0,342) und *T. brucei rhodesiense* (4 Stämme, Mittelwert ± S.E.: 2,973 ± 0,174) gefunden. Es wurden ungereinigte lösliche Zellextrakte verwendet.

Dieser Unterschied ist charakteristisch und wird durch folgende Parameter nicht beeinflußt: Art der verwendeten Stämme, Zahl der Parasiten im Blut und Anzahl Subpassagen in Ratten.

ALAT : ASAT-Quotienten und spezifische ALAT-Aktivitäten in Blut- und Kulturformen von *T. b. brucei* unterschieden sich signifikant voneinander.

Kontrollversuche zeigten den Wert des Tests bei der Verwendung von groben löslichen Enzymextrakten.

Innerhalb der beiden Subspezies wurde eine positive signifikante Korrelation zwischen Pleomorphismus (Prozentsatz Langformen im Blut) und ALAT : ASAT-Quotient gefunden.

Résumé

Au moyen de «Biochemica Test Kombinationen» (Boehringer GmbH, Mannheim) une différence significative a été trouvée dans les quotients alanine aminotransférase : aspartate aminotransférase (ALAT : ASAT) entre les formes sanguicoles de *Trypanosoma brucei brucei* (4 souches, moyenne ± S.E.: 4,203 ± 0,342) et *Trypanosoma brucei rhodesiense* (4 souches, moyenne ± S.E.: 2,973 ± 0,174). Les extraits d'enzymes utilisées étaient solubles et bruts.

Cette différence est caractéristique et n'est pas influencée par le type de souches utilisées, le nombre de parasites dans le sang et le nombre de sous-passages chez les rats.

Les quotients ALAT : ASAT et les activités spécifiques d'ALAT dans les formes sanguicoles et les formes cultivées de *T. b. brucei* sont différents.

Des expériences de contrôle ont démontré la valeur du test pour les préparations enzymatiques solubles brutes des deux sous-espèces de *T. brucei*.

A l'intérieur de chaque sous-espèce on trouve une corrélation positive significative entre le degré du pléomorphisme (la proportion des formes élancées dans le sang) et les quotients ALAT : ASAT.