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NOTES ON THE GROWTH CURVE OF *TRYPANOSOMA CRUZI* CHAGAS 1909 AS DETERMINED BY OPTICAL DENSITY

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SUMMARY

The growth of *Trypanosoma cruzi*, as measured by optical density (OD) and by count, was studied in extensive experiments with the semi-synthetic "T2" culture medium of BONÉ & PARENT. These experiments showed that:

1) The growth of four *T. cruzi* strains in the T2 medium was much improved by the addition of blood-agar extract in Locke's solution; 2) Stearic acid, an essential growth factor in the experiments of BONÉ & PARENT, proved far less indispensable in the present experiments; 3) Subcultures in T2 medium were less successful from T2 cultures than those started from cultures in Tobie's diphasic medium; 4) In spite of difficulties in estimating trypanosome numbers by counting, correlation between counts and OD was generally satisfactory when applied to the growth curve of cultures started at the same time from the same basic culture. But this relationship did not hold when different sets of experiments were compared. Size and stage of development of the individual organisms were probably responsible for this discrepancy; 5) In studies of growth characteristics of trypanosomes, the OD measurement here described is considered the method of choice because it is fast, reliable and it reduces danger of contamination to a minimum.

INTRODUCTION

Many trypanosomes can be successfully cultured in a so-called diphasic culture medium. In the G. W. Hooper Foundation laboratories 10 strains have been maintained for several years: 4 strains of *Trypanosoma cruzi*, 3 of *T. rangeli*, 2 of *T. conorhini*, and 1 of an undetermined trypanosome (LAMBRECHT[4]). Most cultured trypanosomes show essentially the same forms as those encountered in invertebrate vectors.

While diphasic cultures are satisfactory for the maintenance of the trypanosome strains, they present certain experimental disadvantages: (1) The medium contains unknown elements inherent to the solid base composed of blood-agar. (2) The cultures contain unknown amounts of suspended and colored organic matter that may interfere with optical measurements such as those planned for the present series of experiments. A simple and as nearly as possible synthetic medium was desirable. Therefore we decided to use the medium described by BONÉ & PARENT, as its only non-synthetic ingredient was tryptose.

MATERIALS AND METHODS

The composition of the medium of BONÉ & PARENT was as follows: Bacto-tryptose, 15 g; glucose, 2 g; thiamine, 1 mg; folic acid, 3 mg; haemin, 20 mg; sodium stearate, 25 mg; sodium chloride, 4 g; sodium phosphate tribasic, 5 g; potassium chloride, 0.4

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g, and distilled water to bring the final volume to 1,000 ml. The sodium stearate was prepared by the reaction at 70°C of 20 mg stearic acid with 1.7 ml NaOH 0.1 N + 98.3 water for about 20 minutes under constant stirring. The haemin was best dissolved separately in a solution of 5 g sodium triphosphate in 100 ml water, then added to the rest of the medium. The mixture was then adjusted to pH 7.6 and autoclaved for 10 minutes at 15 lb. The resultant dark, straw-colored solution was somewhat clouded. As cloudiness interferes with turbidity measurements, the liquid was Seitz-filtered before autoclaving. Comparison of filtered and unfiltered culture media showed that filtration did not remove essential chemicals.

Tobie's diphasic medium[3] consists of: Senekjie's leishmania blood-agar[5] and an overlay of a Locke's solution made up of: sodium chloride, 8 g; potassium chloride, 0.2 g; calcium chloride, 0.2 g; monobasic potassium phosphate, 0.3 g; dextrose, 2.5 g; and distilled water, 1,000 ml.

Optical densities were measured by means of the Klett-Summerson Photoelectric Colorimeter, using red filter 66. Trypanosomes were cultured in special 300-ml Erlenmeyer flasks with side arm, like those used in bacteriology (Fig. 1A), and maintained in an incubator at 28° C. Before each measurement, the flask was shaken for 30 seconds to insure thorough mixture and to provide aeration. It was then tilted to fill the side arm, which was then placed in the colorimeter well (Fig. 1B). Measurements were taken daily, except Saturday and Sunday, between 7 and 8 a.m.

Actual trypanosome counts were made with the "Spencer Bright Line Improved Neubauer" hemacytometer, for comparison with the OD curve. Samples were taken by simply dipping a Pasteur pipette into the culture to the depth where capillary effect caused the liquid to rise, until a proper amount was drawn. Sampling was of course done under sterile conditions. Trypanosomes could be counted without the addition of a fixative to immobilize the organisms. Because the flagellates were often at different levels, it was necessary to use two focusing levels, but after a little practice it was possible to count all the trypanosomes. One of the main difficulties in counting was the development of "rosette" bundles in many cultures (the daily shaking, recommended above, helped to keep rosette formation at a minimum). It was impossible to estimate accurately the number of trypanosomes in these rosettes; furthermore, their distribution within the counting chamber was irregular and varied also from sample to sample. The presence of rosettes, usually containing large numbers of individual flagellates, greatly influenced the count of the organisms in the counting chamber.

The basic experiments were carrid out with *Trypanosoma cruzi*, "Tulahuen" strain. All experimental cultures were started with washed cells. The starting culture was cen-

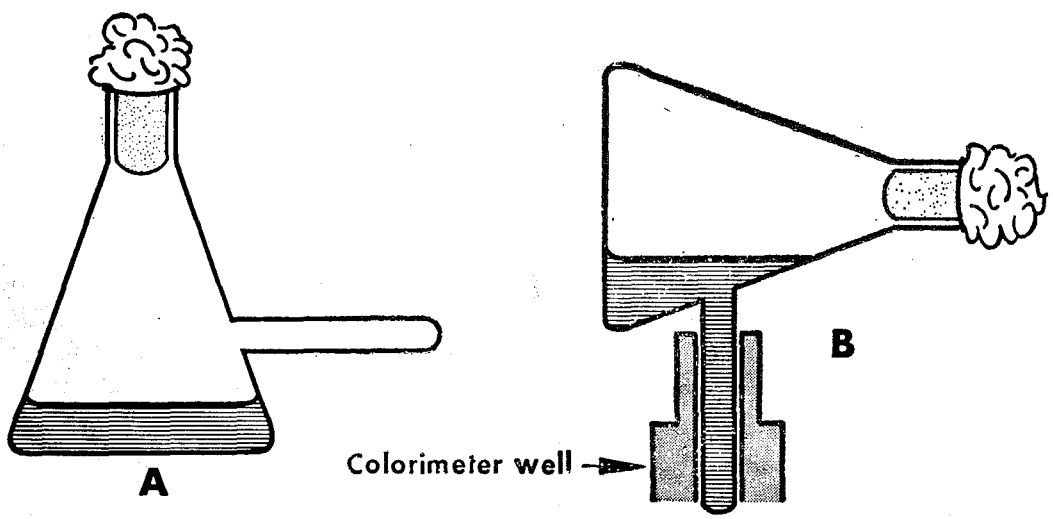

Fig. 1 (A and B)

trifuged at 2,000 r.p.m. for 10 minutes, the supernatant discarded, and the packed cells were resuspended in sterile Locke's solution, to which 200,000 units of penicillin G potassium and 250 mg streptomycin were added per 100 ml of solution. Although these concentrations of penicillin and streptomycin are considerably higher than usually used, the trypanosomes survived and were successfully cultured afterward. Washing and centrifugation were repeated three times to insure the elimination of all metabolites from the previous culture while safeguarding against contamination of the cells. The last suspension was left in contact with the liquid for 2 hours before the last centrifugation.

RESULTS

Growth in T2 medium and modification thereof

First attempts to culture *T. cruzi* in T2 medium resulted in a sluggish start and generally poor development. It was thought that perhaps the cells needed some metabolite from previous cultures, which had been eliminated by the centrifugation and washings. Therefore, a culture was started to which a few milliliters of supernatant from the centrifugation were added. Results were a shorter lag period and appreciable increased development.

In the next experiment, Tobie's modified Locke's solution was left at room temperature in contact with the solid part of her diphasic medium[3] (Senekjie's leishmania agar) for 48 hours and was then added to the T2 medium. A similar result was achieved when the supernatant was used. It was therefore concluded that the active ingredient was not a compound formed during the culturing of the organism in Tobie's medium but something already present in the blood agar and soluble in Locke's solution by extraction. Similar results were obtained for other *T. cruzi* strains: Brazil, Sonora and a strain isolated from a marmoset (*Tamarinus nigricollis*) in 1961 (DUNN et al.[2]).

Typical growth curves of comparative *T. cruzi* Tulahuen cultures are shown in Fig. 2, where "B" represents the growth curve for a T2 culture supplied by slant extract; "C" represents that for a T2 culture supplied with supernatant from a spun-down culture in Tobie's medium.

The improvement of the growth characteristics by the addition of slant extract or supernatant can be appreciated by comparison with curve "A" representing growth in a nonmodified T2 medium.

Growth in stearic-acid-free T2 medium

BONÉ & PARENT[1], who originated and experimented with the T2 medium, observed that stearic acid is essential to growth of *T. cruzi* in that medium. The strain they used had been obtained 5 years before from the Institute of Tropical Medicine, Antwerp.

The poor development of first cultures in T2 medium and their marked improvement by the addition of "slant extract" in my studies led me to question the role of stearic acid in the development of the strains dealt with. A series of cultures was started to compare development in stearic-acid-free T2 medium and in normal T2 medium. In each case development was approximately the same, while control cultures in T2 medium supplied with "slant extract" and run at the

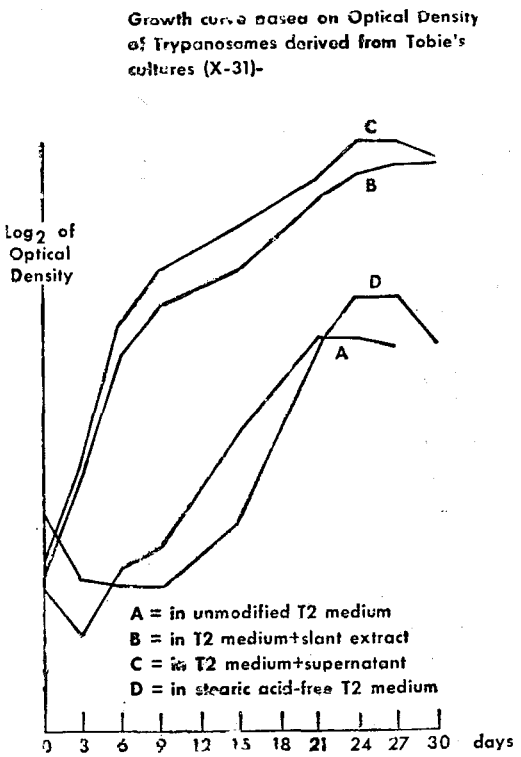

Fig. 2

same time from the same starting culture, once more were superior. In Fig. 2, "D" represents a typical growth curve of a stearic-acid-free medium that was started from the same Tobie culture as the other three cultures in the same experiment. From these curves it is evident that the lag period in the stearic-acid-free cultures was somewhat longer, but subsequent development resembled that in normal T2 medium. Differences in the lag period between such cultures were less noticeable when a transfer was made from a previous culture in T2, as discussed next.

Growth of second-generation T2 transfers

It was thought that the transfer of organisms previously grown in Tobie's culture medium into the very different environment of the T2 medium might result in the selection of adaptable types in the new medium and thus perhaps influence growth characteristics, including the need for stearic acid. Growth curves in Fig. 3 represent organisms separated from previous cultures in T2 and washed and transferred into fresh T2 cultures. In this case, growth in both the normal T2 and in stearic-acid-free T2 was similar, but culture in normal T2 suffered a serious setback the first few days. Once more, the culture supplied with "slant extract" did much better than the others. Generally speaking, the second-generation T2 transfers were less successful than those started directly from Tobie's culture medium.

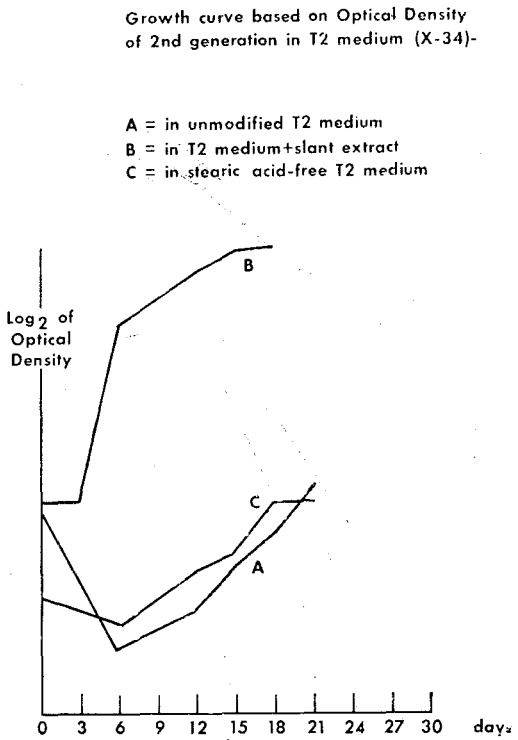

Fig. 3

Correlation between optical density and number of organisms

As previously indicated, the actual trypanosome count in cultures was hampered by certain difficulties related to movement of the organisms, different levels in the counting chamber, and the presence of rosettes. Despite these difficulties, correlation between counts and optical densities was generally satisfactory, but varied from one culture to another so that, while the photoelectric measurement seemed completely reliable to follow the development of a particular culture, it could not be used to predict the actual number of organisms per unit of culture. The most obvious reason for this disparity was variability in size and the optical density related to it from individual to individual and from one stage of development to another of a trypanosome population. Therefore, although one organism represents one unit in the hemacytometer, the size of its optically reactive surface may vary considerably with its developmental stage or with age.

The optical density and trypanosome count for two cultures in experiment X-31 are compared in Fig. 4 and correlation is rather good. Figure 5 shows two growth curves of the same experiment where correlation is less satisfactory but still acceptable. Optical density does not decline immediately at death of the culture, as next discussed, but death is indicated by the negative count of living trypanosomes.

Death of a culture

Death of a culture is spotted immediately by microscopic examination at the time of counting. The cells, or what is left of them, however, do not seem to lyse completely so that structures resembling so many nuclei keep floating in the medium for days. This

is reflected in the measurement of turbidity which drops momentarily but does not follow the downgrade curve so characteristic of the "death-curve" observed at this stage in bacterial cultures. Compared to time of survival, the death of a trypanosome culture in these experiments was relatively rapid and was indicated by microscopical examination rather than by optical density, although of course persistence of the same optical density reading would have indicated nondevelopment.

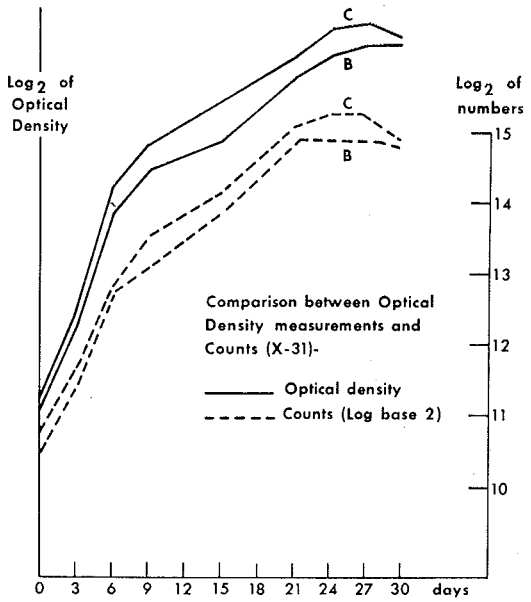

Fig. 4

These experiments did not show a direct relationship between rate of development and time of death; e.g., a culture supporting relatively few trypanosomes did not have a longer life span than one whose development was rapid and whose total yield of organisms was much higher per unit of culture. For instance, in Table I the cultures with the highest (40,000/mm^3) and lowest (3,300/mm^3) trypanosome count both died on the 27th day. This suggests that the *age of the population* of a "closed-circuit" culture, rather than the amount of available nutriment in the culture, is the regulatory factor. This table summarizes 2 sets of 3 experiments with comparative cultures. Many other cultures not included in this table corroborated these results.

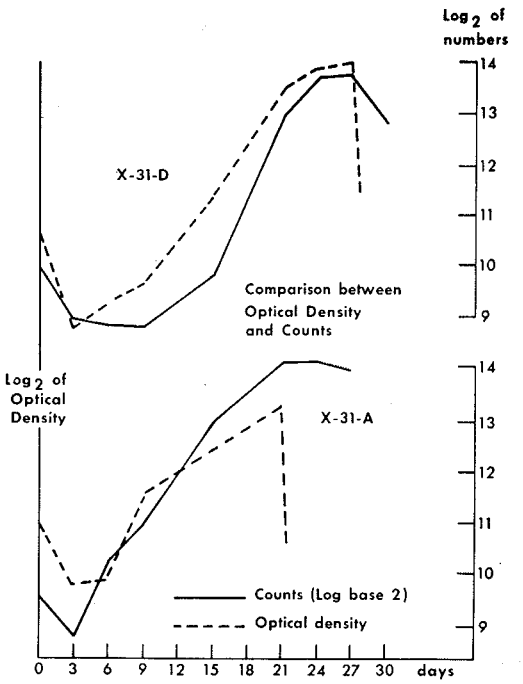

Fig. 5

CONCLUSION

It is felt that carefully planned and executed culture experiments with trypanosomes may lead to useful information regarding important physiological characteristics and possibly to greater knowledge of biological strain variations. Present experiments are a preliminary effort towards more refined culture methods and culture media that may permit attainment of these goals.

Ability to follow regularly the development of a culture is, of course, a primary necessity. For many practical reasons the measurement of turbidity by photoelectric means would seem indicated.

RESUMO

Observações sôbre a curva de crescimento do Trypanosoma cruzi CHAGAS 1909, *determinada pela densidade óptica*

O crescimento do *Trypanosoma cruzi*, medido por densidade óptica (D.O.) e por contagem, foi estudado em extensos experimentos realizados com o meio de cultura semi-sintético T2, de BONÉ & PARENT. Êstes

TABLE I

Comparison of two types of *Trypanosoma cruzi* cultures grown in three different T2 media

	Cultures started from:	Media					
		Nonmodified T2		T2 minus stearic acid		T2 plus blood agar extract*	
		Maximum	Average	Maximum	Average	Maximum	Average
Age in days	Tobie	21	18	27	27	27	27
	T2	24	24	27	27	24	20
Highest number of living trypanosomes per mm³	Tobie	13,200	11,600	13,000	12,100	40,000	35,000
	T2	4,000	4,000	4,000	3,300	12,500	12,500
Highest optical density	Tobie	78	76	99	86	232	208
	T2	29	29	32	30	111	96

* These results are similar to those obtained with Tobie's supernatant

experimentos demonstraram que: 1) O crescimento de quatro "cepas" de *T. cruzi* no meio T2 foi intensificado pela adição de extrato de ágar-sangue em solução de Locke; 2) O ácido esteárico, fator de crescimento essencial nas experiências de BONÉ & PARENT, revelou-se bem menos indispensável em nossos experimentos; 3) Subculturas em meio T2 a partir de culturas no mesmo meio, não apresentam desenvolvimento tão bom quanto aquelas estabelecidas a partir de culturas no meio difásico de Tobie; 4) Apesar das dificuldades na estimativa do número de tripanossomos pela contagem, a correlação entre a D.O. e as contagens foi geralmente satisfatória quando aplicada à curva de crescimento de culturas iniciadas ao mesmo tempo e originárias da mesma cultura básica. Tal correlação não se mantinha, porém, quando se comparavam diferentes séries de experimentos. O tamanho e o estágio de desenvolvimento individual dos organismos foram, provàvelmente, responsáveis por essa discrepância; 5) Nos estudos das características de crescimento de tripanossomos, a medida da D.O. aqui descrita é considerada o método de eleição, por ser rápida, fidedigna e reduzir a um mínimo, o risco de contaminação.

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