Microsomal Hemoproteins in *Trypanosoma cruzi*[1] (35230)

J. F. DE BOISO[2] AND A. O. M. STOPPANI[2]

Instituto de Química Biológica, Facultad de Medicina, Buenos Aires, Argentina

Spectroscopic investigation of *Trypanosoma cruzi*, causative agent of Chagas' disease, shows the absorption spectra of reduced hemoproteins which by similarity of position of bands with respect to the mitochondrial mammalian and yeast cytochromes have been designated cytochromes *a*, *b*, and c_1 [Refs. (1–5)]. Little is known about these pigments and there is no evidence for biochemical similarity with the mammalian and yeast cytochromes (5). Inhibition of the parasite respiration by cyanide and azide (1–7), may be explained by the existence of a terminal oxidase (2, 5, 7) tentatively identified as *T. cruzi* cytochrome *a* [Ref. (5)]. However, in contrast to mammalian cytochrome oxidase, *T. cruzi* oxidase does not oxidize reduced cytochrome *c* (1–4, 7) and does not show light-dependent inhibition by carbon monoxide (2, 4). In the present study the cytochrome system of *T. cruzi* culture forms has been reinvestigated, particularly with respect to its distribution in the cell fractions. Unexpectedly, most of the parasite hemoproteins show properties characteristic of the mammalian microsomal cytochromes.

Materials and Methods. Organism. T. cruzi (Tulahuen strain) was grown in Roux bottles on a diphasic medium at 28°. The solid phase was made of agar (Bacto; 19 g), brain–heart infusion (Difco; 38 g) and water 1 liter. 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 liter. Six days after inoculation, the overlay of several bottles were pooled, the crithidia (epimastigotes) were concentrated by centrifugation at 4°, and the cells were washed three times in the centrifuge with isotonic (0.154 *M*) NaCl solution. Blood was omitted from the culture medium to minimize the interference of spurious heme pigment with the investigation of the parasite hemoproteins.

Other materials. The yeast employed for spectrophotometric control, was a sample of *Saccharomyces cerevisiae* (wild strain, diploid) kindly supplied by Dr. E. H. Ramos. Antimycin A, NADH, cytochrome *c*, phenazine methosulfate and Tris buffer (Trizma) were purchased from Sigma Chemical Co.

Cell disruption and fractionation. The method employed was basically the one described by Agosin and Von Brand (8). The parasites were suspended in a medium containing 0.25 *M* sucrose; 1 m*M* EDTA; 5 m*M* Tris HCl at pH 7.4 and then subjected to 2000 psi in the Ribi refrigerated cell fractionator at 0°. Microscopic control showed that most of the cells were broken. The resulting homogenate was processed as follows: (a) centrifugation at 700*g* for 30 min to sediment nuclei, coarse cell fragments, and unbroken cells; (b) centrifugation of the first supernatant in the Servall RC-2 centrifuge at 10,000*g* for 30 min to sediment mitochondria (heavy) and related fragments ("mitochondrial" fraction); (c) centrifugation of the second supernatant at 105,000*g* in the Spinco Model L centrifuge for 60 min to sediment microsomes and related particles ("microsomal fraction"). The last, clear supernatant ["true supernatant" (8)] was dialyzed over night against distilled water and concentrated by lyophilization. All operations were performed at 4°.

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In a preliminary investigation of the intracellular distribution of hemoproteins in *T. cruzi*, the cells were disrupted by repeated (3 times) freezing (at —18°) and thawing (2); the homogenate was centrifuged at 1000*g* and 4° to eliminate unbroken cells, nuclei, and cell debris, and the supernatant was employed for spectrophotometric investigation.

Spectrophotometric techniques. The absolute spectrum of *T. cruzi* was recorded with a Beckman DK-2 spectrophotometer equipped with a 75-W Model 401 xenon source. The photomultiplier sensitivity was adjusted to 1× and slit-widths were generally <0.2 mm. The *T. cruzi* cells were separated from the suspension medium by centrifugation and the resulting thick pellet was transferred to a 2-mm light-path cuvette made of Plexiglas. Pieces of flat oiled paper were placed in the reference cuvette in order to have an adequate blank absorbance. After a short period in the cuvette, the parasite cytochromes were reduced by the activity of endogenous metabolism and the corresponding absorption bands became apparent and stable. The reliability of the method was established by recording, under identical experimental conditions, the spectrum of a sample of bakers' yeast, the characteristics of which are well known (9).

Difference spectra of cell fractions were measured with the same instrument, in 10-mm light-path standard cuvettes, with the media described under Results. Light-scattering by particles was very great and therefore addition of deoxycholate was essential in order to obtain adequate transparency. Furthermore, the spectrophotometry of mitochondrial hemoproteins was performed with a pH 10 extract (10) of particles sedimenting at 30,000*g* because of the high turbidity of the original suspension. The low-speed sedimenting fraction (10) was examined in the same manner.

Assay of enzyme activities. These were measured with a Gilford 2000 spectrophotometric unit in 10-mm light-path cuvettes, at 30°. The reaction mixtures employed (total vol, 3 ml; 26 μg of active protein) were completed as follows. NADH-oxidase: 0.17 *M* phosphate buffer and 0.23 m*M* NADH,

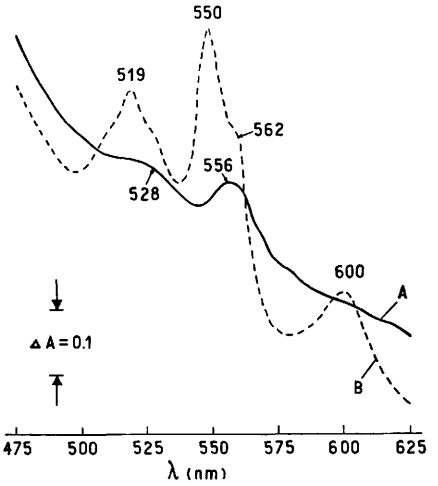

FIG. 1. Absolute spectrum of *T. cruzi* (A) and bakers' yeast (B). Experimental conditions as described under Methods.

pH 7.4. Absorbance was measured at 340 nm. NADH-cytochrome *c* reductase: conditions as above; 40 μ*M* cytochrome *c* was added to the reaction mixture. Absorbance was measured at 340 nm unless stated otherwise. NADH-ferricyanide reductase: conditions as described by Minakami *et al.* (11). Succinate oxidase: 0.3 *M* phosphate buffer, 10 m*M* succinate, pH 7.4. Absorbance was measured at 250 nm (12). Succinate dehydrogenase: conditions as described by Arrigoni and Singer (13); spectrophotometric phenazine methosulfate method, except succinate was 8 m*M* and cysteine sulfinate was omitted 24 μg of active protein.

Protein concentrations in cell fractions were measured with the biuret method (14).

Electron microscopy. Samples were fixed in 3% (w/v) solution of glutaraldehyde and then in 1.5% (w/v) solution of OsO_4. They were dehydrated with ethanol and were embeded in Epon. After staining with 2% (w/v) uranyl acetate, the specimens were observed under a Siemens Elmiscop I.

Results. Figure 1 shows the recorded absolute spectrum of *T. cruzi.* Broad bands with maxima about 556 and 528 nm, respectively, were clearly detected; in some spectra a very weak band appeared about 600 nm. The recorded spectra were in good agreement with spectroscopic observations by Baernstein and Tobie (1), Baernstein (2), Ryley (3),

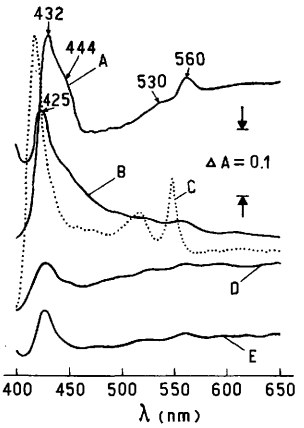

FIG. 2. Difference spectra (reduced minus oxidized) of microsomal (A–D) and mitochondrial (E) fractions of *T. cruzi*: (A) dithionite reduced spectrum; protein conc, 3 mg/ml; 0.08 *M* phosphate buffer, pH 7.3; sodium deoxycholate, 5% (w/v) added to optimal transparency of solution (3 drops). (B) NADH (2.7 m*M*) reduced spectrum; other conditions as in (A). (C) same as (B) added cytochrome *c* (11 μM) to both reduced and oxidized (control) samples. (D) cysteine (20 m*M*) reduced spectrum; conditions as in (A) except reductant. (E) dithionite (reduced minus oxidized) difference spectrum of NaOH extract [pH 10.1; Ref. (12)] of mitochondrial fraction (protein conc, 4.6 mg/ml; other conditions as in (A).

and Fulton and Spooner (4). On the other hand, comparison of *T. cruzi* spectrum with the spectrum of bakers' yeast (Fig. 1B) allowed to exclude the presence of cytochromes *c* and c_1 from the former organism, at least in concentrations detectable with the method employed. Concerning cytochrome *b*, the difference between the trypanosomal and yeast cytochromes may be inferred from the shape of the respective curves and the wavelength of maximal absorbance.

Figure 2A–D shows the (reduced minus oxidized) spectra of the microsomal fraction with different reductants. With dithionite (line A), a cytochrome *b*-type spectrum was obtained, with α, β, and Soret peaks around 560, 530, and 432 nm; the Soret peak showed a shoulder at 444 nm. Addition of cyanide (0.1 m*M*) did not affect the dithionite spectrum. With NADH as reductant (Fig. 2B), a similar spectrum was recorded with peaks at 560 (α), 526 (β), and 425 (Soret) nm but the overall variation of absorbance was less than with dithionite. When cytochrome *c* was added to both the NADH-reduced and reference samples, the characteristic bands of reduced *c* became rapidly apparent (Fig. 2C), while the bands corresponding to the original spectrum (B) disappeared. With cysteine as reductant (line D), the spectrum resembled the one obtained with NADH although the intensity of bands was weaker. Finally, line E is the (dithionite-reduced minus oxidized) spectrum of the "mitochondrial" fraction; peaks corresponding to the mammalian and yeast cytochromes a–a_3, *b*, *c*, and c_1 were absent. Spectra similar to E were obtained with the alkaline extract (10) of the low-speed sedimenting fraction and with the "true supernatant."

The (dithionite reduced minus oxidized) spectrum obtained with the supernatant from cold-treated parasites (2) showed the same pattern as in Fig. 2A, but the amount of pigments extracted was smaller than with the much more efficient Ribi press method.

Figure 3 shows the (dithionite reduced + CO minus reduced) difference spectrum of the microsomal fraction. The spectrum was recorded 30–60 min after the addition of deoxycholate which may be significant for the interpretation of the results obtained. The peak at 420 nm and the trough at 442 nm reveal the presence of a CO-reactive pigment. For comparative purposes, the (re-

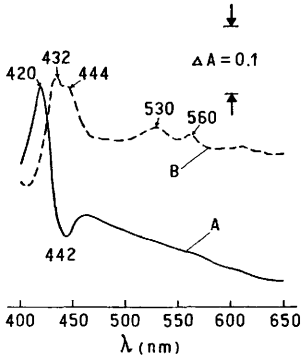

FIG. 3. (A): (Dithionite reduced + CO *minus* reduced) difference spectrum of microsomal fraction of *T. cruzi*. Protein conc, 2.2 mg/ml; 0.08 *M* phosphate buffer; 25% (v/v) glycerol; deoxycholate as in Fig. 2. (B) (dithionite reduced minus oxidized) difference spectrum of the same microsomal fraction (control).

TABLE I. Enzyme Activities in Mitochondrial and Microsomal Fractions of *T. cruzi*.[a]

Fraction	Enzyme reaction	Addition	Enzyme activity[b]
Microsomal	NADH-ferricyanide reductase	None	620
	NADH-oxidase	None	14
	NADH-cytochrome *c* reductase	None	37
	NADH-cytochrome *c* reductase	AA[c]	37
	Succinate dehydrogenase	None	0
Mitochondrial	NADH-ferricyanide reductase	None	350
	NADH-oxidase	None	25
	NADH-oxidase	AA[c]	25
	NADH-oxidase	KCN (1 m*M*)	25
	NADH-cytochrome *c* reductase	None	46
	NADH-cytochrome *c* reductase	AA[c]	46
	Succinate dehydrogenase	None	13
	Succinate oxidase	None	0

[a] Exp. conditions as described under Methods.

[b] Enzyme activities are expressed as nanomoles of substrate oxidized per minute per milligram of protein.

[c] AA, antimycin A (1 μg/ml).

duced minus oxidized) spectrum of the same microsomal fraction (line B) is included. In this case, the addition of glycerol to the solvent mixture allowed a better resolution of superimposed peaks and accordingly, the 444 nm shoulder was more clearly visible than in Fig. 2.

Enzymes activities of fractions. Table I shows the results obtained with the microsomal and mitochondrial particles. With both, the NADH-ferricyanide reductase activity was fairly high. On the other hand, the NADH-cytochrome *c* reductase activities were much less, although well above the blank values; antimycin A (40 μg/mg of protein) did not inhibit the microsomal and mi-

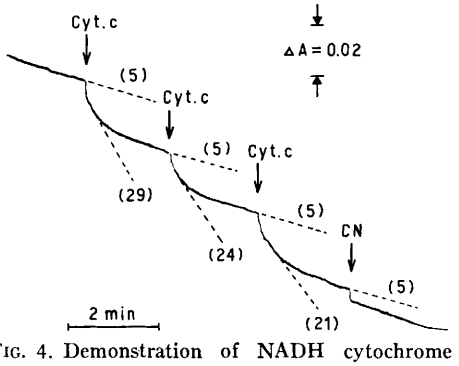

FIG. 4. Demonstration of NADH cytochrome *c* reductase activity of microsomal fraction of *T. cruzi*. Methods for NADH-oxidase assay, except protein concentration (0.53 mg). Where indicated cytochrome *c* (40 μM) and cyanide (1 mM) were added. The values in parentheses represent the rate of NADH oxidation (nmole/min/mg of protein).

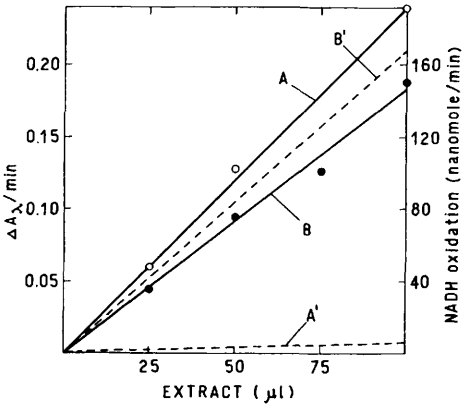

FIG. 5. Effect of protein concentration on NADH-cytochrome *c* reductase (A, A'); and NADH-ferricyanide reductase (B, B') of *T. cruzi* microsomal fraction. Experimental conditions as described under Methods except cytochrome *c* (20 μM) and enzyme preparation which is stated in the abscissa (5.32 mg of protein/ml of extract). Absorbance measured at 420 nm (NADH-ferricyanide reductase) or 550 nm (NADH-cytochrome *c* reductase). Lines A and B are plotted against the left ordinate; A' and B' against the right one.

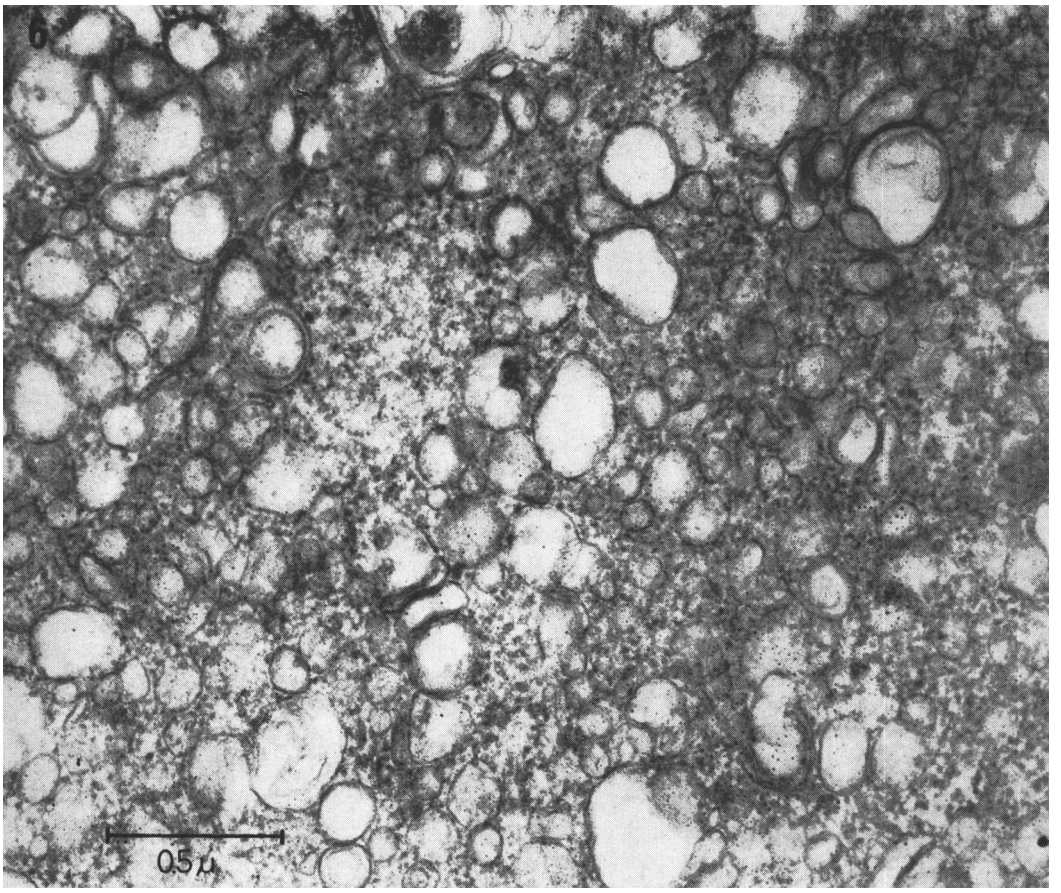

FIG. 6. Electron micrograph of the microsomal fraction of *T. cruzi* in the phase of crythidiae. Vesicular profiles with a mean diameter of 0.23 μ and ranging between 0.12 and 0.35 μ are observed intermingled with numerous ribosomes. No mitochrondrial profiles are observed in this fraction. Glutaralydehyde–osmium fixation; Siemens Elmiskop; 1 × 45,000.

tochondrial NADH-cytochrome *c* reductases. Succinate dehydrogenase activity could be demonstrated only with the mitochondrial fractions. Nevertheless, no succinate oxidase activity was evidenced by these particles.

The kinetics of the microsomal NADH-cytochrome *c* reductase reaction is shown in Fig. 4. Addition of cytochrome *c* elicited a rapid burst of NADH oxidation. When the oxidant was fully reduced, the rate of NADH oxidation returned to the initial level.

Figure 5 shows the effect of enzyme concentration on the NADH-ferricyanide and NADH-cytochrome *c* reductase activities of the microsomal fraction. In both cases a lineal relationship was obtained. It is worth noting the much higher rate of the NADH-ferricyanide reaction, when expressed on the base of substrate (NADH) oxidation.

Electron microscopy. The microsomal and mitochondrial fractions and the whole cells were examined under the electron microscope. The microsomal fraction shed vesicular profiles with a mean diameter of 0.23 μ, intermingled with numerous ribosomes. No mitochondrial profiles were observed (Fig. 6). On the other hand, the mitochrondrial fraction showed mitochondria-like vesicles without christae. Similar structures were observed in whole cells, in agreement with Sanabria's observations (16, 17). A study of *T. cruzi* mitochrondria is beyond the scope of this paper and therefore the respective electron micrographs are omitted.

Discussion. Among the many disintegrating

methods assayed to prepare biologically active fractions from *T. cruzi* (2–4, 7, 8, 15), crushing the organism in the Ribi fractionator was selected by Gonzalez-Cappa *et al.* (15) to obtain antigenic fractions because the parasite cells were broken in an atmosphere ensuring control of temperature and oxidative reactions during preparation. These requirements may also be valid for respiratory particles. The disrupting pressures applied in our experiments were similar to those employed by Agosin and Von Brand (8) which ensured that 100% of the parasite cells were broken.

The results of the present investigation show that culture forms of *T. cruzi* contain microsomal hemoproteins. This is in agreement with the existence of a rough endoplasmic reticulum in the parasite cells (16, 17) as well as the demonstration of microsomal profiles in the corresponding subcellular fraction (Fig. 6). Among the microsomal hemoproteins of *T. cruzi*, the NADH-cytochromes b_5 system can be characterized by comparison with the corresponding liver system, which may be taken as standard (18). The liver system has the following properties: (a) it is attached to the microsomal system; (b) it is composed of the flavoprotein NADH-cytochrome b_5 reductase and cytochrome b_5; (c) the (reduced minus oxidized) difference spectrum of cytochrome b_5 has maxima at 556, 531, and 423 nm; (d) cyanide does not alter the absorption spectrum of cytochrome b_5; (e) cysteine reduces cytochrome b_5; (f) cytochrome *c* is an *in vitro* electron acceptor for the system although the significance of this reaction is unknown; (g) the NADH-dehydrogenase flavoprotein has the highest turnover number with ferricyanide as oxidant. Conditions (a–g) are apparently satisfied by the *T. cruzi* microsomal preparations, as shown in Figs. 2, 4, 5, and Table I.

Besides cytochrome b_5 other hemoprotein(s) may be present in *T. cruzi* microsomes. This assumption is supported by (a) the CO-binding effect (Fig. 3), and (b) the differences between the dithionite and NADH reduced pigments (Fig. 2). The CO-binding effect may be explained by a hemoprotein similar to liver hemoprotein P-420 (19, 20). In liver, P-420 is the solubilized form of hemoprotein P-450 the CO-derivative of which strongly absorbs at 450 nm; agents like deoxycholate (19) rapidly convert P-450 into P-420. A similar change may have occurred with *T. cruzi* microsomes since the preparation which spectrum is shown in Fig. 3A received deoxycholate before the spectrophotometric investigation. Furthermore, when the (reduced minus oxidized) spectrum of *T. cruzi* microsomal fraction was recorded shortly after deoxycholate addition (Figs. 2A, 3B), a shoulder appeared around 444 nm which in other investigations (21) has been attributed to P-450. However, with the preparations employed in the present study, the identifying peak at 450 nm could not be obtained and therefore, the very nature of the microsomal CO-binding pigment must be left unsolved.

The presence of succinate dehydrogenase in the mitochondrial fraction of *T. cruzi* is reliable evidence for the origin of the respective particles. Similar results were reported by Agosin and Von Brand (8). In addition, the fairly active NADH-ferricyanide reductase observed with the same fraction can be tentatively ascribed to the mitochondrial NADH-dehydrogenase. In this regard, observations by Sanabria showed that *T. cruzi* [crithidia in mouse myocardium (16) or the rectum of *Rhodnius prolixus* (17)] do contain mitochondria. However, two essential properties of the mitochrondrial electron transfer system, namely (a) the sensitivity to antimycin A and cyanide, and (b) the presence of cytochromes *b*, *c*, and *a*, a_3 could not be demonstrated with the subcellular fractions employed in the experiments reported in Table I and Fig. 2. The absence of cytochrome *c* is remarkable and confirms previous spectroscopic observations with whole cells (1–5). Those negative results do not necessarily oppose Sanabria's observations (16, 17). In fact, most of the mitochrondrial structures reported by him (16, 17) and the similar ones observed in the present study, had very few (or no) christae, which are known to contain the *a–c* cytochrome system. The metabolic role of these atypical mitochondria remains to be established.

Summary. Spectrophotometry of whole cells, microsomal, mitochondrial, and super-

natant fractions of *Trypanosoma cruzi* (Tulahuen strain, culture forms) shows that the microsomal hemoproteins largely contribute to the heme pigments of the parasite. Both the microsomal and mitochondrial fractions have NADH-ferricyanide reductase, NADH-cytochrome *c* reductase and NADH-oxidase activities, the relative values of which decrease in the order given. Cyanide and antimycin A do not affect the NADH-cytochrome *c* reductase and NADH-oxidase activities. The mitochondrial fraction shows succinate dehydrogenase activity, which is absent from the microsomal fraction. Spectroscopic and kinetic evidence is put forward for the existence of a NADH-cytochrome b_5 reductase system in the microsomal fraction. In addition, the microsomal fraction contains a CO-binding compound which CO-difference spectrum shows strong absorbance at 420 nm. Mammalian type cytochromes *a*, *b*, and *c* are not detectable in the trypanosomal particulate and soluble fractions.

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