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Biogenesis of the glycosome in *Trypanosoma brucei*: the synthesis, translocation and turnover of glycosomal polypeptides

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Glycosomes, the microbodies of *Trypanosoma brucei*, contain a number of enzymes involved in glucose and glycerol metabolism. The biogenesis of three of these enzymes has been studied. Aldolase, D-glyceraldehyde-3-phosphate dehydrogenase and NAD-linked glycerol-3-phosphate dehydrogenase are all synthesized in the cytosol on free rather than on membrane-bound polysomes. *In vitro*, as well as *in vivo*, these polypeptides are synthesized at their mature size, and no evidence was found for any processing upon entry into the glycosomes. Continuous and pulse–chase labelling experiments with procyclic trypomastigotes revealed that the enzymes have a half-life in the cytosol of ~3 min or less, and then turn over rapidly in the glycosomes, with half-lives as short as 30 min.

Key words: Trypanosoma brucei/glycosome/biogenesis/translocation/glycolytic enzymes/degradation

Introduction

The African trypanosomes are members of the Trypanosomatidae, a group of protozoan haemoflagellate parasites responsible for considerable morbidity and mortality in humans and their domestic animals (Molyneux and Ashford, 1983). The Trypanosomatidae combine several unique biochemical peculiarities (Opperdoes, 1985), of which one is the glycosome, a membrane-surrounded organelle that contains several essential metabolic pathways, including glycolysis, carbon dioxide fixation, ether lipid biosynthesis and β-oxidation of fatty acids (Opperdoes *et al.*, 1984a).

The presence of the latter two pathways in these organelles and their morphological resemblance to microbodies have suggested that glycosomes may be related to the peroxisomes and glyoxysomes of other eukaryotic organisms (Opperdoes *et al.*, 1984b).

The occurrence of glycolytic enzymes within glycosomes in the Trypanosomatidae is unique and at complete variance with the organization of this essential pathway in other eukaryotic organisms, where the glycolytic system is invariably found in the cytosol.

Glycosomal glycolytic polypeptides may therefore be expected to possess unique topogenic sequences absent in their cytosolic homologues. Evidence of such differences has indeed been reported in several recent publications (Osinga *et al.*, 1985; Clayton, 1985; Misset *et al.*, 1986; Michels *et al.*, 1986; Wierenga *et al.*, 1987). In order to appreciate the significance of these differences, however, we need information concerning the site of synthesis of the glycosomal enzymes and on their mode of transfer to the glycosomes.

This question has been addressed in the present paper. The results, which concern three distinct glycosomal enzymes in *Trypanosoma brucei*, and partly two others, indicate that these proteins are synthesized on free ribosomes in the cytosol and then transferred to the glycosomes.

Results

Characterization of antibodies

Some of the glycolytic enzyme activities associated with glycosomes, for example, D-glyceraldehyde-3-phosphate dehydrogenase (GAPDH), are present also in the cytosol, where they are represented by a different isoenzyme (Opperdoes *et al.*, 1986; Misset *et al.*, 1987). Thorough characterization of the monoclonal antibodies used was therefore necessary. Monospecific antibodies were selected by means of conventional solid-phase radioimmunoassay (RIA) and affinity column chromatography with immobilized purified glycosomal enzymes (results not shown). The monoclonal antibodies thus obtained were further characterized by dot-blot analysis using purified enzymes, crude homogenates and cytosolic fractions, either native or denatured. As can be seen in Figure 1, two monoclonal antibodies raised against glycerol-3-phosphate dehydrogenase (GPDH) and two against fructose-bisphosphate aldolase (ALDO) recognized only the native proteins, whereas the monoclonal antibodies against GAPDH and α-tubulin recognized both the native and the denatured proteins. Most of the monoclonal antibodies used reacted insignificantly with polypeptides found in the soluble fractions, with the exception of α-tubulin which is indeed a cytosolic protein. Anti-ALDO2 and anti-GPDH2 were found to have lower affinity for protein A, due to their immunoglobulin subclasses

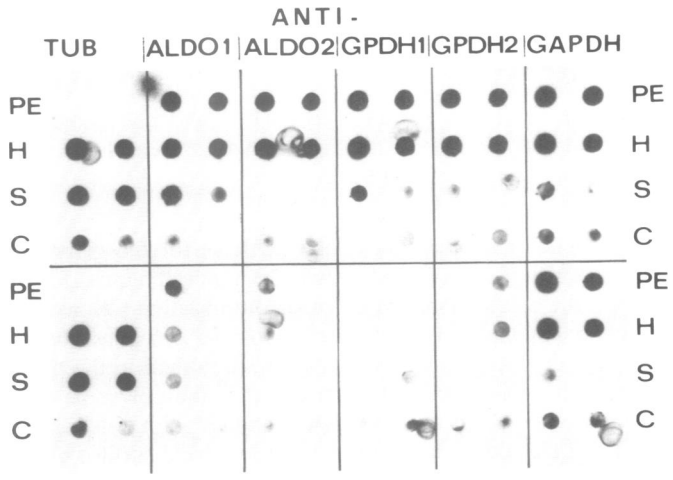

Fig. 1. Dot-blot analysis of antibody specificity. **Upper panel**, native proteins; **lower panel**, denatured proteins. PE: pure enzyme (except for α-tubulin); H: homogenate; S: soluble subcellular fraction; C: control BSA. Duplicates are from two different subcellular fractionations or batches of purified enzyme. Antibodies were anti-α-tubulin, two distinct anti-ALDO and anti-GPDH antibodies and anti-GAPDH.

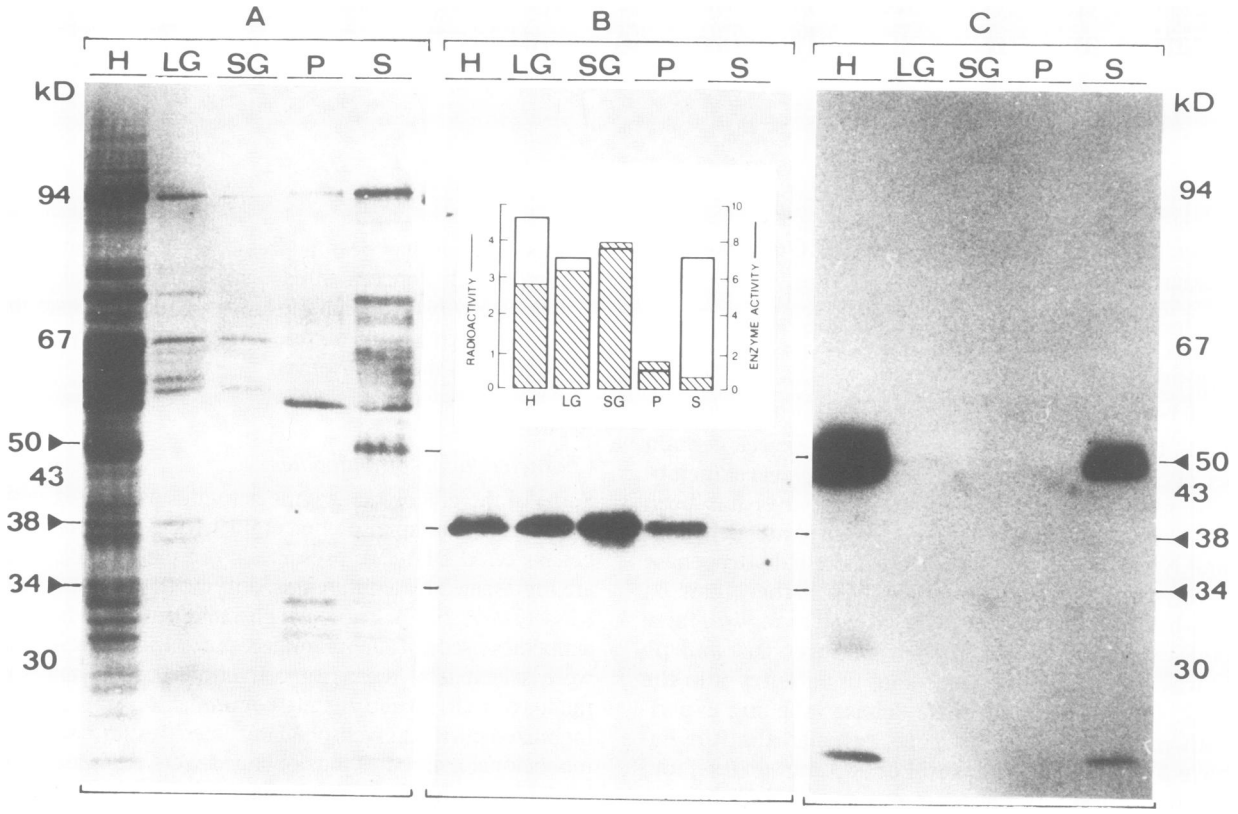

Fig. 2. Western blot analysis of subcellular fractions. (**A**) India ink protein stain. (**B**) Autoradiography of GAPDH revealed by ^{125}I-labelled anti-GAPDH. The inset shows the relative enzyme activity in the subcellular fractions. The corresponding radioactivities derived from densitometric readings of the bands are shown by shaded areas scaled on the assumption that the activities in the three particulate fractions are glycosomal. (**C**) Immunodetection and autoradiography using ^{125}I-labelled anti-α-tubulin. The mol. wts of α-tubulin (50−55 kd), GAPDH (glycosomal 38 kd and cytosolic 34 kd) are shown. H: homogenate; LG: large granule (mitochondrial); SG: small granule (glycosomal); P: particulate (microsomal); and S: soluble (cytosolic).

and were therefore not used in the subsequent immunoprecipitation experiments.

Both the GAPDH and α-tubulin antibodies were further characterized by Western blot analysis (Figures 2 and 3). Subcellular fractions were screened for the distribution of GAPDH and α-tubulin. The former is predominantly localized in the small granular fraction, while the latter is found exclusively in the soluble fraction. As shown in Figure 2, <5% of the glycosomal GAPDH (mol. wt. 38 kd) was found in the soluble fraction, whereas the cytosolic isoenzyme (mol. wt 34 kd) (Opperdoes *et al.*, 1986; Misset *et al.*, 1987) was not recognized by our antibody in spite of the high activity present in the soluble fraction (see insert Figure 2).

Localization of mRNAs

The subcellular localization of the mRNAs for the glycolytic polypeptides was investigated by two different techniques. Figure 4 shows the results obtained by *in vitro* translation followed by immunoprecipitation. For all three glycosomal enzymes studied, there was distinctly more mRNA in the free than in the membrane-bound polysomes. The efficiency of the polysome fractionation is indicated by the results obtained with α-tubulin, a product of free polysomes, and with the variable surface glycoprotein (VSG) 118, which is made by bound polysomes. Similar results were obtained with recombinant DNA probes for GAPDH (Michels *et al.*, 1986), and for two other glycosomal enzymes, phosphoglycerate kinase (Osinga *et al.*, 1985) and triosephosphate isomerase (Swinkels *et al.*, 1986), applied to dot-blotted RNA preparations. Table I summarizes the quantitative results obtained with the two techniques. On average, mRNA for glycosomal polypeptides was >3 times more abundant in the free than in the bound polysome preparations whereas the mRNA for α-tubulin was 7−8 times more abundant in the former.

Absence of longer precursors

As already shown in Figure 4, and as seen more clearly in Figure 5, the *in vitro* translated products recognized by anti-ALDO, anti-GAPDH and anti-GPDH antibodies are the same size as the native polypeptides present in the glycosomes. It appears, therefore, that these glycosomal enzymes are not made with cleavable leader sequences.

Biogenesis and turnover of glycosomal polypeptides

The subcellular distribution of newly synthesized GPDH in *T. brucei* culture forms incubated with [^{35}S]methionine for increasing lengths of time can be seen in Figure 6. Labelled GPDH first appeared in the soluble fraction and then almost immediately after in the small and, to a lesser extent, large granular fractions, where it continued to accumulate. The distribution of enzyme activity (Figure 7) clearly shows that GPDH is primarily located in the small granular fraction, with some activity also found in the large granular fraction, and virtually none in the microsomal or soluble fractions. It should be noted that the apparent mol. wt of GPDH in the cytosolic fraction is the same as that of the particulate enzyme. The incorporation of radioactivity in GAPDH and ALDO was also examined, with similar results. Quantification of the radioactivity in the respective bands was achieved by densitometric scanning of the autoradiographs and the

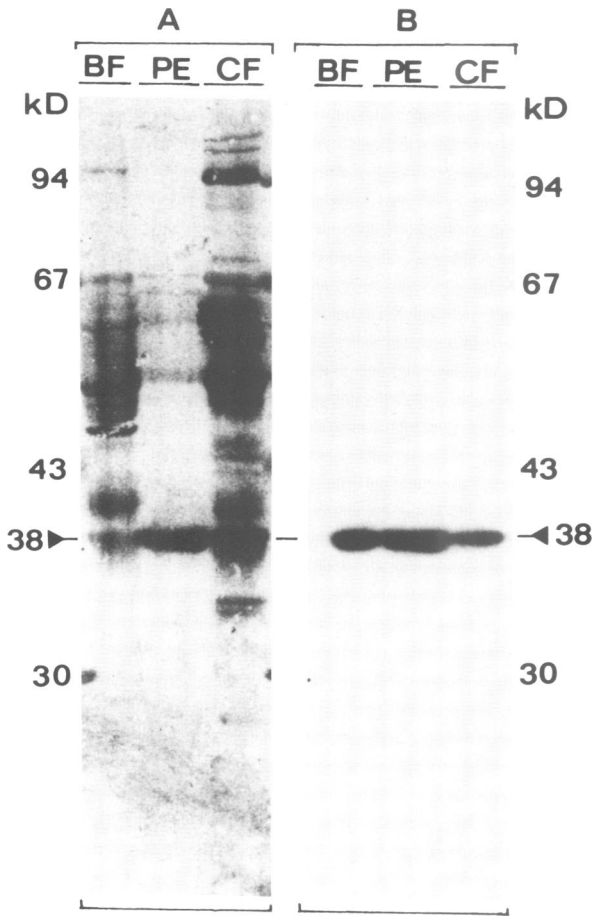

Fig. 3. Western blot analysis of glycosomes and pure GAPDH. (**A**) India ink-stained proteins; (**B**) immunodetection and autoradiography using anti-GAPDH. BF: bloodstream form; CF: culture form; PE: pure enzyme (GAPDH).

results for the three enzymes fitted very well with the mathematical model described in Materials and methods, which assumes synthesis in the cytosol followed by transfer to the glycosomes. The best curve fit for the experimental data for each of the enzymes is shown in Figure 8, and the derived constants are listed in Table II.

A similar set of experiments was carried out on cells pulsed for 5 min and then chased for up to 650 min. The results, illustrated in Figure 9, also fitted the mathematical model (Figure 10). Table II lists the values of the constants arrived at by this technique. Although there are some variations in the results obtained by the two approaches, they lead basically to the same conclusion. The newly synthesized glycosomal proteins appear first in the cytosol, from where they are rapidly transferred to the glycosomes, with half-lives of the order of 1 min or 3 min, depending on the technique used. Once in the glycosomes, the enzymes suffer rapid degradation, with half-lives of the order of 30 min under pulse–chase conditions. The corresponding values obtained by continuous labelling are more variable, but are similarly short.

Discussion

Several distinct conclusions emerge from the experimental results described in this paper. Firstly, the glycosomal enzymes studied are all most likely synthesized in the cytosol by free polysomes, and then rapidly transferred post-translationally into the

Table I. The ratio of mRNA found in the free and bound mRNA fractions

	mRNA ratio (free/bound)			
	Culture form		Bloodstream form	
	cDNA probe	Ab probe	cDNA probe	Ab probe
Phosphoglycerate kinase	2.5	ND	3.7	ND
ALDO	ND	2.4	ND	2.9
GAPDH	3.5	3.0	5.7	4.1
GPDH	ND	2.9	ND	3.5
Triosephosphate isomerase	2.8	ND	4.0	ND
VSG (118)	NA	NA	±0.2	0.3
α-Tubulin	NA	8.0	NA	7.1

ND = not determined
NA = not applicable

glycosomes. This conclusion is strongly supported by the kinetic findings and confirmed by the observed distributions of the mRNAs, as detected with two different probes. The only alternative explanation compatible with the evidence would require assuming some sort of fragile biosynthetic site that breaks up upon homogenization of the cells to release polysomes and their products in the manner found. This hypothesis would seem entirely gratuitous but for one odd observation: ~25% of the five glycosomal mRNAs studied consistently came down with the bound polysome fraction, as opposed to little more than 10% for the tubulin mRNA. There is at present no explanation for this difference. It could reflect an adsorption artifact due to a peculiarity of the glycosomal polypeptides, for example, their strongly basic character (Misset *et al.*, 1986).

Secondly, our results show that the glycosomal GAPDH, GPDH and ALDO are all three synthesized at their mature size and suffer no detectable proteolytic processing upon transfer into the glycosomes. This agrees with previous findings showing that the mature sizes of the proteins are those predicted from the nucleotide sequences of their respective genes (Misset *et al.*, 1986). Furthermore, sequencing of the N-terminal part of GAPDH (Michels *et al.*, 1986) and three-dimensional structure analysis of triosephosphate isomerase (Wierenga *et al.*, 1987) have indicated that the N terminus of these proteins is not subjected to any form of processing. It must be concluded, therefore, that all the information required for import of the glycosomal proteins – at least those studied so far – into their host organelles resides in the primary sequences of the proteins themselves. Recent results suggest that a special configuration of basic residues on the surface of the molecules may play a critical role in this respect (Wierenga *et al.*, 1987).

Our findings recall the numerous observations that have been made on the biogenesis of peroxisomes and glyoxysomes (Kindl, 1984; Lazarow *et al.*, 1982; Lazarow and Fujiki, 1986; Borst, 1986). Like the glycosomal enzymes, the enzymes associated with these particles are made by free polysomes, mostly at their mature size, and they are conveyed to their host particles without proteolytic processing and without apparent requirement for any other secondary modification, such as glycosylation or the covalent linkage of lipids (Borst, 1986). These similarities are supportive of a kinship between glycosomes and peroxisomes. It would, therefore, be interesting to know, in this respect, whether peroxisomal enzymes share the configuration of positive residues suspected of playing a role in the targetting of the glycosomal enzymes. Information on this subject is lacking.

The low steady-state concentration maintained for the

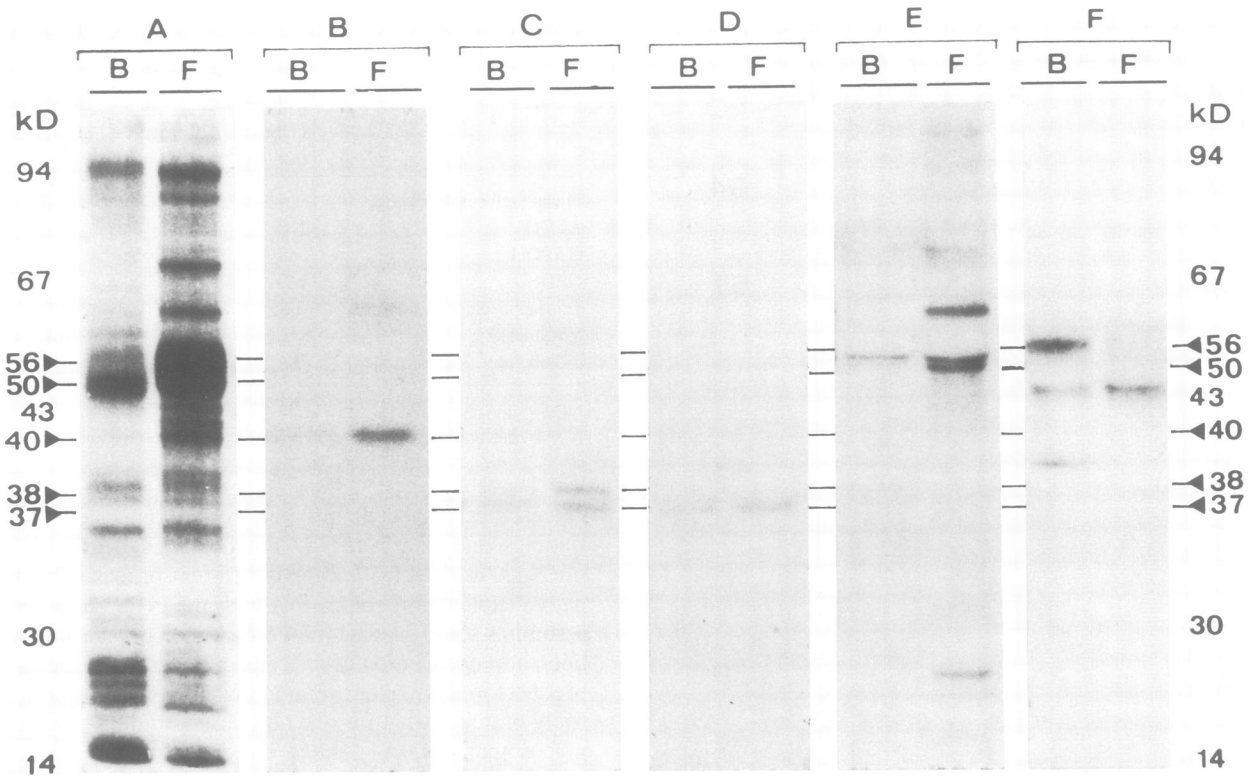

Fig. 4. Immunoprecipitation of *in vitro* translation products from free and bound mRNA. (**A**) Autoradiography of the total *in vitro* translation products; (**B, C, D, E** and **F**) immunoprecipitation of ALDO, GAPDH, GPDH, α-tubulin and VSG 118. B: bound mRNA; F: free mRNA. In each case total RNA was used for translation [i.e. not a poly(A)-enriched fraction].

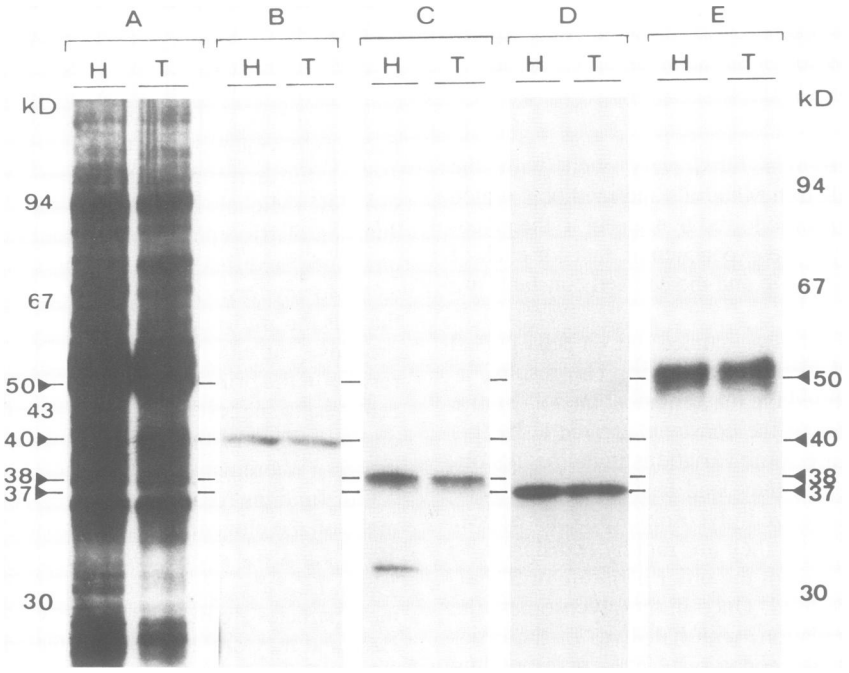

Fig. 5. Immunoprecipitation of *in vivo* and *in vitro* synthesized proteins. Autoradiographs, after SDS−PAGE separation, of labelled proteins produced *in vivo* and *in vitro*. (**A**) Total homogenate (H) and translation mixture (T), (**B, C, D** and **E**) ALDO (40 kd), GAPDH (38 kd), GPDH (37 kd) and α-tubulin (50−55 kd) immunoprecipitated from the homogenate (H) or the translation mixture (T).

glycosomal enzymes in the cytosol in spite of their high rate of synthesis suggests that their import into the glycosomes is a speedy and efficient process. Our value of ~3 min, or perhaps even as small as 1 min, for the half-life of glycosomal proteins in the cytosol is comparable to the fastest translocation rates described for several rat-liver (Lazarow *et al.*, 1982) or yeast peroxisomal proteins (Miura *et al.*, 1984).

One important characteristic that clearly differentiates

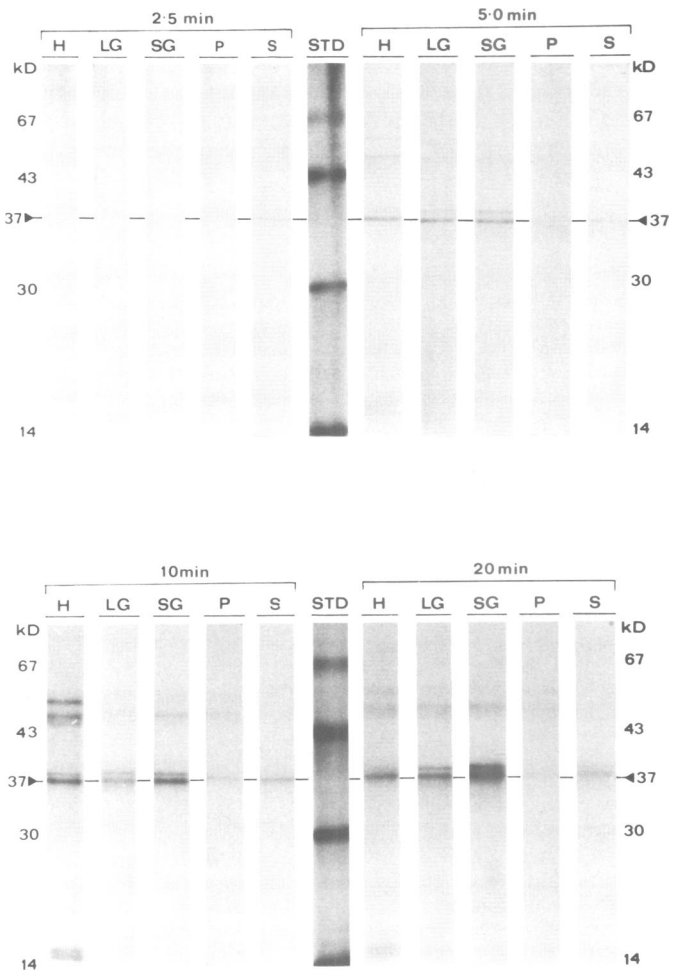

Fig. 6. *In vivo* incorporation of radioactivity into GPDH by continuous labelling. Autoradiography of immunoprecipitated GPDH in subcellular fractions from cells disrupted after 2.5, 5, 10 and 20 min incubation in the presence of [^{35}S]methionine. H: homogenate; LG: large granule (mitochondrial); SG: small granule (glycosomal); P: particulate (microsomal); and S: soluble (cytosolic).

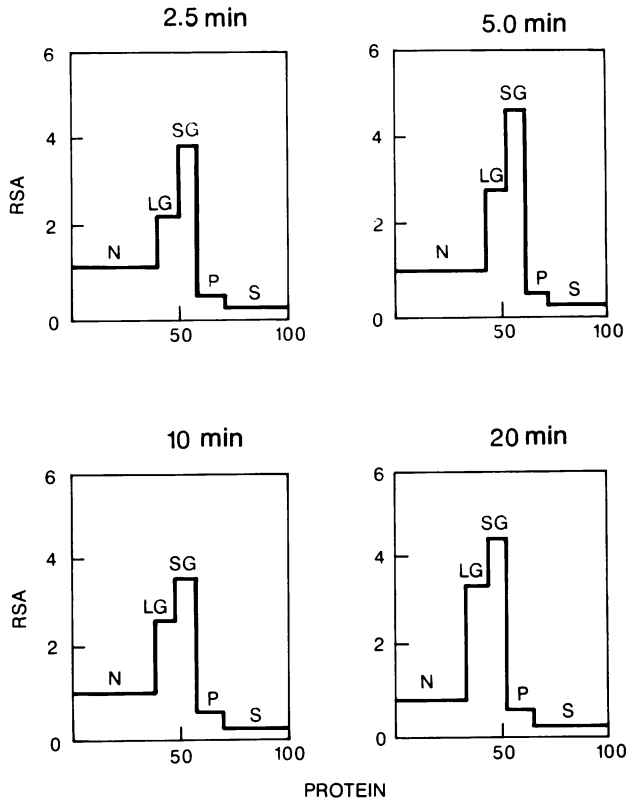

Fig. 7. The relative specific activity of GPDH in the subcellular fractions. Distribution of GPDH activity in fractions of Figure 6. Ordinate: relative specific activity of enzyme; abscissa: % protein.

glycosomes from the peroxisomes of other eukaryotes is the high turnover rate of their enzymes. Our half-life values are of the order of 30 min for all three enzymes in the pulse−chase experiments. They are more variable, but still very low, in the continuous labelling experiments. In contrast, the half-life of rat-liver peroxisomal proteins has been estimated at 1.5 days (Poole *et al.*, 1969). It is tempting to infer from the fact that the three glycosomal enzymes turn over at the same rate under pulse−chase conditions that *T. brucei* glycosomes are destroyed as whole units, as is believed to be the case for rat-liver peroxisomes (Poole *et al.*, 1969). However, the situation seems to be different under continuous labelling conditions. It is, of course, possible, with such a fast-evolving system, that significant cellular heterogeneity may occur within the population of cells used in the experiment.

In order to verify the internal consistency of our kinetic values, we have taken advantage of the detailed molecular information available on ALDO to estimate the real rate of synthesis of these enzymes from the observed k_s values. This calculation is as follows: with a rate of methionine incorporation of 0.9×10^{-15} mol/min/μg and 10 methionine residues per subunit, the aldolase synthesis amounts to $0.9 \times 10^{-15} \times 10^{-1} \times 6.2 \times 10^{23} = 5.6 \times 10^{7}$ subunits/min/μg (Equation 1). According to Misset *et al.* (1986), bloodstream form trypanosomes contain 240 glycosomes per cell and 1720 native aldolase molecules per glycosome. Since each aldolase consists of four subunits, one bloodstream form trypanosome contains $1720 \times 4 \times 240 = 1.6 \times 10^{6}$ subunits per cell. Since culture form trypanosomes have only 3.5% of the aldolase activity of the bloodstream form (Hart *et al.*, 1984), it is assumed that culture form cells contain only $1.6 \times 10^{6} \times 0.035 = 5.6 \times 10^{4}$ subunits/cell. Since one cell represents 10^{-5} μg of protein, there are $5.6 \times 10^{4} \times 10^{5} = 5.6 \times 10^{9}$ subunits/μg (Equation 2). Combining Equations 1 and 2 shows that 1% of the total amount of aldolase is synthesized per min. Of this, 0.115% is stored (assuming exponential − or linear, the difference is hardly significant − growth with a doubling time of 10 h). This leaves 0.885% to be destroyed per min, which amounts to a turnover time of 113 min, or a half-life of 78 min. Although indeed small, this value is nevertheless significantly higher than the values listed in Table II. Quite possibly, our premises do not exactly apply to the cells under study. An alternative possibility is that the labelled methionine suffers significant intracellular dilution by unlabelled molecules arising from endogenous protein breakdown. With proteins turning over as rapidly as they do in *T. brucei* − even tubulin has a high turnover − this would not be at all surprising. In any case, our conclusion that glycosomal enzymes have a notably short half-life is clearly borne out by their high rate of synthesis.

This rapid turnover of glycosomes may be necessitated by transformational requirements during the parasite's life cycle. Bloodstream forms may undergo a relatively rapid transformation to procyclic trypomastigotes (Bienen *et al.*, 1981; Overath

et al., 1983) and the energy metabolism of both life cycle stages differs dramatically. The metabolic differences are concomitant with the differences found in the polypeptide composition of the glycosomes of these two stages (Hart *et al.*, 1984). The abundance of aldolase, for example, in procyclics is at least 20- to 30-fold lower than in bloodstream forms, whereas several other enzymes, including malate dehydrogenase, are much more abundant in the procyclics. Although nothing is known about the metabolic changes taking place during the transformation from the procyclic trypomastigote stage to the epimastigote stage in the tsetse fly, it is quite likely that such a transformation would also require a rapid adaptation of glycosomal metabolism and therefore a high rate of turnover of the organelles.

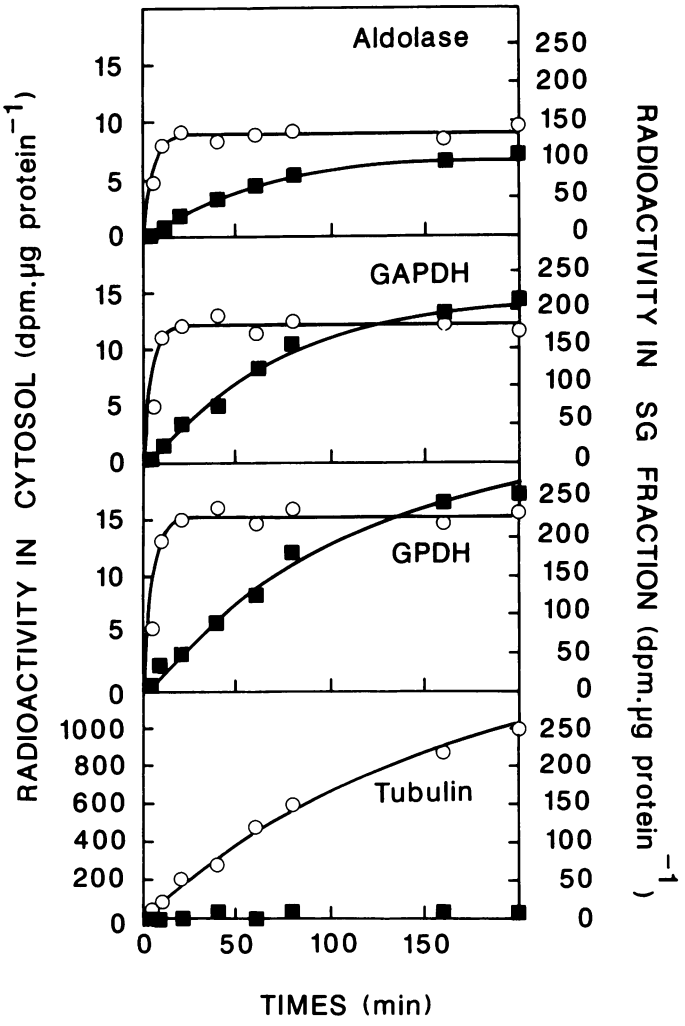

Fig. 8. Kinetics of incorporation of radioactivity into immunoprecipitated glycosomal polypeptides and tubulin upon continuous labelling. ○: cytosolic fraction; ■: small granule fraction. The curves illustrate best fit of experimental points to Equations 3 (○) and 4 (■). Constants are given in Table II.

Materials and methods

Trypanosomes

Both bloodstream forms and procyclic trypomastigotes were obtained and harvested as previously described (Hart *et al.*, 1984). For the experiments of biogenesis and degradation kinetics, procyclic forms were maintained in RPMI 1640 defined medium (Flow Laboratories) with haemin (Sigma Chemicals) at 5 µg/ml. Under these conditions the medium supported uninhibited growth for at least two further cell divisions.

Subcellular fractionation

Subcellular fractions were prepared exactly as described by Steiger *et al.* (1980), except that 10 mM non-radioactive methionine was included in the harvesting and fractionation buffers. The fractions were characterized as described by Opperdoes *et al.* (1981) by the following marker enzymes: oligomycin-sensitive ATPase (mitochondrion), hexokinase (glycosome), acid phosphatase (flagellar pocket) and glucose-6-phosphate dehydrogenase (cytosol). All enzyme activities were determined as described previously (Hart *et al.*, 1984).

Antibody production and selection

For the preparation of monoclonal antibodies directed against individual glycosomal enzymes, mice were injected with highly purified glycosomes (Opperdoes *et al.*, 1984a), together with Freund's complete adjuvant, and hybridomas were produced and cloned according to Shulman *et al.* (1978). Antibodies were selected by solid-phase RIA using both total glycosomes and pure enzymes (Misset and Opperdoes, 1984; Misset *et al.*, 1986).

Affinity columns were used to verify the monospecific nature of each antibody. The dot-blot technique of Jahn *et al.* (1984) was modified so that native (sonicated with 2% Triton X-100) and denatured (boiled for 2 min with 0.5% SDS and 1 mM dithiothreitol) samples could be screened to determine whether the antibody epitopes were structural or non-structural (i.e. present on denatured polypeptides). A 5-fold excess of Triton X-100 over SDS was used to allow binding of the denatured samples to the nitrocellulose filters. Samples of pure enzyme (± 1 µg), homogenate (15 µg) or cytosolic (30 µg) fractions were dot-blotted alongside bovine serum albumin (BSA) (30 µg) control dot-blots to evaluate a specific binding. A Bethesda Research Laboratories dot-blotter was used and an 'in-well' screening with the first and iodinated second antibodies (Amersham International) was developed.

Western blot analysis of the non-structural antibodies (i.e. recognizing epitopes on denatured proteins) was done as described by Burnette (1981), except that 20% methanol was used for the transblotting and all blots were baked in a vacuum oven at 80°C for 5–10 min. Radioactive and non-radioactive molecular weight standards (Amersham International and Pharmacia Fine Chemicals) were used and proteins were visualized by India ink staining as described by Hancock and Tsang (1983). Thanks to the baking of the blots, BSA blocking was completely reversible after washing (50 mM Tris-HCl, 150 mM NaCl and 2.0% Tween 20). Blots used for screening and autoradiography could subsequently be stained without significant background. The monoclonal anti-α-tubulin used was purchased from Amersham International, and the polyclonal antiserum against the VSG 118 was kindly supplied by Professor P.Borst, NKI, Amsterdam.

Table II. Summary of the synthesis rates and half-lives

	Rate of synthesis k_s[a] (fmol meth/min/µg cell protein)		Half-life in cytosol (min)		Half-life in glycosomes (min)	
	Continuous labelling	Pulse–chase labelling	Continuous[b] labelling	Pulse–chase labelling	Continuous labelling	Pulse–chase labelling
ALDO	0.9	1.0	3.1	1.2	35	30
GAPDH	1.4	3.5	2.7	1.3	49	33
GPDH	1.4	8.0	3.4	1.0	71	30
α-tubulin	4.0	3.8	115	130	–	–

[a]Assuming that the specific radioactivity of methionine in the cytosol is the same as in the extracellular pool. In the case of intracellular dilution of the precursor, the k_s values will be proportionately higher. Total methionine incorporation was 32 fmol/min/µg protein.
[b]For the half-life in the cytosol, values obtained by continuous labelling are more accurate, since for pulse–chase labelling the estimates are based mainly on two points of a steep curve.

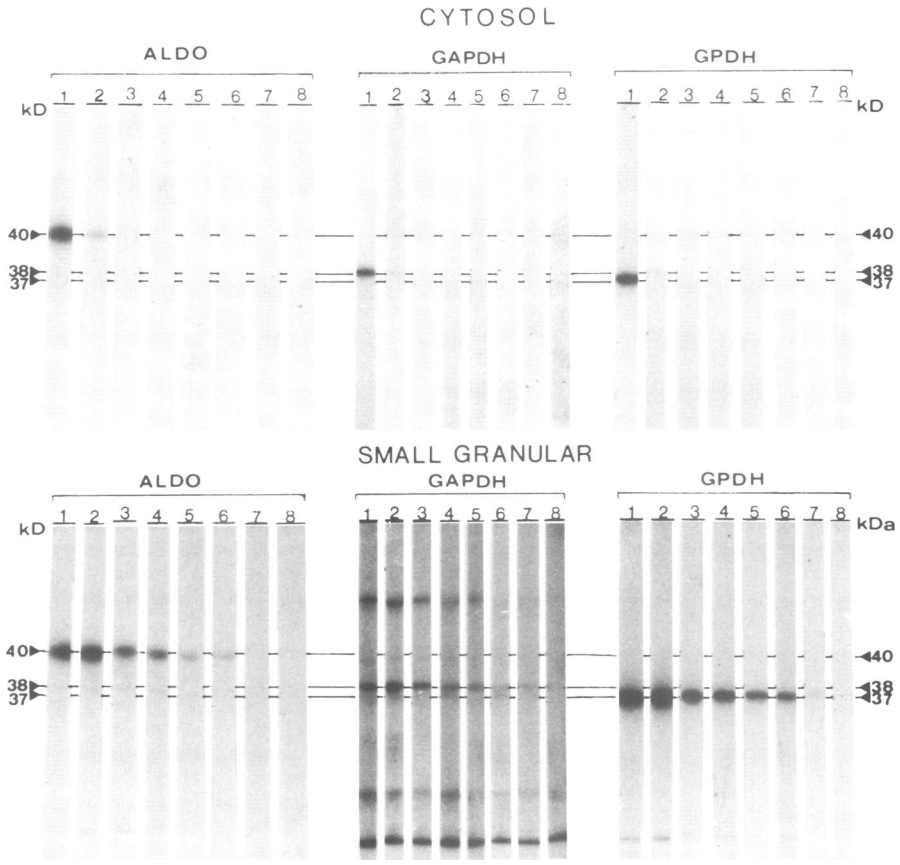

Fig. 9. Radioactivity in soluble and particulate glycosomal polypeptides after a pulse–chase. Autoradiography of ALDO, GAPDH and GPDH immunoprecipitated from the cytosolic fraction and from the small granule fraction, separated from cells pulsed with [^{35}S]methionine and chased 5 min later. Cells were disrupted at 5 (1), 10 (2), 20 (3), 40 (4), 60 (5), 80 (6), 160 (7) and 200 (8) min.

RNA preparation and translation in vitro

Total RNA was prepared by the lithium chloride/urea method of Auffray and Rougeon (1980). Poly(A)-enriched RNA was prepared as described by Maniatis *et al.* (1982). Free and bound rRNAs were prepared by the methods of Ramsey and Steele (1976) and Mechler and Rabbitts (1981). The latter method was modified to include a 1.8 M sucrose layer to improve separation and the discontinuous gradients were centrifuged at 48 000 r.p.m. for 12 h. Translation *in vitro* of total and poly(A)-enriched RNA was done in both rabbit reticulocyte lysates (Jackson and Hunt, 1983) and wheat germ systems (Erickson and Blobel, 1983). Both systems were kindly supplied by Dr M.Robbi (ICP, Brussels). Yeast tRNA (Boehringer) was added (10 μg/ml) and both systems were optimized for K^+, Mg^{2+}, polyamine and RNA levels as described by Jackson and Hunt (1983) and Erickson and Blobel (1983). Translations in the wheat germ system yielded higher quantities of the glycolytic polypeptides, whereas α-tubulin and the VSG polypeptides were equally well translated in either system (results not shown).

cDNA hybridization selection of mRNA and screening of free and bound RNA

Recombinant DNA (cDNA) for three glycolytic enzymes (Osinga *et al.*, 1985; Michels *et al.*, 1986; Swinkels *et al.*, 1986) was used to select their respective mRNA populations (Miller *et al.*, 1983). *In vitro* translation of these RNA populations and immunoprecipitation of the products served to authenticate the cDNAs. Nick-translated cDNA probes were hybridized against RNA isolated from free and membrane-bound ribosomes that had been dot-blotted. The RNA dot-blots were prepared as described by Thomas (1980) with 50 ng of total RNA and screened with serial halving dilutions of RNA in adjacent wells. Total rat-liver RNA was used as a control and heparin (500 μg/ml) was added to reduce non-specific hybridization (Singh and Jones, 1984).

Continuous labelling

Continuous labelling with [^{35}S]methionine (sp. act. 1220 Ci/mmol and ± 10 μCi/mg cell protein) of procyclic trypomastigotes was achieved in methionine-deficient RPMI 1640 medium (Flow Laboratories). Labelling was stopped at various times by the addition of an excess of non-radioactive methionine (10 mM final concentration) and the cells were then fractionated as described above. The incorporation of radioactivity into glycosomal and control polypeptides was followed by immunoprecipitation at times up to 200 min. By the end of the incubation period, <35% of the available radioactivity had been incorporated.

Pulse–chase labelling

Pulse–chase labelling was carried out like continuous labelling except that the radioisotope was diluted with an excess of non-radioactive methionine after 5 min. For measurement of their turnover, glycosomal and control polypeptides in the subcellular fractions were separated by immunoprecipitation for up to 650 min, followed by SDS–PAGE. Correction was made for the increase in total protein mass due to cell multiplication.

Immunoprecipitation

The protocol of Anderson and Blobel (1983) for immunoprecipitation from cell-free translations was followed, except that the SDS denaturation step was omitted and replaced by sonication in the immunoprecipitation buffer with 1% Triton X-100 and 500 mM NaCl to prevent co-precipitation of glycosomal polypeptides. Under conditions of low salt (250 mM NaCl) GAPDH and GPDH tend to co-precipitate as shown in Figures 4 and 6, whereas with high salt no co-precipitation occurred (see Figure 9). Non-radioactive methonine (1 mM), BSA (0.1%) and additional proteinase inhibitors [phenylmethylsulphonyl fluoride, leupeptin and aprotein (± 1 μM)] were routinely incorporated in all buffers.

Other methods

SDS–PAGE was carried out on 10–15% slab gels as described by Hart *et al.* (1984). Fluorography was carried out with Amplify (Amersham International), Dupont Lighting Plus Screens (Dupont) and X-omat AR X-ray film (Kodak). A Chromoscan 3 (Joyce Loebl Ltd, UK) densitometer was used to quantify the radioactivity in the respective bands of the autoradiographs. Proteins were measured by the fluorescamine method with BSA as standard (Stein *et al.*, 1973). Western blots were stained with India ink as described by Hancock and Tsang (1983).

Mathematical model

The model assumes that an enzyme is synthesized at a constant rate in the cytosol and subsequently transferred to glycosomes with first-order kinetics. Synthesis is measured by the incorporation of a labelled amino acid assumed to be present at constant specific radioactivity in the cytosolic pool (in equilibrium with the

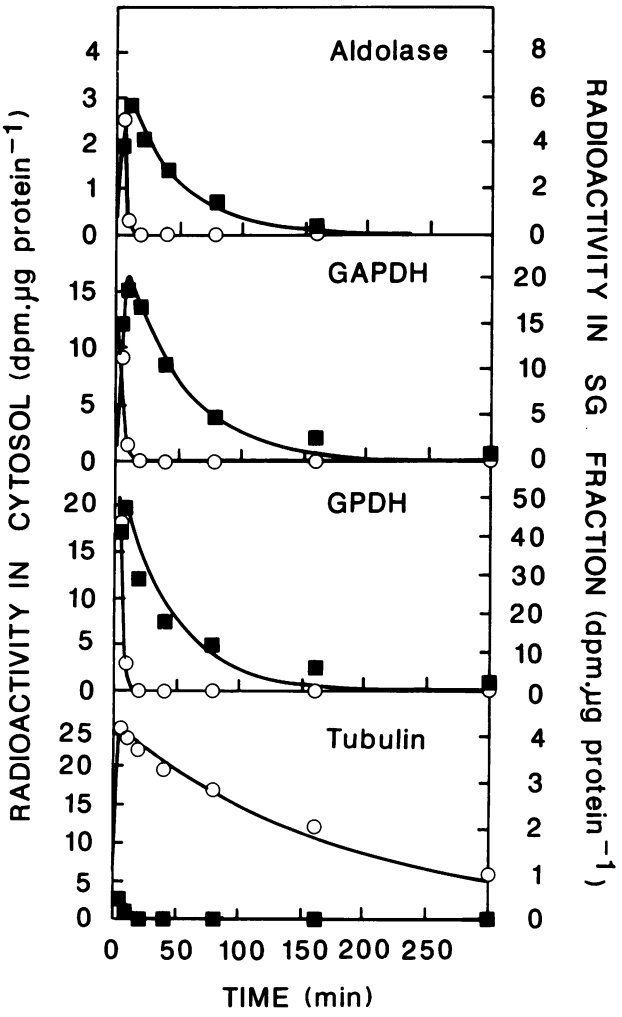

Fig. 10. Kinetic behaviour of glycosome polypeptides and tubulin after pulse–chase labelling. The points shown represent the radioactivities of the immunoprecipitates of Figure 9. ○: cytosolic fraction; ■: small granule fraction. The curves illustrate best fit of experimental points to Equations 6 (○) and 8 (■). Constants are given in Table II.

extracellular pool). Hence, we have for the rate of change of newly synthesized enzyme in the cytosol:

$$\frac{dE_c}{dt} = k_s.S - \frac{E_c}{T_c} \quad (1)$$

in which E_c is the amount of radioactive enzyme in the cytosol (d.p.m./µg cell protein), t the time (min), k_s the rate of incorporation of the precursor amino acid (mol/min/µg cell protein), S the specific radioactivity of the precursor amino acid in the cytosol pool (d.p.m./mol) and T_c the turnover time (half-life/0.693) of the enzyme in the cytosol (min).

It is supposed that the enzyme is degraded with first-order kinetics in glycosomes. Degradation is here defined as the loss of immunological reactivity. It is further assumed that re-utilization of the label can be neglected. Under these conditions, we get for the glycosomal compartment:

$$\frac{dE_g}{dt} = \frac{E_c}{T_c} - \frac{E_g}{T_g} \quad (2)$$

where E_g is the quantity of newly synthesised enzyme in glycosomes (d.p.m./mg cell protein) and T_g the turnover time of the enzyme in glycosomes (min).

Upon integration, Equation (1) yields:

$$E_c = k_s.S.T_c\,(1 - e^{-t/T_c}) \quad (3)$$

Introducing the value of E_c from Equation (3) in Equation (2) we obtain by integration:

$$E_g = k_s.S[T_g\,(1 - e^{-t/T_g}) - \frac{(e^{-t/T_c} - e^{-t/T_g})}{(1/T_g - 1/T_c)}] \quad (4)$$

Equations (3) and (4) can be used directly for the interpretation of experiments in which incorporation of the label is followed over a long period of time. If a pulse–chase experiment is performed, these equations remain valid during the pulse ($0 < t < t_p$). During the chase, we have:

$$\frac{dE_c}{dt} = -\frac{E_c}{T_c} \quad (5)$$

since S becomes 0. Defining $E_{c,p}$ as the quantity of labelled enzyme in the cytosol at time t_p one gets for $t > t_p$:

$$E_c = E_{c,p}.e^{-(t-t_p)/T_c} \quad (6)$$

For evaluating the quantity of labelled enzyme in glycosomes, we have to combine Equations (2) and (6). One then obtains:

$$\frac{dE_g}{dt} = \frac{1}{T_c}.E_{c,p}.e^{-(t-t_p)/T_c} - \frac{E_g}{T_g} \quad (7)$$

which yields upon integration:

$$E_g = \frac{1}{(T_c/T_g - 1)} E_{c,p}\,(e^{-(t-t_p)/T_c} - e^{-(t-t_p)/T_g}) + E_{g,p}\,e^{-(t-t_p)/T_g} \quad (8)$$

where $E_{g,p}$ is the quantity of labelled enzyme in glycosomes at time t_p.

For curve fitting, a simple program for a personal computer was written for displaying graphically the experimental points and for computing the curves after determining k_s, T_c and T_g. The parameters were adjusted so as to obtain visually the best possible fit.

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