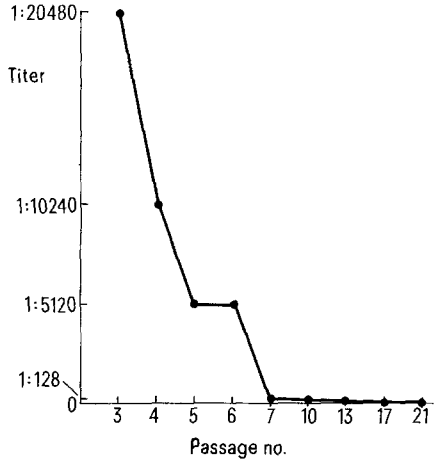
per culture. Out of 192 cultures 51 showed growth of hybrid cells. In 21 cultures hybrid cells secreted antibodies specific for SRBC. The culture with the highest titer of anti-SRBC antibody was selected and cloned twice under limiting dilution conditions. Then the clone was expanded to mass culture and injected i.p. to BALB/c mice. Testing ascites fluids two weeks later by Ouchterlony analysis showed that the clone was secreting IgG_1 antibody. Antibody titer was assessed after subsequent in vivo passages of hybridoma cells. After a number of passages it was found that the hybrid clone produced progressively less antibody (fig.). Ascites fluid from the third passage had the titer of 1:20,480 which dropped to 1:5120 after the next three passages. After seven passages, that is approximately 4.5 months after fusion, the antibody titer decreased gradually. Finally after the 13th passage, that is six months after fusion, the antibody titer was negligible. Eight months after fusion (21st passage) the antibodies to SRBC were not detectable in ascites fluids.



Karyotype of hybridoma cells producing antibody to SRBC, compared with the antibody titer

Number of metaphases analyzed	Chromosome number	Passage number	Antibody titer
50	104 ± 4*	3	1:20,480
47	104 ± 7	6	1:5120
50	94 ± 4**	13	1:4
49	96 ± 7**	21	0

* Mean value ± SD. ** $p < 0.001$ vs. the chromosome number in the passage No. 3.

It is of interest that the decrease of antibody titer was accompanied by a decrease in the chromosome number in the hybrid cells. Chromosomal analysis showed that the hybridomas secreting specific antibodies had a mean chromosome number of 104 ± 4 (table). Six months after fusion, the chromosome number dropped to 94 ± 4 and did not change significantly in the following two months (21st passage; table). Loss of antibody production by hybridoma cells is probably due to a number of factors. The simplest situation is when the antibody forming hybridoma is one among a number of non-antibody-secreting hybrids in a given culture. When the antibody producing hybridoma is growing more slowly than non-producing hybrids, then it may rapidly be overgrown by the other cells in the culture. This possibility was excluded in the present study by cloning twice the hybrid clone producing antibodies to SRBC. Loss of antibody production may also be due to segregation of the genes for antibody heavy and/or light chains[6,7]. On the basis of the data presented here it may be postulated that the observed loss of antibody production is most probably due to chromosome loss in the hybrid line studied.

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On the mechanism of killing of *Trypanosoma cruzi* by human polymorphonuclear leukocytes[1]

R.L. Cardoni[2], E.H. de Titto and R. Docampo

Instituto Nacional de Diagnóstico e Investigación de la Enfermedad de Chagas 'Dr. M. Fatala Chabén', Av. Paseo Colón 568, 1063 Buenos Aires (Argentina), and Department of Health and Human Service, National Institute of Environmental Health Science, Research Triangle Park (North Carolina 27709, USA), 7 February 1984

Summary. The temperature-dependence of some processes involved in the killing of sensitized *T. cruzi* epimastigotes by human polymorphonuclear leukocytes (PMN) was determined. The rate of the reactions was related to the temperature of incubation according to the Arrhenius equation and the apparent energies of activation (Ea) were calculated. The Ea values separated these complex reactions into two groups: one with Ea of about 10 kcal/mol for the phagocytosis of the parasites and the release of lysosomal enzymes by PMN, and the other with Ea of about 22 kcal/mol for the cytotoxicity against sensitized *T. cruzi*, the rate of oxygen consumption by PMN, and the lysis of the parasites with added hydrogen peroxide.
Key words. Trypanosoma cruzi; polymorphonuclear leukocytes.

Peripheral human polymorphonuclear leukocytes (PMN) are able to kill in vitro the epimastigote and amastigote forms of *T. cruzi* in the presence of antibody directed against the parasite[3-6]. At present only the characteristics of the killing of the epimastigote forms of *T. cruzi* had been extensively documented[3,4,6-10]. It had been demonstrated that the interaction between the Fc receptor of PMN and the Fc from the antibody bound to *T. cruzi* epimastigotes leads to phagocytosis and par-

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asite lysis[6]. During the process PMN increase their oxygen consumption and release lysosomal enzymes and reactive oxygen species[7,9,10]. Although previous reports supported the view that the lysosomal enzymes from PMN were essential to the killing of phagocytized cells[11] we provided evidence that the release of lysosomal enzymes was related to the attachment of sensitized *T. cruzi* to the effector cell membranes and does not seem to be involved in the rapid destruction of the parasites[9]. More recently the production of oxygen intermediates was considered as the crucial point in the killing of engulfed bacteria and protozoa[12]. According to that, our previous results indicated that the killing of sensitized *T. cruzi* was mainly due to the hydrogen peroxide generated during the increased oxygen uptake[7,10]. In the present work, the killing of sensitized *T. cruzi* has been studied from a physicochemical point of view determining the apparent energies of activation of various associated reactions. This methodologically simple approach gives a solid framework for interpreting the overall process.

Methods. PMN were purified from EDTA-supplemented venous blood by dextran sedimentation of pellets obtained after Ficoll-Hypaque separation. Epimastigotes of *T. cruzi,* Tulahuén strain, grown in Warren's medium with or without ^{3}H-uridine were used *(T. cruzi* or ^{3}H-*T. cruzi)*[13]. They were sensitized with sera from rabbits immunized with total homogenates of *T. cruzi* epimastigotes *(T. cruzi*-Ab or ^{3}H-*T. cruzi*-Ab). Cytotoxicity was evaluated as the percentage of radioactivity released by ^{3}H-*T. cruzi*-Ab incubated with 4×10^5 PMN at a 10:1 effector to target cell ratio[3]. Phagocytosis was evaluated morphologically as the percentage of PMN containing intracellular parasites after incubation with *T. cruzi*-Ab at a 1:1 effector to target cell ratio[8]. Oxygen consumption by PMN was measured in a Gilson K-IC oxygraph with a Clark-type electrode; oxygen uptake by 10^7 PMN was recorded in resting conditions and after the addition of 10^7 *T. cruzi*-Ab[7]. Release of lysosomal enzymes, lysozyme and β-glucuronidase, from 2.5×10^6 PMN was determined in cell free supernatants after incubation with 2.5×10^6 *T. cruzi*-Ab[9,14,15]. Percentage of lysis of 2.5×10^5 ^{3}H-*T. cruzi* with 200 mM hydrogen peroxide was determined as the percentage of radioactivity released. Kinetic studies showed that the reactions proceed linearly until 1 h of incubation; thus the percentage of cytotoxicity, phagocytosis, lysis of ^{3}H-*T. cruzi* with hydrogen peroxide and lysosomal enzymes release measured after 1 h of incubation may be regarded as the initial reaction velocity. Reactions were carried out at temperatures between 4 °C and 39 °C as indicated in figure 1.

Apparent energy of activation of the reactions involved in the killing of *T. cruzi* epimastigotes by human PMN

	Ea (kcal/mol)[a]	Slope (K^{-1})[b]	S_{YX}	r[c]
Cytotoxicity	21.9 ± 0.4	4790 ± 90	0.21	0.976
O_2 consumption	20.0 ± 0.7	4370 ± 160	0.11	0.949
Lysis with 200 mM H_2O_2	23.1 ± 0.3	5060 ± 60	0.15	0.977
Phagocytosis	11.6 ± 0.2	2550 ± 173	0.05	0.982
Release of lysozyme	9.9 ± 0.1	2160 ± 30	0.07	0.970
Release of β-glucuronidase	9.5 ± 1.0	2080 ± 220	0.10	0.934

Parameters of the lines depicted in the insets of figure 1. [a] Apparent energy of activation ± SDM. [b] Slope values ± SDM. The covariance S_{YX} and the SDM of the estimated slope (b) was calculated as:

$$S_{YX} = \sqrt{\Sigma Y^2 - a\Sigma Y - b\Sigma XY/N\text{-}2} \qquad SDM = \pm \frac{t}{N\text{-}2}(S_{XY}/S_X)$$

where $S_X = X^2/N$ and the t value was in the 95% confidence interval for N-2 d.f. To test the differences between slopes, t values were calculated as:

$t = (b\text{-}b')\sqrt{N\text{-}2/(1\text{-}r^2)}$.

The slope values for cytotoxicity, oxygen consumption and lysis with hydrogen peroxide are significant · different from the slope values of phagocytosis and release of lysosomal enzymes ($p < 0.05$). [c] Correlation coefficient between Y and X.

The logarithmic form of the Arrhenius mathematical expression for the temperature-dependence of the rate of a reaction was used in order to fit the experimental values into a straight line equation[16]:

$$\log k = \log A - \frac{Ea}{2{,}303\ R} \times \frac{1}{T}$$

where A is a constant factor termed the preexponential factor; R is 1.987 cal; T is the absolute temperature in K; and Ea, in cal/mol, is the Arrhenius energy of activation. It can be written as: $Y = a + bX$ (where $Y = \log k$, $a = \log A$, $X = 1/T$, and $b = -Ea/2{,}303\ R$).

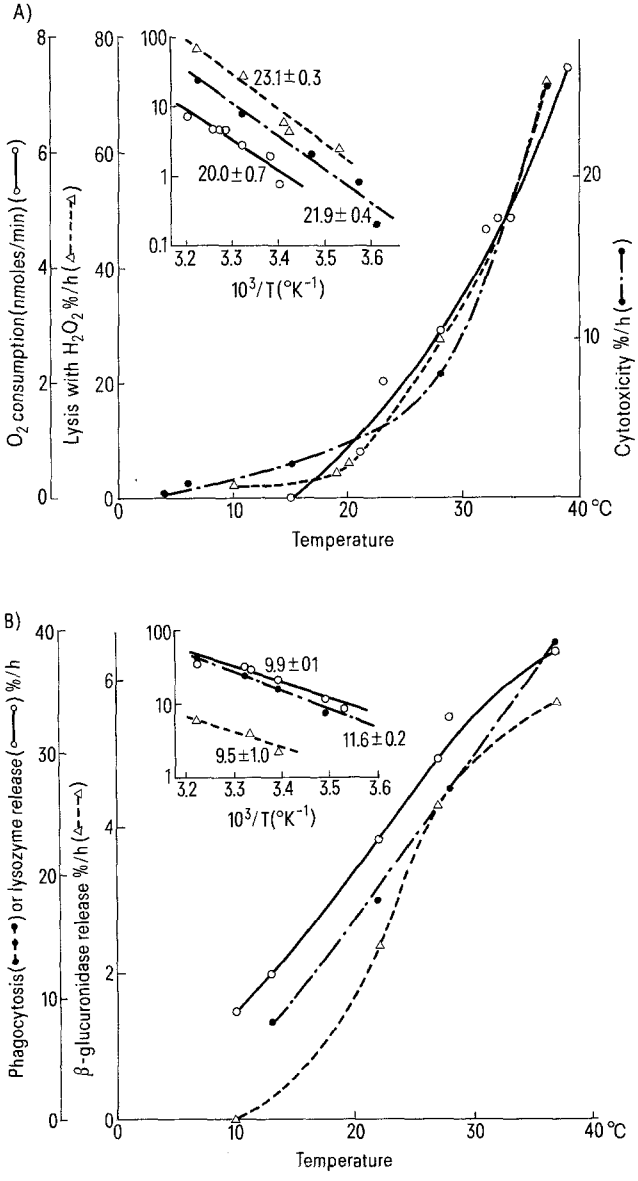

Figure 1. Effect of temperature on the killing of *T. cruzi* by human polymorphonuclear leukocytes or by hydrogen peroxide.

The initial rate of cytotoxicity (^{3}H-*T. cruzi*-Ab lysed %/h), oxygen consumption by PMN (nmoles/min), lysis of ^{3}H-*T. cruzi* with hydrogen peroxide (lysis %/h) (A), phagocytosis (PMN with intracellular parasites %/h) and release of lysosomal enzymes (lysozyme or β-glucuronidase released %/h) (B) were plotted against the temperature of incubation. Insets: Arrhenius plots, regression line of the logarithm of the rates vs. 1/T (K^{-1}). Parameters of the lines are detailed in the table. The numbers represents Ea calculated from the slopes values ± SDM.

Results and discussion. In order to characterize the different processes that lead to the destruction of sensitized *T. cruzi* epimastigotes by PMN we have determined the temperature-dependence of different in vitro reactions (table). Since previous results indicated that the H_2O_2 generated by PMN during the increased O_2 uptake was involved in the killing of the parasites[7] we have included in this study the lysis of ^{3}H-*T. cruzi* with H_2O_2 in the absence of PMN. The concentration of H_2O_2 required to lyse the parasites in the absence of cells, i.e. 200 mM, is similar to the one estimated within the phagocytic, vacuoles[17]. As shown in figure 1, the velocity of the reactions studied increased exponentially as the temperature of incubation was raised. The transformation of the results to the logarithmic form of the Arrhenius expression[16], as shown in the corresponding insets in figure 1, led us to calculate the energies of activation, which indicate the temperature-dependent increase of the rate of the reaction and characterize each process. The regression lines obtained do not show discontinuity in the slopes, indicating that the process considered took place as though Ea remained constant in the range of temperature explored.

The estimated Ea values indicated that the processes involved in the killing of sensitized *T. cruzi* by human PMN can be separated into two different groups (table). Ea values for the reaction of cytotoxicity, oxygen consumption and lysis with added H_2O_2 were about 22 kcal/mol, while the reactions of phagocytosis and lysosomal enzymes release had an Ea value of about 10 kcal/mol. The velocity of the reactions of cytotoxicity, oxygen consumption and lysis with H_2O_2 were increased about 3-fold, whereas phagocytosis and release of lysosomal enzymes increased nearly 2-fold on raising the temperature by 10 °C. Mahoney et al.[18] reported Ea values of about 55 kcal/mol for the Fc-mediated phagocytosis of red cells by mouse peritoneal macrophages, the differences from our results may be ascribed to differences in both the effector and target cells used.

Each process is certainly a multi-step one, and our results do not allow us to determine whether in each group of reactions the overall activation energy represents a large contribution by a single component or the addition of smaller contributions. In either case, similarity between Ea would imply the sharing of rate-limiting steps among the various reactions measured. The Ea of the cytotoxic reaction is close to both the Ea of the oxygen uptake and of the H_2O_2-induced parasite lysis. This process seems to be closely related to the sequence: O_2 uptake→O_2^- formation→H_2O_2 release→$HO^\cdot$ generation→parasite lysis. The first step corresponds to the O_2^- formation by the superoxide anion synthetase of PMN plasma membrane[19]; H_2O_2 formation is catalyzed by both leukocyte and parasite superoxide dismutase[20, 21] and the hydroxyl radicals are probably generated by the cytochrome-catalyzed scission of H_2O_2. The presence of KCN inhibited the lysis of *T. cruzi* with added hydrogen peroxide (fig. 2) indicating that parasite constituents, in addition to those from effector cells, transform H_2O_2 and were involved in the membrane disruption. Our data allows us to discard phagocytosis and enzymatic release as the rate-limiting step for parasite lysis since they have a different Ea from the main process.

The killing of sensitized *T. cruzi* by human PMN can be visualized as the combination of two different processes. Phagocytosis of the parasites and the release of the constituents of lysosomal granules seems to reflect membrane processes, such as parasite attachment, endocytosis and phagolysosome formation, with lower apparent activation energies. The second type of process could reflect chemical reactions, such as O_2 uptake, H_2O_2 formation and parasite lysis by a free radical reaction, with higher apparent activation energies. According to the data on the rate of phagocytosis and cytotoxicity the lysis of the parasites occurs mainly intracellularly. An early endocytosis of the parasites preceding the slower lysis with H_2O_2 may contribute to the efficiency of the system, since the clearance of the parasites prevents the involvement of additional antibody and cells during the ongoing killing of the parasites.

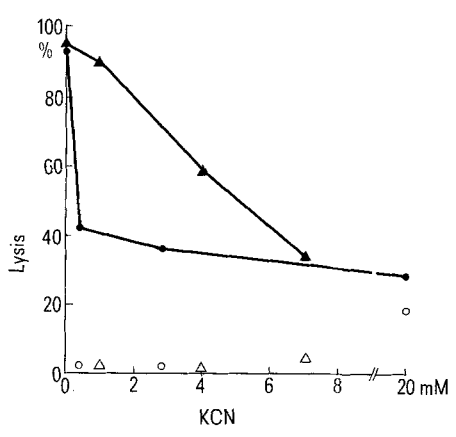

Figure 2. Effect of the presence of cyanide on the lysis of *T. cruzi* by hydrogen peroxide.
The parasites were incubated with H_2O_2 25 mM at 28 °C (●) or with H_2O_2 1.5 mM at 37 °C (▲) or in the absence of H_2O_2 ((○) 28 °C; (△) 37 °C) for 3 h varing the concentrations of KCN as indicated in the figure.

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2 Address all correspondence to: R.L. Cardoni, Instituto Nacional de Diagnóstico e Investigación de la Enfermedad de Chagas 'Dr. M. Fatala Chabén', Av. Paseo Colón 568, 1063 Buenos Aires, Argentina.
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