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COMPARISON OF AFRICAN TRYPANOSOMES OF DIFFERENT ANTIGENIC PHENOTYPES, SUBSPECIES AND LIFE CYCLE STAGES BY TWO-DIMENSIONAL GEL ELECTROPHORESIS

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High resolution two-dimensional polyacrylamide gel (2D gel) electrophoresis and autoradiography were used to analyze the protein gene products of African trypanosomes biosynthetically labelled with [^{35}S]methionine. Using cloned populations of parasites it was found that: (1) antigenically different bloodstream trypanosomes from the same serodeme differed only in their variant surface glycoproteins; (2) *Trypanosoma brucei, T. b. rhodesiense* and *T. b. gambiense* subspecies could be distinguished on the basis of differences in expressed proteins; (3) transformation from bloodstream trypomastigotes to procyclic epimastigote culture forms was accompanied by loss of variant surface glycoproteins and several other qualitative and quantitative changes in minor proteins. The results indicate that 2D gel analysis may allow improved classification of African trypanosomes (based on the observation of hundreds of protein markers) and may also provide a general technique for the identification of lifecycle stage specific markers.

Key words: African trypanosomes; Procyclic culture forms; Protein gene products; Two-dimensional gel electrophoresis; *Trypanosoma brucei* spp.

INTRODUCTION

African trypanosomes are protozoan parasites which have a complex life cycle involving both tsetse fly vectors and mammalian hosts. Because of their importance as disease causing organisms and as model eukaryotes for basic research these parasites have received increasing attention in the past few years. Nevertheless, at the molecular level, relatively little is known about many aspects of their biology including antigenic variation, speciation and differentiation during the life cycle [1].

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Abbreviations: VSG, variant surface glycoproteins; 2D gels, two-dimensional polyacrylamide gels.

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One particularly important problem in studying African trypanosomiasis is the difficulty encountered in distinguishing the subspecies of the *Trypanosoma brucei* group of organisms. The relationships between the human-infective parasites *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* and the cattle-infective organism *Trypanosoma brucei brucei*, defined as non-infective for humans, are not understood. Indeed, recent observations that human-infective organisms can arise in a serodeme defined as *T. b. brucei* [2], reports on the extreme instability of trypanosomes to human serum sensitivity [3] and the increasing evidence that game and domestic animals act as reservoir hosts for *T. b. rhodesiense* and *T. b. gambiense* [3] suggest that the subspecies identification is not as straightforward as previously thought. Tests which rely on interspecific enzyme and isoenzyme polymorphism [4,5] and recently on DNA hybridization [6,7] have allowed preliminary taxonomic characterization of trypanosome stocks. Such tests sample a very small proportion of expressed genetic material, which may or may not be representative of the trypanosome genome or, in the case of DNA hybridization, do not measure expressed gene products. A more definitive approach to this problem is clearly required in order to delineate the relationships between these so-called subspecies.

The approach we have taken is to use multiple two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) as performed using the ISO-DALT system [8,9] which is designed to allow highly accurate comparisons of protein gene products in various biological samples. Using this system we have analyzed the proteins of various species and subspecies of African trypanosomes biosynthetically labelled to a high specific activity with [^{35}S]methionine and have performed an initial visual analysis of differences observed. In addition, we have examined a class of stage-specific protein markers.

MATERIALS AND METHODS

Parasites. Bloodstream form trypomastigotes and coatless procyclic culture forms of various species of African trypanosomes were used for biosynthetic labelling and high-resolution 2D-PAGE. Cloned populations of parasites were used throughout. *T. b. brucei* parasites of the IsTat 1 serodeme [10] were cloned populations derived from the original stock EATRO 164. *T. b. brucei* IsTat 1.1a (designated 164-1a) is a fast growing clone. *T. b. brucei* IsTat 1.1.3c (designated 164-21a) is a slow growing clone which is antigenically different from 164-1a. *T. b. brucei* LUMP 1026 (designated 1026) is a stabilate prepared at the London School of Hygiene and Tropical Medicine from the stabilate *T. b. brucei* EATRO 1244. This organism was passaged in rats several times as an uncloned population [11] and then cloned in the laboratory of Dr. Ken Stuart, Issaquah, Washington. All *T. b. brucei* organisms were obtained as frozen stabilates from Dr. Stuart. *T. b. rhodesiense* WRATat 3 [12] was also obtained from Dr. Stuart. *T. b. rhodesiense* ViTat 1.1 was cloned in our laboratory in Victoria from an uncloned population of *T. b. rhodesiense* EATRO 1895 [13] obtained from Dr. George Hill,

Nashville, Tennessee. *T. b. gambiense* ViTat 1.1 was cloned in our Victoria laboratory from a cloned population of *T. b. gambiense* TTrT-1 [14] obtained from Dr. Dick Seed, Chapel Hill, North Carolina. *T. b. gambiense* TREU 1285 (designated U2) and TREU 1257 (designated L2) [15] were obtained from Dr. Jack Doyle, Nairobi, Kenya via Dr. Ken Stuart. *T. congolense* IL588 was a cloned population from *T. congolense* stock 5E-12 [16].

Bloodstream forms of the trypanosomes were grown in cyclophosphamide suppressed BALB/c mice [17] and were aseptically purified from heparinized blood by chromatography on diethylaminoethyl cellulose [18]. Procyclic culture forms of trypanosomes were established from cloned bloodstream populations and maintained in culture in vitro at 26°C in SDM-79 medium according to established methods [19].

Internal labelling of parasites. Harvested organisms were adjusted to 10^7 ml^{-1} in sterile phosphate-buffered saline containing 1% glucose and 0.5 ml was added to a 35 ml tissue culture flask containing 4.5 ml of RPMI 1640 medium containing 10% dialyzed, heat-inactivated fetal calf serum (FCS), only 5% of the normal methionine concentration and 200 μCi of [^{35}S]methionine (Amersham, SJ204, Oakville, Ontario). The flasks were gassed with 5% CO_2 in air, placed horizontally and incubated for 16 h at 37°C.

After incubation the parasites were centrifuged (500 × *g*, 20 min), resuspended in 400 μl of RPMI 1640 medium containing 10% FCS and placed in a Sarstedt capillary bottom tube (No. 72.702, Sarstedt, Princeton, NJ). The trypanosomes were pelleted by centrifugation for 1 min in a microcentrifuge and the overlying medium was removed using a 0.25 ml Hamilton syringe (Hamilton Inc., Reno, NV). The pellet was solubilized in 40 μl of pH 9.0 solubilization buffer: 9 M urea, 5% 2-mercaptoethanol, 4% Nonidet P-40 (v/v), 2% pH 9–11 ampholines (LKB, Bromma, Sweden). The surface of the pellet was slowly eroded with the solubilization buffer using the Hamilton syringe. The use of this solubilization buffer minimizes degradation due to proteolysis since 9 M urea and the high pH render most proteases inactive. After 15 min at room temperature the sample was centrifuged (11 400 × *g*) for 30 sec in a microcentrifuge to remove debris and the supernatant placed in a fresh capillary-bottom tube. The tubes were stored at −70°C for a maximum of three weeks before use.

2D-PAGE. ^{35}S-labelled detergent lysates were thawed and centrifuged for 5 min (11 400 × *g*) in a microcentrifuge immediately prior to 2D gel analysis in order to remove aggregates. For each sample, 1–2 × 10^6 cpm (approximately 5 μl) were applied to isoelectric focusing gels in the ISO-DALT system [8,9] and run for 14 000 or 18 000 V h. Ampholines in the first dimension tube gels were a mixture of 60% Pharmalyte pH 5–8, 20% Pharmalyte pH 3–10, 20% Servalyte pH 3–10 (Figs. 1–5) or a mixture of 90% LKB pH 3.5–10, 10% LKB pH 2.5–4 (Fig. 6). Gradient gels (9–18% acrylamide) were used for the second dimension. Gels were dried and autoradiographed for 1–3 weeks using Kodak X-OMAT AR film.

Comparison of 2D gel patterns. Autoradiographs were compared by placing one autoradiograph over another on an X-ray light box. Superimposition of gel spot constellations allowed easy detection of qualitative differences in gel spots and in some cases permitted detection of quantitative differences. This simple procedure is accurate and reliable because the ISO-DALT multiple 2D gel system allows 20 gels (poured from the same batch of reagents) to be run simultaneously, thus all samples can be electrophoresed in one run. In addition, in this particular study the parasites used were all closely related and shared most proteins, thus allowing correct alignment of the gel autoradiographs by matching of conserved spot constellations. Interpretations of gel spot patterns were performed using previously published criteria [20,21].

RESULTS

Comparison of T. b. brucei clones within a serodeme. Protein gene products of two trypanosome clones with antigenically different variant surface glycoprotein (VSG) phenotypes (as tested by monoclonal antibody surface immunofluorescence) were compared using *T. b. brucei* 164.1a and 164.21a bloodstream organisms from the IsTar 1 serodeme. The 2D gel autoradiographs are shown in Fig. 1. The only qualitative differences observed were in the spots which were designated as VSGs by virtue of their apparent molecular weight, abundance, microheterogeneity [28] and stage-specific expression (Fig. 1A and 1B). A third organism (*T. b. brucei* 1026) which is also antigenically distinct but from a different serodeme differs from the other two *T. brucei* clones in two spots (probably by charge polymorphism) and again in VSG (Fig. 1C) (no VSG spot appears on the gel, probably because the 1026 VSG is too basic to focus in this 2D system). Indeed, Coomassie blue stained gels of this organism showed that the VSG spot was absent from the gels. Thus the absence of VSG from the gel autoradiographs was not due simply to the absence of methionine from (and lack of labelling of) the VSG. Quantitative differences seen among these three different clones were minimal. The prominent spot (labelled Q) on the extreme basic (right) side of all three autoradiographs of *T. b. brucei* was not found in 2D patterns of any other species of trypanosome or in procyclic culture forms (see below) and therefore probably represents a stage-specific subspecies marker.

Comparison of different trypanosomes within a species. Three different clones designated *T. b. gambiense* from different origins and thus likely of different serodemes were compared (Fig. 2A, B and D). The L2 and U2 clones appear extremely similar,

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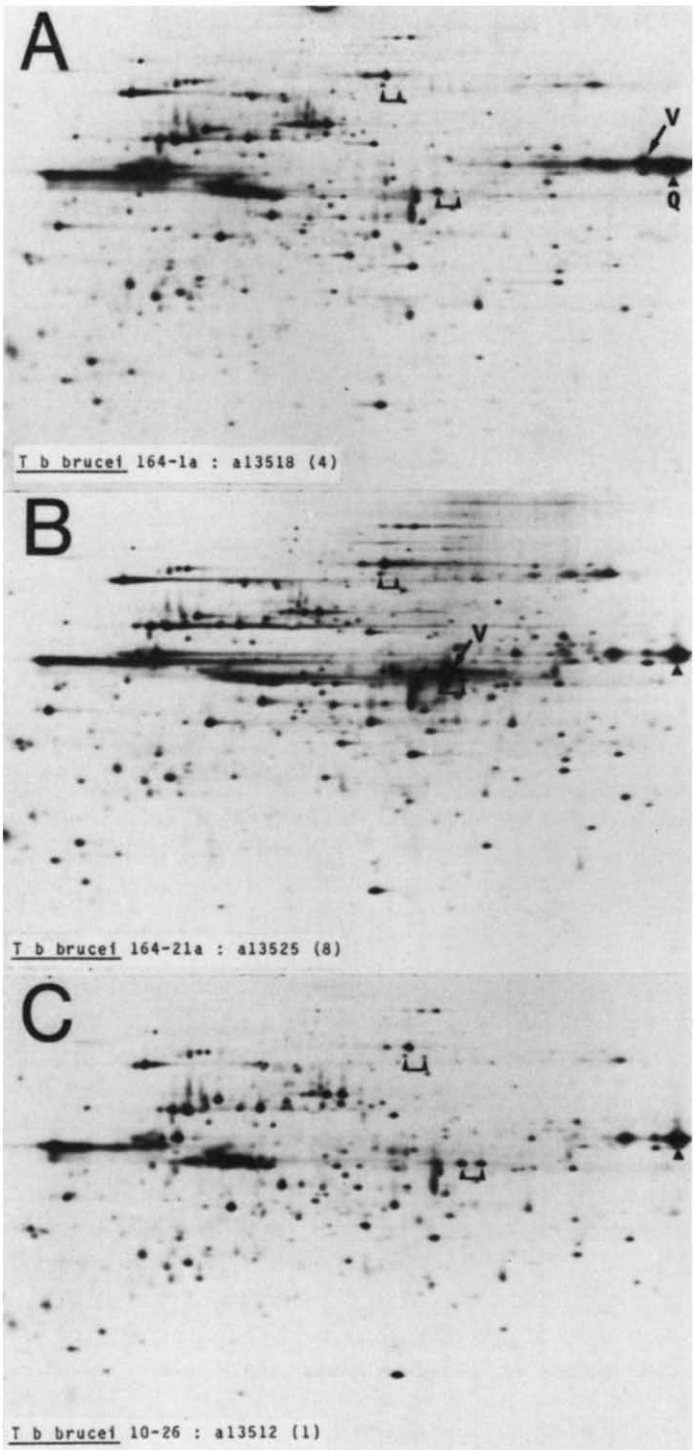
Fig. 1. 2D gel autoradiograms of [^{35}S]methionine-labelled *T. b. brucei* bloodstream clones of different antigenic phenotypes. (A) *T. b. brucei* 164-1A; (B) *T. b. brucei* 164-21A; (C) *T. b. brucei* 1026. The V indicates the main VSG spot for each clone and Q indicates a constant 'VSG-like' spot. The connected vertical arrows indicate putative charge shift allelic proteins which show changes between clones. All gel autoradiographs are shown with the acid end to the left and increasing M.W. from bottom to top.


A
V
Q
T b brucei 164-1a : a13518 (4)
B
V
T b brucei 164-21a : a13525 (8)
C
T b brucei 10-26 : a13512 (1)

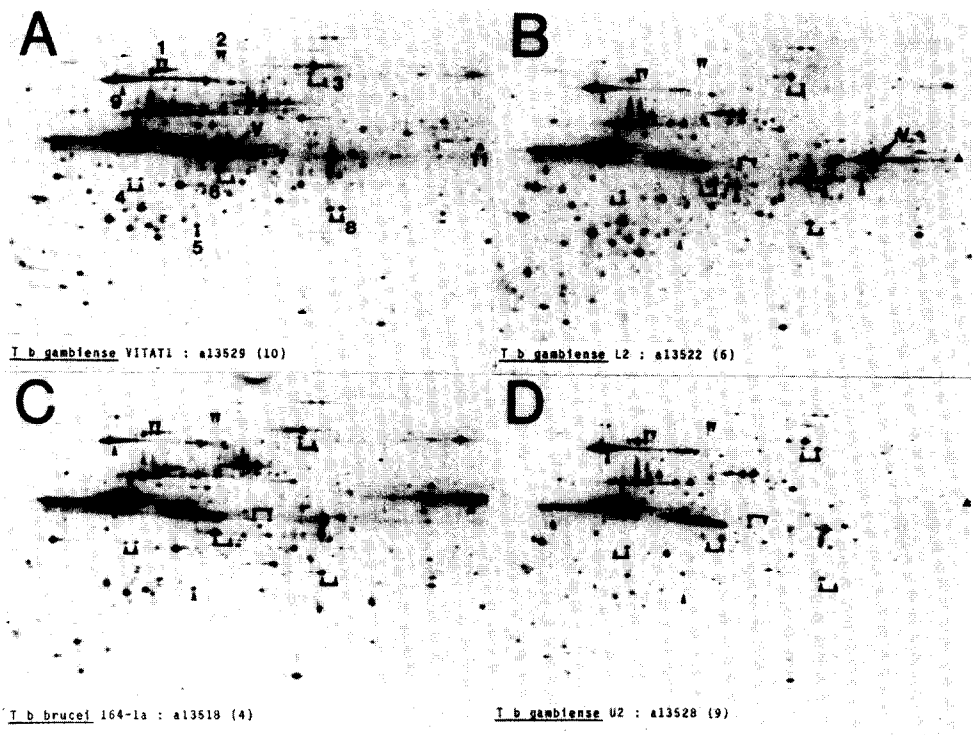


Fig. 2. 2D gel autoradiograms of [^{35}S]methionine-labelled *T. b. gambiense* bloodstream clones: comparison with *T. b. brucei*. (A) *T. b. gambiense* ViTat 1.1; (B) *T. b. gambiense* L2; (C) *T. b. brucei* 164-1A; (D) *T. b. gambiense* U2. Connected arrows indicate putative charge-shift protein polymorphisms differing between clones. Single arrows indicate proteins which are qualitatively different between clones and for which no other 'allele' has been identified.

differing only by the putative VSG spots and three large spots which appear under the VSG in the L2 organism (Fig. 2B). Both *T. b. gambiense* L2 and U2 organisms differ qualitatively from *T. b. brucei* (see Fig. 2C) in eight different spots, some of which (spots 1–4,6,7) are probably charge-shift polymorphisms, while the others are completely missing in some of the organisms or present in others (spots 5, 9). The 2D pattern of the *T. b. gambiense* ViTat 1.1 organism (Fig. 2A) resembles the pattern of *T. b. brucei* (Fig. 1C) much more closely than it resembles those of *T. b. gambiense* L2 and U2. Indeed, spots 1–6 and 9 are identical in Fig. 2A and 2C, thus these patterns show a difference only in VSG spots and in spots 7 and 8 which likely represent charge polymorphisms. Only the configuration of spot 7 is shared with U2 and L2; *T. b. gambiense* ViTat 1.1 has a unique configuration of spot 8 not shared by U2, L2 or *T. b. brucei*. The conventional classification of *T. b. gambiense* ViTat 1.1 is here considered suspect.

Fig. 3 shows 2D gel maps of two different clones of *T. b. rhodesiense*. The spot profiles

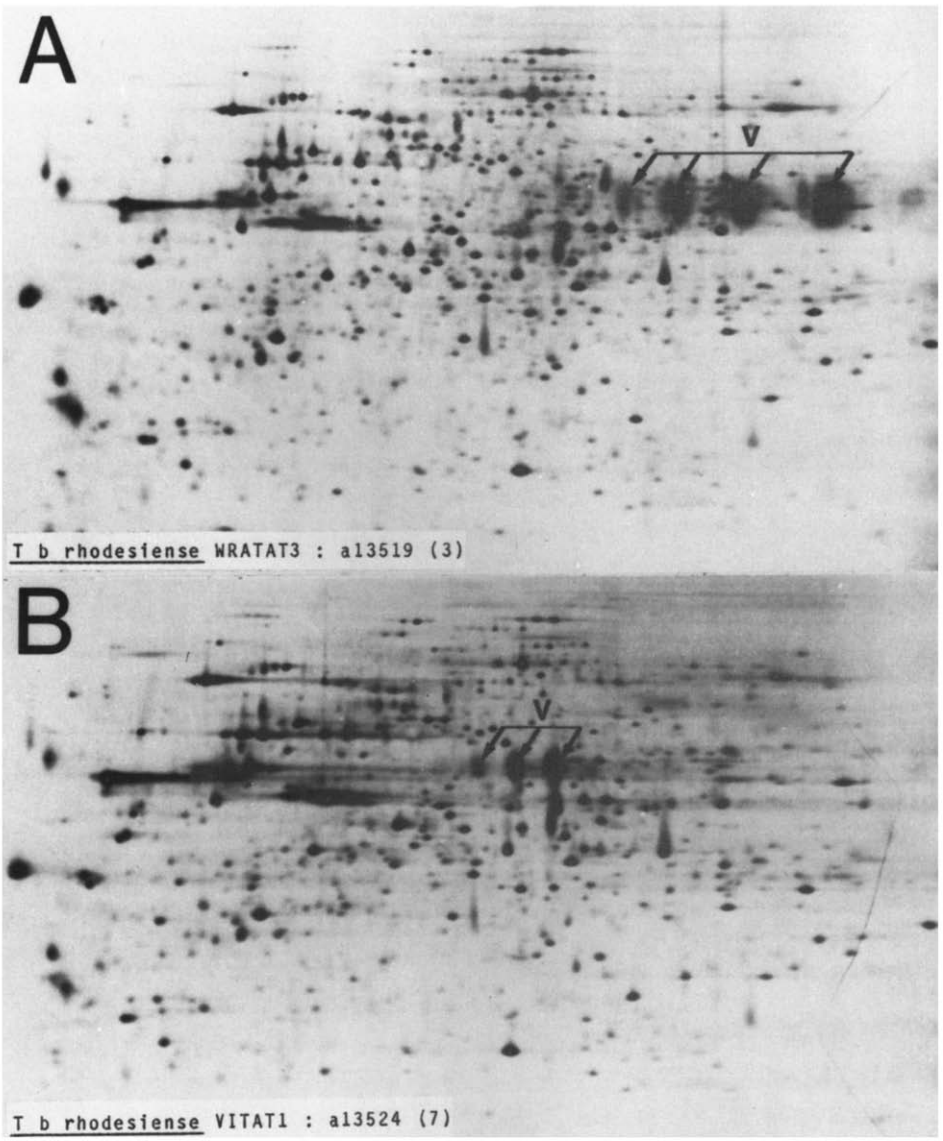
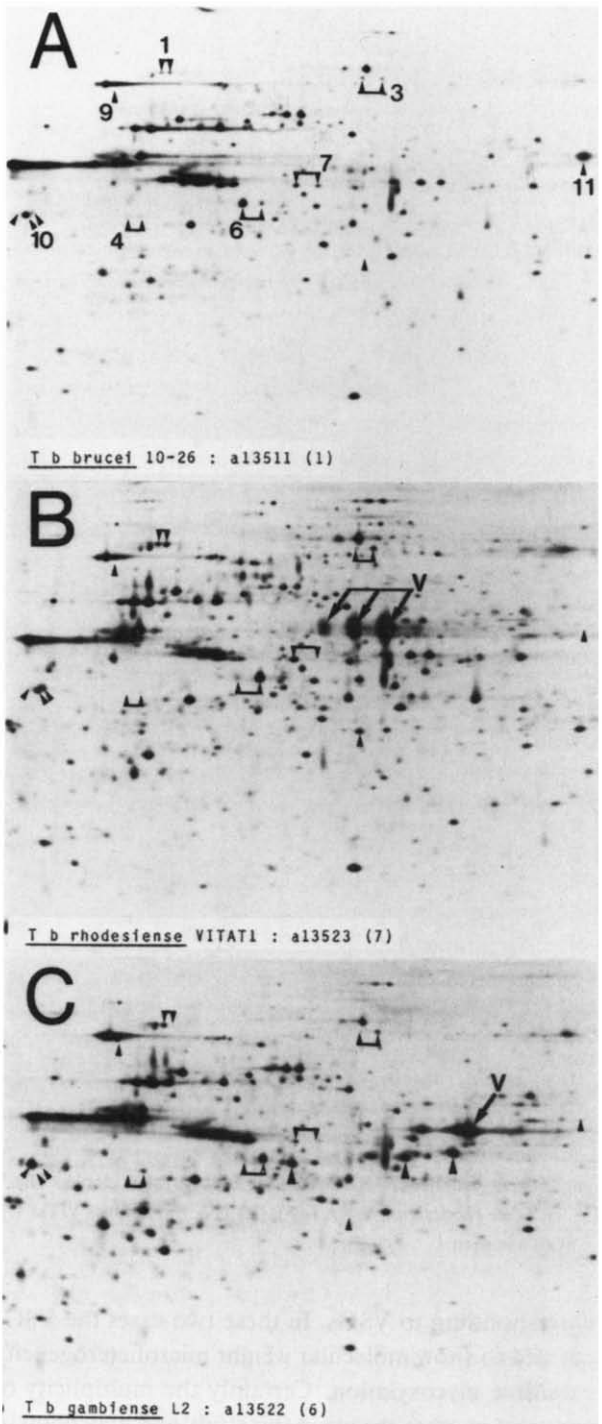


Fig. 3. 2D gel autoradiograms of [^{35}S]methionine-labelled *T. b. rhodesiense* bloodstream clones. The V indicates the charge-shifted VSG proteins. (A) *T. b. rhodesiense* WRATat 3; (B) *T. b. rhodesiense* ViTat 1.1. No qualitative differences (aside from VSGs) are seen.

differ only in a series of spots corresponding to VSGs. In these two cases the VSGs appear to be charge heterogeneous and to show molecular weight microheterogeneity [20] probably associated with extensive glycosylation. Certainly the multiplicity of VSG spots is greater than those observed in other species in this study and may point to a characteristic feature of *T. b. rhodesiense* VSGs.


A
1
3
9
7
11
10
4
6
T b brucei 10-26 : a13511 (1)
B
V
T b rhodesiense VITAT1 : a13523 (7)
C
V
T b gambiense L2 : a13522 (6)

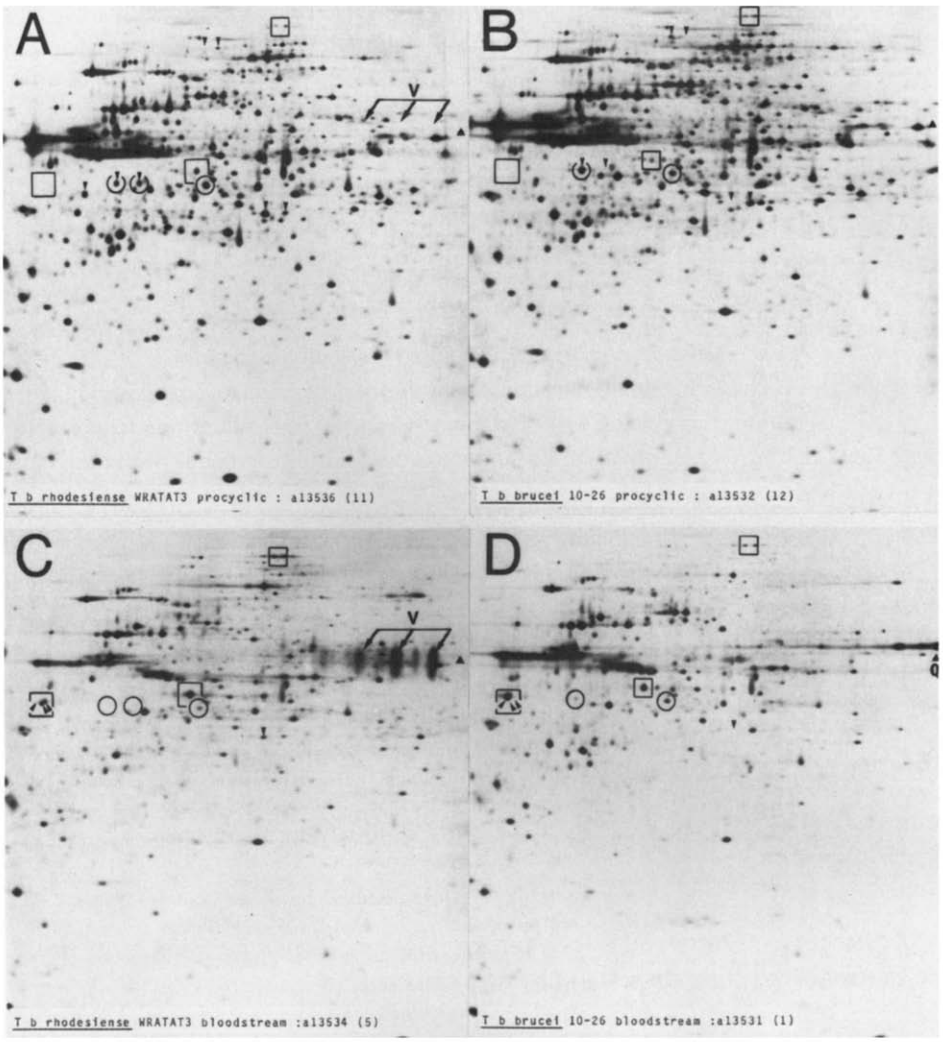


Fig. 5. 2D gel autoradiograms of [^{35}S]methionine-labelled bloodstream clones of *T. b. brucei* and *T. b. rhodesiense* and their corresponding procyclic culture forms. (A) *T. b. rhodesiense* WRATat 3 procyclic culture form; (B) *T. b. brucei* 1026 procyclic culture form; (C) *T. b. rhodesiense* WRATat 3 bloodstream form; (D) *T. b. brucei* 1026 bloodstream form. The outlined areas and arrows indicate proteins which differ qualitatively or quantitatively between the gel patterns.

Fig. 4. 2D gel autoradiograms of [^{35}S]methionine-labelled bloodstream clones of the three *T. brucei* subspecies. (A) *T. b. brucei* 1026; (B) *T. b. rhodesiense* ViTat 1.1; (C) *T. b. gambiense* L2. The connected arrows indicate charge-shift polymorphisms which differ between clones; single arrows indicate proteins which are qualitatively different between clones and for which no other 'allele' has been found. Markers are numbered consistently with Fig. 2.

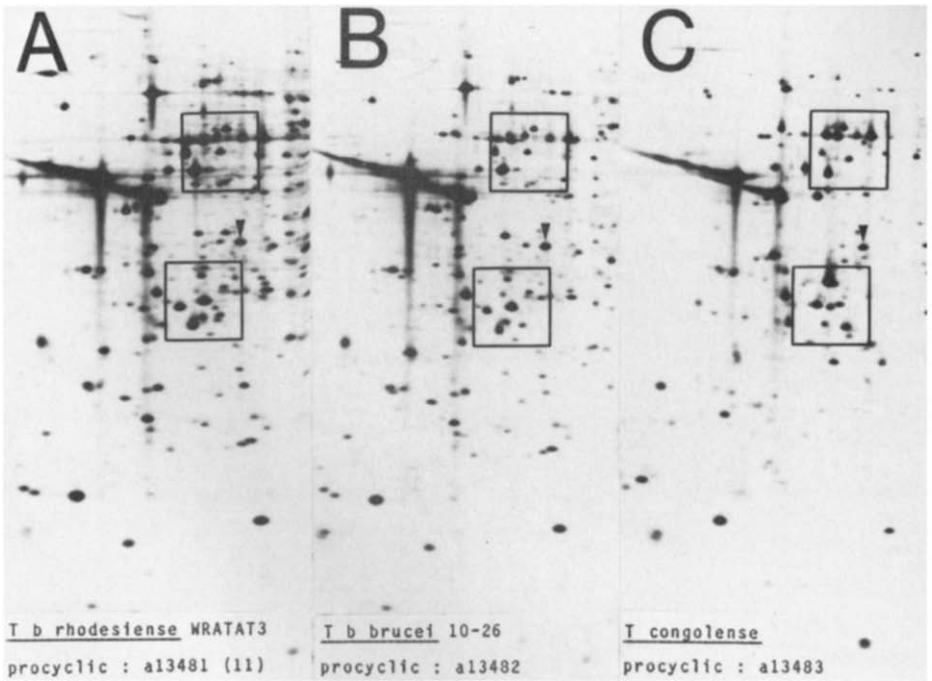


Fig. 6. 2D gel autoradiograms of [^{35}S]methionine-labelled procyclic culture forms of cloned trypanosomes of different species or subspecies. The outlined areas and arrows serve to orientate the reader. Within the boxes, *T. b. rhodesiense* and *T. b. brucei* are essentially identical, while *T. congolense* is completely different (in the sense that no spots perfectly coelectrophorese with a *T. brucei* spot). One spot (marked by the arrow) does appear to migrate identically in all three cases.

All three patterns shown by *T. b. brucei* (Fig. 1) clones are extremely similar, with only one protein spot (other than VSG) being different.

Comparison of T. b. brucei, T. b. rhodesiense and T. b. gambiense. Since within a species (with the three *T. b. brucei*, two *T. b. rhodesiense* and two *T. b. gambiense* tested here, discounting the suspect *T. b. gambiense* ViTat 1.1) the 2D gel protein gene product autoradiographs were remarkably similar (Figs. 1–3) it was of interest to compare the three *T. brucei* spp. with each other. Fig. 4 shows such a comparison. It is clear that the gel patterns differ in more than VSG spots since changes in spots attributable to charge-shift polymorphisms and in appearance or disappearance of spots can be seen. A similarity matrix comparing Fig. 4A, B and C drawn using a small set of conspicuous qualitative markers is presented in Table I. Comparison of markers in Fig. 4A (*T. b. brucei*) with Fig. 4B (*T. b. rhodesiense*) shows 7/15 identities, Fig. 4A (*T. b. brucei*) with 4C (*T. b. gambiense*) 3/15 similarities, Fig. 4B (*T. rhodesiense*) with 4C (*T. b. gambiense*) 9/15 similarities. Given the small number of markers used and the

TABLE I

Similarity matrix comparing proteins in the *T. brucei* group of African trypanosomes[a]

	T. b. brucei	*T. b. rhodesiense*	*T. b. gambiense*
T. b. brucei	15/15		
T. b. rhodesiense	7/15	15/15	
T. b. gambiense	3/15	9/15	15/15

[a] Marker proteins and data determined using autoradiograms shown in Fig. 4. Here a set of the most conspicuous qualitative differences between subspecies has been used. Each subspecies is homogeneous in the appearance of each of these markers except for marker 3 in *T. b. brucei* (where *T. b. brucei* 1026 differs from the other *T. b. brucei* in having both putative forms of the marker) and discounting *T. b. gambiense* ViTat 1.1 as a possible misidentification. The matrix values count the presence of spots; hence the pair of spots likely to be charge-shift alleles in markers 1, 3, 4, 6, 7 and 10 are counted separately for this comparison.

range of similarity values obtained, it is not clear which are more closely related; *T. b. brucei* and *T. b. gambiense* seem the most different from each other and *T. b. rhodesiense* and *T. b. gambiense* the most related.

Comparison of bloodstream and procyclic trypanosomes. Autoradiographs of 2D gels of cloned bloodstream form trypanosomes of two subspecies and the corresponding procyclic culture forms derived from them are shown in Fig. 5. Upon transformation to procyclic forms, the major VSG spots were lost from *T. b. rhodesiense* WRATat 3 (compare Fig. 5C and 5A). Similarly, although the VSG was not visible in the map of bloodstream *T. b. brucei* 1026 (Fig. 5D) the major set of basic spots (labelled Q) characteristic of all three *T. b. brucei* clones examined (see Fig. 1) disappeared upon transformation to the procyclic forms (Fig. 5B). Several other minor qualitative or quantitative differences were seen, including both increases and decreases upon transformation. One further set of subspecies markers (circles with arrowheads in panels A and B, Fig. 5) was discovered in the procyclic form that appears absent in bloodstream trypanosomes. The coregulation of both spots in *T. b. rhodesiense* (absence in bloodstream, presence in procyclic), the presence of approximately twice as much density in the single spot present in *T. b. brucei* procyclics and the separation of the two spots by a perfectly horizontal (purely p*I*) displacement strongly suggest that these two proteins are allelic products and hence that *T. b. rhodesiense* WRATat 3 is heterozygous at this locus.

Comparison of two *T. brucei* procyclic culture forms with *T. congolense* procyclics showed that a large number of proteins were qualitatively different between species (compare *T. congolense* procyclics in Fig. 6C with *T. b. rhodesiense* and *T. b. brucei* procyclics in Fig. 6A and B, respectively). In fact, the downward pointing arrow shows the only protein shown by careful comparison to perfectly co-electrophorese between *T. brucei* and *T. congolense*. Only minor differences were seen between subspecies (Fig. 6A and B).

DISCUSSION

2D-PAGE [22] is the most highly resolving technique available for analysis of the protein gene products in biological materials. The development of the ISO-DALT gel system which allows multiple isoelectric focusing gels and gradient slab gels to be poured and then run simultaneously enables extremely accurate comparisons between 2D gels run in the same batch [8,9], overcoming one of the major drawbacks to single gel or dual gel systems. Indeed, in pilot studies with replicate samples of lysates of [^{35}S]methionine-labelled African trypanosomes, the 2D gel autoradiograph patterns which showed more than 400 spots could be perfectly superimposed. The power of this electrophoresis system for intercomparison of gel patterns is evident from the autoradiographs of the three *T. b. brucei* clones shown in Fig. 1. Of more than 250 clearly visible spots only two spots attributable to the VSGs and one quantitative difference in a minor spot were different between antigenically distinct trypanosomes from the same serodeme. Thus the only major protein difference between antigenic variants was in the VSG molecules as had been reported previously [23]. The clones used in the current study also showed greatly different growth rates in vivo (K. Stuart, personal communication). The lack of non-VSG protein differences between these organisms implies that if proteins are rate determining then either the VSG is important in determination of growth rate in host animals or that slight differences in protein molecules were not detected. Only one spot other than VSG differed in the *T. brucei* clone isolated from a different serodeme demonstrating the high degree of similarity of organisms within the *T. b. brucei* species. Several major protein spots on the basic (right) side of the gels are possible markers for *T. b. brucei* as they were not observed on 2D autoradiographs of any other trypanosome species. These same protein spots were not seen in autoradiographs from procyclic culture forms of the same bloodstream clones and are thus stage-specific markers as well. It is possible that this basic protein is either associated with VSG molecules, or else is the result of persistent expression of a 'frozen' VSG. However, at this time the only relationship which can be drawn between them is their coexpression on the bloodstream stage of the parasite.

Comparison of the autoradiographs of two antigenically different clones of *T. b. rhodesiense* and three of *T. b. gambiense* again point to a remarkable similarity within a species. Indeed, the only spots which were not shared between the *T. b. rhodesiense* organisms were attributable to VSGs. The *T. b. gambiense* L2 and U2 clones differed only in VSG and in three spots which appeared only in the L2 organisms in the region of the VSG and may be VSG breakdown products. The *T. b. gambiense* ViTat 1.1 organism, which was cloned in our laboratory in Victoria from the TTrt-1 stabilate [14], had a 2D pattern which strongly resembled those of the *T. b. brucei* clones. This trypanosome has been carried as a laboratory strain for more than 70 years, is extremely virulent for laboratory rodents and its species identity has been questioned recently due to restriction mapping data (unpublished data from the WHO Scientific Working Group on African Trypanosomiases, Glasgow, 1982). Because marker 8

(Fig. 2) nevertheless distinguishes *T. b. gambiense* ViTat 1.1 from all three strains of *T. b. brucei* examined, it seems very unlikely to represent a case of recent laboratory contamination. More likely *T. b. gambiense* ViTat 1.1 represents a separate, mischaracterized isolate of *T. b. brucei*.

Together these data make it evident that within a species of trypanosome there is little heterogeneity in proteins detected by 2D gel autoradiography of [^{35}S]methionine-labelled parasites and that each of the three subspecies examined has a characteristic set of marker proteins. For example, *T. b. brucei* organisms may be identified on the basis of the major basic protein on the right hand side of the gel ($\simeq$M.W. 60 000, labelled Q in Fig. 1) or using the markers numbered 3, 4, 6, and 7 (Fig. 2) which probably represent charge polymorphisms. Similarly, *T. b. gambiense* may be identified using markers 1 and 2 or using the absence of markers 5 and 9. Only one unique marker protein appeared to be specific for *T. b. rhodesiense* (the most basic (right-hand) member of complex 10, Fig. 4). It is interesting, in addition, that the VSGs from *T. b. rhodesiense* showed multiple charge heterogeneity, unlike the VSGs from any of the other parasites in this study. Whether or not this isoelectric point heterogeneity is generally characteristic of *T. b. rhodesiense* is as yet unknown.

Determination of the relatedness of the different species of trypanosomes based on their 2D patterns remains somewhat equivocal using the limited numbers of marker proteins and trypanosome clones analyzed. However, it is interesting that *T. b. brucei* and *T. b. gambiense* only shared 3/15 marker proteins whereas *T. b. rhodesiense* and *T. b. gambiense* shared 9/15, implying that the human pathogens are more closely related. This conclusion is at variance with studies using restriction endonuclease analysis of genomic DNAs in distinguishing trypanosome species [6,7] which showed that *T. b. brucei* and *T. b. rhodesiense* subspecies constitute a group which is different from *T. b. gambiense*, at least with respect to VSG genes. The sharing of expressed protein gene products between organisms which have adapted to life in human hosts could perhaps explain the similar 2D gel patterns seen with the human pathogens. Thus gene regulation and expression may be of more value than DNA sequence data from one or a small number of genes in discriminating among the so-called subspecies, since host range and geographic distribution of the three groups of parasites was the primary basis for their ordering into subspecies [24] and this would be reflected in the 2D patterns.

Those proteins that differ between subspecies illustrate clearly major genetic changes accumulating during evolution of the different organisms. In Fig. 2, seven of the nine markers occur as pairs of adjacent spots (or spot micropatterns) of identical SDS-molecular weight. The horizontal (p*I*) distances between members of such pairs are consistent with p*I*-shifts associated with single unit changes in protein charge, attributable to a single charge-change amino acid replacement caused by a single base change at the DNA level. Random base substitutions in DNA produce either no change in the coded protein, an amino acid change conserving charge, an amino acid change altering charge, or a change in protein length (alteration of a termination or

initiation codon) with relative frequencies of 0.24, 0.42, 0.26, 0.08 [26]. Since only the latter two changes are generally visible in the 2D system, we would expect to see charge shifts and size jumps in a ratio of about 3:1. This is consistent with the observed ratio of 7 charge shifts in 9 markers, and suggests that approximately $1/0.26 + 0.08$ [9] $\simeq 26$ total single base changes have occurred in the DNA coding for the observed proteins ($\approx$ 200 kb) during divergence of *T. b. brucei* and *T. b. gambiense*. Similar results are obtained in comparisons of isolates of *Leishmania* [27]. Examination of larger numbers of proteins would provide a more stringent test of this hypothesis. If the spot pairs indeed represent allelic products, then the presence of two alleles at similar abundance in a cloned organism (markers 4, 7 and 8, Fig. 2) argues strongly for heterozygosity at these loci and hence for a diploid genome.

Transformation in culture of bloodstream trypomastigotes to procyclic forms was accompanied by the complete loss of VSG. This confirms at a molecular level the absence of VSG in procyclics as detected by electron microscopy and immunological techniques [25]. Remarkably few qualitative changes in non-VSG proteins occurred upon in vitro transformation although some quantitative changes did occur.

Comparison of *T. brucei* procyclics with *T. congolense* procyclics showed the highest level of difference observed in this study; although the protein patterns were generally similar in distribution of major proteins, essentially no proteins were identical between species. This result shows that *T. brucei* and *T. congolense* are far more divergent at the sequence level than, for instance, man and the apes.

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