The effect of 3 different incubation media on the rate of pyruvate synthesis from D-glucose metabolism in T. brucei cells permeabilized with 60 /micro/g digitonin/10^8 trypanosomes was investigated. Pyruvate synthesis was maximal in the medium containing K⁺ and Mg²⁺, while it was very low in the medium containing Na⁺ as the only metal cation. By titrating glycolysis in T. brucei incubated in the medium containing K⁺ and Mg²⁺ with adenosine triphosphate (ATP) pyruvate synthesis was stimulated but later inhibited when the molar ratio of ATP to Mg²⁺ was greater than unity; when D-glucose was replaced by fructose 1,6-diphosphate (FDP) as substrate, adenosine diphosphate (ADP) could replace ATP. This confirms the requirement for ATP in the conversion of D-glucose to FDP and the requirement of ADP during substrate-level phosphorylation in glycolysis. Addition of 1 mM exogenous reduced nicotinamide adenine dinucleotide (NAD⁺) to the incubation medium had no significant effect on the metabolism of D-glucose to pyruvate. The results of this investigation indicate that permeabilization of T. brucei cells with digitonin causes the leakage of some Mg²⁺, K⁺, ATP and ADP, but not NAD⁺. It is concluded that ADP, Mg²⁺ and ATP have a different microenvironment in the cell from that occupied by NAD⁺. Some advantages for this cellular organization are discussed.