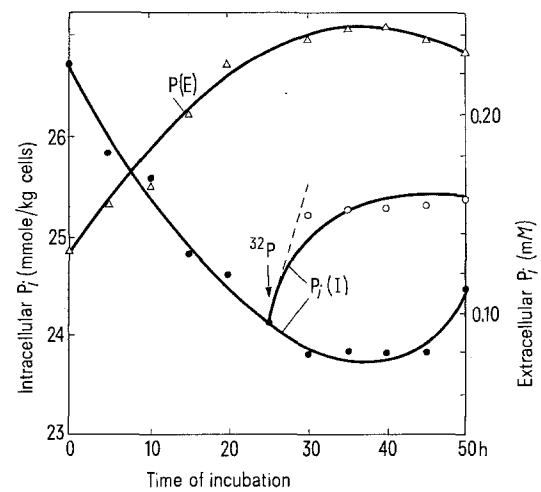
Phosphate Transport in *Trypanosoma cruzi*

Culture forms of *Trypanosoma cruzi* (the agent of Chagas disease) are capable of assimilating P_i[1] and incorporating it into the intracellular phosphates[2,3]. The results reported here show that an inward P_i transport contributes in an essential manner to preserve P_i concentration in *T. cruzi*.

Material. T. cruzi (Tulahuen strain) was grown in Roux bottles on a biphasic medium at 28 °C. The solid phase was made of agar (Bacto; 19 g), brain-heart infusion (Difco; 38 g) and water 1 l. The overlay contained yeast extract (Difco; 5 g), glucose (5 g), neopeptone (5 g), NaCl (7 g), inactivated calf-serum (80 ml) and water 1 l. 4 days after inoculation, the overlay of several bottles was pooled. The cells were collected by centrifugation at 4 °C, washed 3 times in the centrifuge with isotonic (0.154 *M*) NaCl solution and resuspended in the same medium. The final concentration of the *T. cruzi* suspension was estimated by measuring the weight after drying the washed epimastigotes at 100–105 °C for 24 h (the dry weight was about 15% of the wet weight). 1 mg (dry weight) of cells contained 4.5×10^7 epimastigotes. Orthophosphate-^{32}P was supplied carrier-free by CNEA (Argentina); the final specific activity is indicated in each case.

Method. Incubations were carried out in Warburg manometers or in 2×16 cm test tubes placed in a shaking-water bath, under air, at 30 °C. All measurements were done in duplicate. The cells were suspended in a standard saline solution made up as follows: 92 m*M* KCl, 31 m*M* NaCl, 36 m*M* *Tris*-HCl buffer, and additions as stated in each case; the final pH was 7.4 and the ionic strength was 0.15. The total volume varied in accordance to the experimental conditions.

After incubation with $^{32}P_i$ the epimastigotes were centrifuged at $3000 \times g$ and 4 °C. The supernatants were kept for the measurement of extracellular P_i and the cells were washed twice with 0.5 ml of 0.154 *M* KCl. The cells were resuspended in 2 ml of 0.154 *M* KCl and samples of these suspensions were used for the determination of P_i. Extraction and fractionation of P compounds were performed as described before[4]. The cells were extracted for 1 h with TCA (10% (w/v)), at 4 °C. The residues were extracted twice more with TCA-solution and the extracts were combined (P_{as} fraction). The cell residues were extracted with ethanol-ethyl ether mixture (3:1 (v/v)) to isolate lipid P (P_l). P_i was determined by the method of ERNSTER et al.[5] P_7 was hydrolyzed with 1 *N* H_2SO_4 for 7 min at 100 °C.



Comparison of outward and inward phosphate fluxes. Epimastigotes were grown in the PD_1 medium. 4 h before harvest, 5 mC phosphate-^{32}P was added. The cells (160 mg) were washed 3 times with 0.5 ml 0.154 *M* NaCl, at 4 °C. 20 samples of cells (6.8 mg each) were suspended in 1.5 ml of standard saline solution containing 10 m*M* glucose. Incubation of first 5 (duplicate) samples was interrupted at the intervals stated in the abscissa. Where indicated by the arrow, 5 of the remaining samples were added ^{32}P-phosphate (final concentration, 5.3 m*M*; specific activity, 4.2×10^9 cpm/m atom g ^{32}P) and the other 5 samples were used as controls. ^{32}P-added and control samples were further incubated for the time stated in the abscissa and $^{32}P_i$ distribution was then measured. Extracellular $^{32}P_i$ was determined with the chemical[5] and the radiochemical methods, on the base of ^{32}P specific activity in the intracellular $^{32}P_i$. Other experimental details were as described in Text and Table I. P(E), extracellular P_i; P(I), intracellular P_i.

[1] The abbreviations used were: P_i, inorganic orthophosphate; P_7, heat and acid-labile phosphate, TCA-soluble phosphate; P_{as}, total TCA-soluble phosphate; P_o, P_{as} less $P_i + P_7$; P_l, lipid phosphate; P_r, TCA-insoluble phosphate less P_l; P_s, total phosphate; TCA, trichloracetic acid.

[2] J. F. ROBERTS, J. Protozool. *14*, 385 (1967).

[3] J. F. DE BOISO, B. F. CAZZULO and A. O. M. STOPPANI, Revta Soc. argent. Biol. *44*, 112 (1968).

[4] A. O. M. STOPPANI, A. BENNUN and E. M. DE PAHN, Archs Biochem. Biophys. *108*, 258 (1964).

[5] L. ERNSTER, R. ZETTERSTRÖN and R. LINDBERG, Acta chem. scand. *4*, 942 (1950).

Table I. Effect of phosphate limitation on P content and $^{32}P_i$ uptake by resting epimastigotes

Culture medium	Total P in medium (m*M*)	P in epimastigotes $\left(\frac{\text{mmole}}{\text{kg}}\right)$		^{32}P-Phosphate uptake by epimastigotes[a] $\left(\frac{\text{mmole}}{\text{kg}^b/\text{h}}\right)$	
		P_i	P_t	Less glucose	Plus 15 m*M* glucose
Complete (see text)	11	38	113	0.10	0.16
Brain-heart infusion (50%) and calf-serum omitted (PD_1)	6.8	21	79	—	—
Brain-heart infusion and calf-serum omitted (PD_2)	0.7	16	73	1.35	1.77

[a] Epimastigotes (42 mg) suspended in standard saline solution; after thermal equilibration, 0.7 m*M* ^{32}P-phosphate (specific activity, 2.4×10^{10} cpm/m atom g ^{32}P) was added. Total volume, 14.2 ml. Incubation for 1 h in a Gyrotory Water Bath, at 30 °C, under air. Other experimental condition as described under methods. [b] Wet-weight.

Table II. Phosphate fractions in epimastigotes: labelling after absorption of ^{32}P-phosphate[a]

P fraction	Phosphate concentration in initial fractions ($\frac{\text{mmole}}{\text{kg cells}^b}$)	^{32}P-phosphate uptake in fractions ($\frac{\text{mmole}}{\text{kg cells}^b}$)	($\frac{\text{cpm } (\times 10^5)}{\text{m atom g } ^{32}\text{P}}$)
P_i	21.3[c]	3.4	293
P_7	17.8	0.3	39
P_o	17.7	0.8	90
P_l	21.2	0.3	32
P_r	39.0	0.6	39

[a] Phosphate-deficient (PD_1) epimastigotes (42 mg) were incubated with 0.7 mM ^{32}P-phosphate and 15 mM glucose as described in Table I (legend[a]). [b] Wet-weight. [c] This value is not very different from P_i concentration in the cell water content of epimastigotes that was 85% of the wet-weight.

P_{as}, P_l and P_t were determined by the method of BARTLETT[6]. ^{32}P activity was measured in isobutanol-benzene extracts of phosphomolybdates complexes[5] (P_i and P_7), total cell suspensions (P_t) and TCA- or lipid-extracts (P_{as} or P_l, respectively). ^{32}P activity was measured in flow-counter in accordance to standard methods. Uptake of ^{32}P-phosphate by epimastigotes was calculated on the base of ^{32}P activity in phosphate fractions and the specific activity of ^{32}P in the added phosphate.

Results and discussion. Table I shows that epimastigotes cultured in the standard medium took up very little $^{32}P_i$, irrespective of the addition of an energy source, such as glucose. On the other hand, a significant uptake of $^{32}P_i$ occurred when the cells were grown in phosphate-limited media and, accordingly, were phosphate deficient.

Table II shows P pools and ^{32}P distribution after uptake of $^{32}P_i$ by the phosphate-deficient epimastigotes. The soluble fraction, that included about half of the cell P, was constitued by P_i (40%), P_7 (30%) and P_o (30%). The P_i fraction had the fastest turnover as shown by the specific activity values. Omission of glucose decreased by 50% total $^{32}P_i$ uptake but did not alter the distribution pattern of ^{32}P. It is worth noting that uptake of $^{32}P_i$ occurred against a steep concentration gradient, as results from comparison of the extra- and intracellular concentrations of P_i (0.7 and near 21 mM, respectively).

The ability of epimastigotes to maintain the P_i gradient is further demonstrated in the Figure. In this experiment, the maximum in the P(E) plot and the symmetric minimum in the P(I) plot indicated that as soon as the extracellular P_i reached a critical concentration, P_i efflux was counterbalanced by P_i influx. In good agreement with that assumption, addition of a small amount of highly radioactive $^{32}P_i$ determined a significant uptake of $^{32}P_i$ by epimastigotes. The rate of $^{32}P_i$ influx was calculated from the slope of the plot of intracellular P_i after the addition of ^{32}P-phosphate (broken line) while the rate of P_i efflux was calculated from the increase of extracellular ^{32}P in the first 5 min of incubation. From data in the Figure resulted the following values for P_i fluxes (in nmole $P_i \times kg^{-1}$ cells $\times min^{-1}$): 1.00 (influx) and 0.16 (efflux). The relatively higher value of the influx rate is remarkable when compared with P_i concentrations in epimastigotes (near 24 mM) and medium (5.5 mM after addition of ^{32}P).

Table III shows that treatment with 3 mM iodoacetate disrupted the permeability barriers for P_i. The effect of iodoacetate was demonstrated by a) the exit of nearly all of the cell P_i and b) by the increase of $^{32}P_i$ uptake. In addition, iodoacetate caused dephosphorylation of organic compounds and an expected[7,8] inhibition of respiration. The 2 latter effects would reflect the inhibition of glycolysis which in *T. cruzi* is a main pathway of glucose oxidation[9,10]. However, neither the variations of P_i and P_7

[6] G. R. BARTLETT, J. biol. Chem. *239*, 466 (1959).
[7] J. F. RYLEY, Biochem. J. *62*, 215 (1956).
[8] R. ZELEDON, Revta Biol. trop. *8*, 181 (1960).
[9] T. VON BRAND, *Biochemistry of Parasites* (Academic Press, New York 1966).
[10] T. VON BRAND, in *Medicina Tropical* (Ed. A. ANSELMI; Editorial Fournier, México D. F. 1967), p. 261.

Table III. Increased permeability of epimastigotes after treatment with 3 mM iodacetate[a]

Iodoacetate (mM)	P_i efflux ($\frac{\text{mmole}}{\text{kg cells}^c}$)	Diminution of cell phosphates ($\frac{\text{mmole}}{\text{kg cells}^c}$)				^{32}P-Phosphate uptake in fractions[b] ($\frac{\text{mmole}}{\text{kg cells}^c}$)		Qo
		P_i	P_7	P_o	P_t	P_i	P_l	
0	−2.0	−1.0	0	−2.0	0	0.11	0.25	7.0
3.0	34	16	14	2.0	35	0.33	0.81	0.5
		(18)[d]	(15)[d]	(12)[d]	(74)[d]			

[a] Epimastigotes (15 mg; PD_1) were suspended in standard saline solution and incubated in Warburg manometers for 3 h with iodoacetate as stated above. ^{32}P-phosphate was then added (final concentration, 3.9 mM; specific activity, 3.42×10^9 cpm/m atom g ^{32}P) and after further incubation for 2 h, the reaction mixtures were analyzed for phosphate distribution. Other experimental details were as in Table I. [b] The amount of assimilated phosphate was calculated on the base of the specific activity of the added ^{32}P-phosphate corrected for phosphate concentration in the extracellular space at the time of ^{32}P-phosphate addition. [c] Wet-weight. [d] Initial concentration.

nor the metabolic inhibition did per se promote phosphate extrusion because antimycin (1 µg/ml) and iodoacetate (0.1 m*M*) inhibited respiration and increased the intracellular concentration of P_i without stimulating P_i efflux[11]. The effect of 3 m*M* iodoacetate on P_i efflux would, therefore, imply a selective alteration of P_i transport. Iodoacetate (3 m*M*) caused also visible alterations of epimastigotes, namely, abnormal round shapes and loss of mobility that would involve important osmotic changes in the cells.

Very little is known about ion transport in *T. cruzi* and its modification by enzyme inhibitors[9,10]. The results described here, particularly the effect of iodoacetate, suggest that exploration of that aspect of the parasite biology might have interesting implications for the chemotherapy of infections by *T. cruzi*[12].

Zusammenfassung. Epimastigoten von *Trypanosoma cruzi* sind für Orthophosphate (P_i) relativ undurchlässig. Man kann trotzdem bei Zellen, die aus Ortophosphat armen Kulturen stammen, einen signifikanten Übertritt von Ortophosphat ins Zellinnere messen. Bei diesen Zellen ist die Geschwindigkeit des P_i-Transportes ins Zellinnere trotz eines diesem Vorgang entgegenwirkenden Phosphat-Konzentrationsgradienten etwa 5 mal grösser als die der P_i-Abgabe aus dem Zellinnern des umgebenden Mediums. Die Durchlässigkeitsschranke für P_i wird durch 3 m*M* Jodacetat zerstört.

J. F. DE BOISO[13] and A. O. M. STOPPANI[13]

Cátedra de Química Biológica, Facultad de Medicina, Paraguay 2155, Buenos Aires (Argentina), 22 March 1972.

[11] J. F. DE BOISO and A. O. M. STOPPANI, Revta. Soc. argent. Biol. *46*, 124 (1970).

[12] This work was supported by grants of Consejo Nacional de Investigaciones Científicas y Técnicas (Argentina), the Instituto Nacional de Farmacología, and the Wellcome Trust.

[13] Career Investigator, Conicet.

Aktivität einiger Transaminasen in den mit SO_2 behandelten Erbsen-Keimpflanzen

In vorgehenden Mitteilungen haben wir über den Einfluss des SO_2 auf den Gehalt freier Zucker und Aminosäuren sowie Ketosäuren in Erbsen-Keimpflanzen berichtet[1,2]. Weiter haben wir die Bildung der Bisulfit-Addukte von Carbonylverbindungen (Glyceraldehyd, α-Ketoglutarat, Pyruvat, Oxalacetat) in den mit SO_2 begasten Erbsen-Keimpflanzen bewiesen[2]. Durch Entstehung dieser Addukte kann die Erniedrigung des Gehalts freier Ketosäuren erklärt werden[2]. Zugleich erklärt diese Tatsache die Veränderung des Gehalts einiger freier Aminosäuren (Alanin, Glutaminsäure) in den Organen der mit SO_2 behandelten Erbsen-Keimpflanzen[1]. Auch bei Kaninchen verursacht SO_2 eine Erniedrigung freier Aminosäuren (Threonin, Histidin, Methionin) im Blut[3]. Alle diese Tatsachen sprechen für die Beeinflussung der Transaminierungsvorgänge durch SO_2 in Pflanzen und wahrscheinlich auch in Tieren. Um diese Voraussetzung zu bestätigen, haben wir die Aktivität einiger Transaminasen (Glutamat-Oxalacetat- und Asparagat-Pyruvat-Transaminase) in den mit SO_2 begasten Erbsen-Keimpflanzen näher untersucht.

Die Versuche wurden mit 15tägigen grünen Erbsen-Keimpflanzen (*Pisum sativum* L., Sorte Orlík), die bei Licht gepflanzt wurden, durchgeführt. Die Kultivierung sowie Intoxikation mit 1% gasförmigem SO_2 geschah auf dieselbe Weise wie in den früheren Versuchen[1,2]. Nach der 48stündigen Intoxikation wurden die Keimpflanzen (Kontroll- sowie Versuchspflanzen) auf Kotyledonen, Wurzeln und Achsen verteilt und diese Organe dann getrennt verarbeitet.

Die Bestimmung der Transaminase-Aktivität geschah nach einer modifizierten Methode von GREBINSKIJ et al.[4]. Das Pflanzenmaterial (5 g) wurde mit wenig Glaspulver in 30 ml eiskalten K_2HPO_4-Puffer (pH 8,0) im Porzellan-Mörser unter Kühlung im Eisbad fein homogenisiert und dann sofort in der Kühlzentrifuge (Janetzki K 14) bei 700 *g* 10 min zentrifugiert. Die Inkubationsmischung war wie folgt zusammengesetzt: 5 ml Enzymextrakt und 2,5 ml 0,06 *M* Na-Pyruvat- bzw. 0,06 *M* Oxalessigsäure-Lösung. Diese Mischung wurde 10 min bei 37 °C unter mildem Schütteln im Dubnoff-Inkubator inkubiert, dann 2,5 ml 0,06 *M* Asparaginsäure bzw. 0,06 *M* Glutaminsäure zugegeben und unter denselben Bedingungen 20 min weiter inkubiert. Die enzymatische Reaktion wurde mit 96%igem Äthanol (25 ml) unterbrochen. Mit jedem Enzymextrakt wurden 3 Parallelversuche durchgeführt; in zwei weiteren Proben (ohne Zugabe von Keto- und Aminosäure) wurde der endogene Gehalt der Aminosäure bestimmt. Eine eventuelle nichtenzymatische Reaktion der Substrate wurde durch eine weitere Probe mit Äthanol-Zugabe am Beginn der Inkubation geprüft.

Nach Zugabe des Äthanols wurde der Eiweisstoff-Niederschlag abzentrifugiert und im Vakuum auf dem Wasserbad abgedampft. Der Rückstand wurde dann in 1 ml 80%igem Äthanol gelöst und auf Whatman Nr. 1 aufgetragen und mittels zweidimensionaler Papierchromatographie getrennt[5,6]. Die Aminosäuren wurden mittels 0,2%iger Ninhydrin-Lösung im Aceton (60 °C, 10 min) sichtbar gemacht. Die Flecke des Alanins und der Asparginsäure wurden dann ausgeschnitten und mit Methanol 3mal unter ständigem Schütteln extrahiert. In den Extrakten wurde die entsprechende Aminosäure mit Ninhydrin und Cadmium-Acetat geprüft und die Absorbanz der orange-roten Cadmium-Komplexe bei 500 nm mit 'Spekol ZV' (C. Zeiss, Jena, DDR) gemessen[5,6].

Die Ergebnisse sind in der Tabelle angegeben. Wegen der nicht ganz befriedigenden Trennung von Asparagin- und Glutaminsäure in den Kotyledonen, wurde die Konzentration der Asparaginsäure nicht bestimmt. Die Mengen der durch die Transaminierung entstehenden Asparaginsäure bzw. des Alanins wurden als Differenz zwischen

[1] J. KOŠTÍŘ, I. MACHÁČKOVÁ, V. JIRÁČEK und E. BUCHAR, Experientia *26*, 604 (1970).

[2] V. JIRÁČEK, I. MACHÁČKOVÁ und J. KOŠTÍŘ, Experientia *28*, 1007 (1972).

[3] G. BOMPART, Y. CREACH und P. LEVY, Compt. r. Soc. biol. Paris *161*, 2297 (1967).

[4] S. O. GREBINSKIJ, N. M. FORNJAK und M. S. GARGOLA, Radiobiologija *6*, 631 (1966).

[5] V. JIRÁČEK, J. KOŠTÍŘ und B. JIRSÍKOVÁ, Sb. čsl. Akad. zeměd. věd. *13*, 165 (1967).

[6] V. JIRÁČEK, J. Chromat. *33*, 312 (1968).