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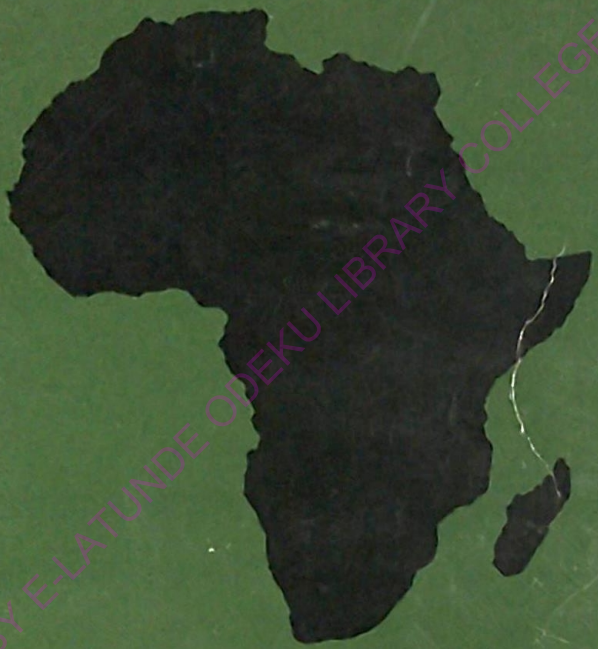

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A modified medium for the *in vitro* cultivation of trypanosomes

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Abstract

A modified medium (ME-99) for the *in vitro* cultivation of trypanosomes was developed and evaluated in our laboratory. The medium comprised of Eagle MEM as a base and various components of medium 199 not found in MEM. *Trypanosoma brucei brucei, T. b. gambiense* and *T. vivax* were grown in ME-99 and various other media combinations such as MEM/RPMI 1640, MEM/BCM, MEM/199, RPMI 1640/BCM, RPMI 1640/199, BCM/199 for comparison. Both growth and infectivity of these parasites were compared in these media. Parasites grew better and maintained their infectivity for longer periods in ME-99 than in any other medium or combination of media.

Resume

Un milieu modifie (ME-99) pour le cultivation *en vitro* de trypanosomes etait develope et evalue dans nos laboratoire. Le milieu etait compris d'Eagle MEM comme base et de divers constituent tel que le MEM/RPMI 1640, MEM/BCM, MEM/199, RPMI 1640/BCM, RPMI 160/199, BCM/199 pour comparison. La croissance et l'intectivite de ces parasites etaient compare dan ces diverse milieu. Les parasites poussaient mieux et entretaient leur infectivites pour les longues periodes dans ME-99 plus que dans un ou bien une combination de milieux.

Introduction

Media used for the *in vitro* cultivation of trypanosomes require either whole or haemolysed red blood cells on biphasic plants. Although the blood-based media have been widely used to support the cultivation of trypanomastigotes for many of the studies on the biochemistry and immunology of trypanosomes, most of these media are noted to have serious deficiencies which limit their application to these areas[8]. These include cultivation of the parasites with antigens derived from erythrocytes, inconsistent growth of different species and strains of trypanosomes, relatively low yields of organisms, and rate of parasite differentiation. It has been shown that the rate of differentiation of circulating blood *T.b. brucie* parasites from rapidly dividing slender forms to immediate and stumpy forms[1] influence the parasite population, growth rate, and the kinetics of host antibody responses[16].

T. vivax causes disease in livestock and is, therefore, of considerable economic importance in Africa. Whereas, the *in vitro* cultivation of trypanosomes has recently been achieved[4,11], attempts to cultivate animal infective *T. vivax* have been relatively unsuccessful[13,18]. Rats are usually refractory to infection with *T. vivax*. Hence, minimal research has been carried out on it. Although, a lot of work has been done on the *in vitro* cultivation of trypanosomes using various media, the problem still remains that the media so far developed, have not been particularly suitable for culture of the *T. b. brucei* strains of trypanosomes[3] and have also not been suitable for growth, survival, and infectivity of *T. vivax*[13].

This study was undertaken to develop a medium that would adequately support the growth of Nigerian strains of trypanosomes with respect to duration of continuous culture, yield of large quantities of the parasites with retained infectivity for long periods of time.

Materials and methods

Parasites

T. b. brucei strains 8/18, *T. b. gambiense* and *T. vivax* were obtained from the Nigerian Institute for Trypanosomiasis Research (NITR), Vom, Jos. The parasites were maintained in albino mice and rats by serial passage, at the Department of Medical

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These parasites were then isolated from the mouse blood according to the method of Hirumi *et al*[11] and counted in the new improved Neubauer haemocytometer using the method for counting the white blood cell.

Isolation of parasites from mouse blood and inoculation of cultures

Parasites 10^3 mice were isolated from heavily infected mice and were inoculated into each flask containing 5ml of medium and mouse kidney cells. The study was repeated.

Cells and culture system

Mouse kidney cells isolated from 14-day-old mice were used in the study. Method for the isolation is standard. Sub-cultures were carried out after trypsinication and resuspension of the feeder layer cells. The pH in the medium was adjusted to 7.4 with Hepes. Washed trypanosome suspension (1ml) containing 10^3 parasites was inoculated into each culture flask[11] which contained 24-hr-old sub-cultured M K cells. All cultures were maintained at 37^c (95% relative humidity with 5% CO_2 and 95% air).

Light microscopy

Smears were made from cultures and animal blood. They were air dried, fixed in absolute methanol, and 10% buffered formalin and stained with Gurr Improved Giemsa. The slides were examined under oil immersion (X100) light microscope.

Preparation of MEM base

This was carried out according to manufacturer's instruction.

Other media combinations

The media Roswell Park Memorial Institute 1640 (RPMI 1640), Basal culture medium (BCM), Tissue Culture 199, Minimum Essential Medium (Eagle's) and sera were obtained in powder from GIBCO, Burlington, Ontario, Canada, and made up according to manufacturer's instructions. The various media combinations were made up by adding in a 1:1 ratio, one medium to the other just before use. The media were supplemented with 20% fetal calf serum.

The modified ME-99 medium

The modified medium ME99 comprised of Eagle's Minimum Essential Medium as a base, and the various components of tissue culture medium 199, not found in MEM. The method involved the preparation of:

(a) Minimum Essential Medium
(b) The various components of 199 not found in MEM
(c) Mixing the two prepared media in a 1:1 ratio.

Preparation of components of M199 not found in Minimum Essential Medium

Eight stock stolutions were prepared thus:

			mg/l
Stock solution (1)	Hypoxanthine	—	10
	Thymine	—	10
	Uracil	—	10
	NH_4OH	—	0.175ml

Components were added to 100ml bi-distilled water and the mixture was warmed (in a warm bath), filtered and distributed into sterile containers - 30ml/container.

Stock solution (2)	Guanine	—	3.0
	Guanosine	—	3.0

These two substances were dissolved in 28ml bi-distilled water and .083ml concentrated NH_4OH was added to aid dissolution. The mixture was heated at 100°C, then cooled and brought up to 30ml with bi-distilled water. It was prepared just before use.

Stock solution (3) Biotin — 1.0

It was dissolved in 7.5ml bi-distilled water and then 1.0 ml 1/HNCl was added and the mixture was made up to 10ml with bi- distilled water, filtered and distributed into sterile test-tubes (1ml/test-tube). The tubes containing the mixtures were stored at 4°C.

Stock solution (4) Tocopherol — 1.0

This components was dissolved in 10ml bi-distilled water, filtered and distributed into 1ml portions, in sterile test-tubes and stored at 4°C.

Stock solution (5) Menadione — 10ml/l

This vitamin was dissolved in 10ml bi-distilled water, agitated and left to stand for 18 hours at 37°C. It was later filtered and divided into 1 ml portions in sterile containers and stored at 4°C.

Stock solution (6) Adenine — 4.0mg/1
Adenosine — 4.0mg/1

This purine and it's sugar derivative were dissolved in 10ml bi-distilled water. The mixture was filtered and dispensed into sterile containers (5ml/container) and stored at 4°C.

Stock solution (7) P-aminobenzoic acid — 5.0mg/1

This component was dissolved in 20ml bi-distilled water, filtered and distributed into sterile test-tubes, in 2ml portions. The above mixture was stored at 4°C and protected from light.

Stock solution (8) Calciferol — 1.0mg/1
Cholesterol — 2.0mg/1

The calciferol was dissolved in 2ml 95% alcohol. Cholesterol (2mg) was added and the solution was thoroughly mixed with the aid of a sterile magnetic stirrer. 3ml of 5% tween 80 was added and the final mixture was filtered and distributed into sterile test-tubes at 5ml/container. A mixture of (a) Serine (b) Aspartic acid, (c) Alanine, (d) Proline and (e) Glycine (aminoacetic acid) was prepared. These components were weighed out and set aside for later use.

The final mixture of this part of the modified ME-99 was made up as follows: A double neck bottle of 2 litre volume was washed and autoclaved. One neck was fitted to a mixer. The stock solutions and other components were added in the following concentrations:

Stock Solution	*Amount*
Stock Solution 1	30ml
NaCl	80,000mg
Stock Solution 2	30ml
KCl	4,000ml
$MgSO_4.7\ H_2O$	2,000ml
KH_2PO_4 (60mg) dissolved in bi-distilled water	
$NaHPO_4$ (60mg) and made up to	100ml
CaCl (1400mg) dissolved in bi-distilled water	
(anhydrous) and made up to	200ml
Stock Solution 7	2ml
Stock Solution 3	1ml
Stock Solution 8	5ml
Stock Solution 4	1ml
Stock Solution 5	1ml
Stock Solution 6	5ml

The volume of the above mixture was brought up to 900ml and the water mixture of serine, aspartic acid, alamine, proline and glycine previously weighed and set aside was added. The final mixture of components of MI99 was well shaken and stored at 4°C for 18 hours, to ensure complete dissolution of the components. At the end of 18 hours period, the mixture was removed from 40° temperature and allowed to return to room temperature. Thereafter, it was made up to 1 litre with double distilled water, filtered and distributed into sterile bottles 100ml/bottle and stored at 4°C.

The following components were made up and added before use of medium 199 components:

L-Cysteine	5mg/1
Glutathione	5mg/1

These two components were dissolved in 50ml bi-distilled water. Vitamin A (10mg) dissolved in 1ml ethyl alcohol and tween 80 (5%) were added to the solution of Cysteine and Glutathione. Finally, Adenosine triphosphate 200mg, dissolved and made up to 100ml with bi-distilled water was added to the whole mixture. The mixture was then filtered and stored at 4°C. However, the mixture of L- Cysteine, glutathione, Vitamin A, tween 80 and Adenosine triphosphate was made up fresh before use, by adding 100ml of the stock solution to 8000l bi-distilled water, 1ml of the solution containing cysteine, glutathione, tween 80, vitamin A and adenosine triphosphate. The solution was brought up to 1 litre with bi-distilled water. The pH was adjusted to 7.4 and the solution was stored for not longer than 2 months.

Preparation of ME-99

The method for using ME-99 was found to be best when MEM base and already prepared 199 component mixture were added thus:

In the tissue culture flask, 2.5ml of Eagle Minimum Essential Medium (already made up according to manufacturer's instructions) were added. To the same flask 2.5ml 199 componen• mixture earlier prepared was added. 20% fetal bovine serum was used as supplement.

Infectivity Tests

10^3 parasites from the following cultures, utilizing the various media combinations and our modified ME-99 were inoculated into groups of mice.

(a) MEM-RPMI 1640 + Mouse Kidney cells + 20% FCS

(b) MEM + M 199 + Mouse Kidney cells + 20% FCS
(c) MEM + BCM + Mouse Kidney cells + 20% FCS
(d) RPMI 1640 + BCM + Mouse Kidney cells + 20% FCS
(e) RPMI 1640 + 199 + Mouse Kidney cells + 20% FCS
(f) BCM + 199 + Mouse Kidney cells + 20% FCS
(g) ME-99 + Mouse Kidney cells + 20% FCS.

Results

Growth of T. b. brucei in the various media combinations

T. b. brucei grew best and maintained its infectivity in all the various media combinations (Tables 1) when compared with the other Trypanosoma species tested.

Apart from media RPMI 1640/BCM, the pattern of growth of *T. b. brucei* in the various media combinations evolved a gradual but steady increase of the parasites from month 1 until month 4 (Table 1). The growth of the parasites thereafter remained constant until month 9. The infectivity was not lost throughout the study (Table 5). The maximum parasite count observed for *T. b. brucei* in MEM/RPMI 1640 media combinations were 2×10^7, MEM/BCM 2×10^6, for MEM/199 it was 2×10^6, while for RPMI 1640/BCM it was 2×10^4 and for RPMI 1640/199 a maximum parasite count of 2×10^8 was observed. For BCM/199, the maximum parasite count observed was 2×10^6.

Table 1: Growth of Trypanosoma brucei brucei in various combinations of growth media evaluated

Media Combination (50/50 v/v)		Number of Parasites Harvested/ml culture fluid								
Medium 1	Medium 2	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
MEM	RPMI 1640	2×10^4	2×10^5	2×10^6	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7	2×10^7
MEM	BCM	2×10^3	2×10^4	2×10^5	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6
MEM	199	2×10^5	2×10^6	2×10^7	2×10^9	2×10^9	2×10^9	2×10^9	2×10^9	2×10^9
RPMI 1640	BCM	2×10^3	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4
RPMI 1640	199	2×10^4	2×10^5	2×10^7	2×10^8	2×10^8	2×10^8	2×10^8	2×10^8	2×10^8
BCM	199	2×10^4	2×10^5	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6

Growth of T. b. gambiense in the various media combinations

The pattern of growth of this species of trypanosoma in the media combinations was demonstrated by an initial lag of 2 months post-inoculation. The parasite did not begin to multiply until the 3rd month (Table 2). After the 4th month, the parasite growth tapered off. There was no more growth after the 9th month. However, for media combination MEM/199, an increase in the parasite number was observed in the 1st month and thereafter, there was no increase in the parasite number until month 3 and an increase of month 4. After the latter month, increase in parasite number tapered off. As regards media combination RPMI 1640/BCM, a decrease in the parasite number between month 1 and 2 was observed. However, increase occurred in the parasite number from the 3rd month. Thereafter, from month 6, the increase in parasite number became constant (Table 2). The infectivity of the parasite was lost after month 3 in all the media combinations except MEM/199 (Table 6). The maximum parasite count observed in MEM/RPMI 1640 was 2×10^5, for MEM/BCM 2×10^5, for MEM/199 2×10^6, for RPMI 1640/199 2×10^5, for BCM/199 2×10^5.

Table 2: Growth of Trypanosoma brucei, gambiense in various combinations of growth media evaluated

Media Combination (50/50 v/v)		Number of Parasites Harvested/ml culture fluid								
Medium 1	Medium 2	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
MEM	RPMI 1640	2×10^3	2×10^3	2×10^4	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5
MEM	BCM	2×10^3	2×10^3	2×10^4	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5
MEM	199	2×10^4	2×10^4	2×10^5	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6	2×10^6
RPMI 1640	BCM	2×10^2	2×10^1	2×10^3	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4
RPMI 1640	199	2×10^3	2×10^3	2×10^4	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5
BCM	199	2×10^3	2×10^3	2×10^4	2×10^6	2×10^5	2×10^5	2×10^5	2×10^5	2×10^5

Growth of T. vivax in the various media combinations tested

T. vivax did not growth well in the various media combinations when compared with the growth responses obtained for *T. b. gambiense* and *T. b. brucei*. The pattern of growth of *T. vivax* involved in gradual increase from month 1 until month 2, when no further increase was observed (Table 3). The level tampered off until month 9 in some of the media combinations and decreased after month 3 for others. However, for media combination MEM/BCM, and BCM/199 the parasites in the media started dying from month 5 and by month 6, no living parasites could be seen in the media. For RPMI 1640/BCM same observations were made from month 4 until month 5.

Table 3: Growth of Trypanosoma vivax, in various combinations of growth media evaluated

Media Combination (50/50 v/v)		Number of Parasites Harvested/ml culture fluid								
Medium 1	Medium 2	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
MEM	RPMI 1640	2×10^3	2×10^4	2×10^4	2×10^3	2×10^3	2×10^3	2×10^3	2×10^3	2×10^3
MEM	BCM	2×10^3	2×10^4	2×10^4	2×10^3	2×10^2	0	0	0	0
MEM	199	2×10^4	2×10^5	2×10^5	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4	2×10^4
RPMI 1640	BCM	2×10^3	2×10^4	2×10^4	2×10^2	0	0	0	0	0
RPMI 1640	199	2×10^3	2×10^4	2×10^4	2×10^3	2×10^3	2×10^3	2×10^3	2×10^3	2×10^3
BCM	199	2×10^3	2×10^4	2×10^3	2×10^3	2×10^2	0	0	0	0

The maximum parasite count recorded for *T. vivax* in MEM/RPMI 1640 media combination was 2×10^4, for MEM/BCM, 2×10^4, for RPMI 1640/199, 2×10^4, and for BCM/199 2×10^4. *T. vivax* in MEM/RPMI 1640 maintained its infectivity in culture up till month 4 and lost it thereafter (Table 7). Parasites cultured in MEM/BCM lost their infectivity after month 3. In MEM/199, the infectivity was

maintained up to the 9th month. In RPMI 1640/BCM media combination, infectivity was lost after 3 months of culturing while in RPMI 1640/199 and BCM/199, infectivity of the parasites was lost after month 4.

Growth of T. b. brucei in ME-99

The results are summarized in Table 4. Growth of *T. b. brucei* in ME-99 was found to be very good. There was a 2-fold increase every 2 days and the parasites kept multiplying until the 8th day when the parasite number became constant. The parasites also remained infective throughout the study (Table 8). The maximum parasites count recorded was 2×10^{10}.

Growth of T. b. gambiense in ME-99

This parasite like the *T. b. brucei*, gave the best result in ME-99 (Table 4). There was a 2-fold increase every month until the 3rd month when the parasite number became constant and the maximum parasite count observed was 2×10^{9}. The parasites also remained infective throughout the study (Table 8).

Table 4: Growth of T. B. brucei, T. B. Gambiense and T. vivax in ME 99

Specie of Parasite	Number of Parasite Harvested								
	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
T. b. brucei	2×10^{5}	2×10^{7}	2×10^{9}	2×10^{10}	2×10^{10}	2×10^{10}	2×10^{10}	2×10^{10}	2×10^{10}
T. b. gambiense	2×10^{5}	2×10^{7}	2×10^{9}	2×10^{9}	2×10^{9}	2×10^{9}	2×10^{9}	2×10^{9}	2×10^{9}
T. vivax	2×10^{3}	2×10^{4}	2×10^{5}	2×10^{6}	2×10^{6}	2×10^{6}	2×10^{6}	2×10^{6}	2×10^{6}

Table 5: Viability of Trypanosoma brucei, brucei in various media combinations

Media Combinations (50/50 v/v)										
Medium 1	Medium 2	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
MEM	RPMI 1640	+	+	+	+	+	+	+	+	+
MEM	BCM	+	+	+	+	+	+	+	+	+
MEM	199	+	+	+	+	+	+	+	+	+
RPMI 1640	BCM	+	+	+	+	+	+	+	+	+
RPMI 1640	199	+	+	+	+	+	+	+	+	+
BCM	199	+	+	+	+	+	+	+	+	+

Key: + = Patent

Table 6: Viability of Trypanosoma brucei gambiense in various combinations of growth media evaluated

Media Combinations (50/50 v/v)										
Medium 1	Medium 2	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
MEM	RPMI 1640	+	+	+	—	—	—	—	—	—

MEM	BCM	+	+	+	—	—	—	—	—	—
MEM	199	+	+	+	+	+	+	+	+	+
RPMI 1640	BCM	+	+	+	—	—	—	—	—	—
RPMI 1640	199	+	+	+	—	—	—	—	—	—
BCM	199	+	+	+	—	—	—	—	—	—

Key: + = Patent
— = Non-patent

Growth of T. vivax in ME-99

Growth of *T. vivax* in ME-99 was found not as good as *T. b. brucei* and *T. b. gambiense*, but better in ME-99 then in other media combinations (Tables 1, 2, 3, and 4). A one-fold increase was observed every month until month 4 when the parasite number became constant. The maximum parasite count recorded was 2×10^6. The growth rate of this parasite in other media combinations was below 50% as opposed to growth in ME-99 where it scored 60%.

Table 7: Infectivity of trypanosoma vivax in various combinations of growth media evaluated

Media Combination (50/50 v/v)		Month	Month	Month	Month	Month	Month	Month	Month	Month
Medium 1	Medium 2	1	2	3	4	5	6	7	8	9
MEM	RPMI 1640	+	+	+	+	+	+	—	—	—
MEM	BCM	+	+	+	+	+	+	—	—	—
MEM	199	+	+	+	+	+	+	+	+	+
RPMI 1640	BCM	+	+	+	—	—	—	—	—	—
RPMI 1640	199	+	+	+	+	—	—	—	—	—
BCM	199	+	+	+	+	—	—	—	—	—

Key: + = Patent
— = Non-patent

Table 8: Infectivity of *T. B. Brucei, T. B. Gambiense* and *T. vivax* in ME-99

Specie of Parasite	Month 1	Month 2	Month 3	Month 4	Month 5	Month 6	Month 7	Month 8	Month 9
T. b. brucei	+	+	+	+	+	+	+	+	+
T. b. gambiense	+	+	+	+	+	+	+	+	+
T. vivax	+	+	+	+	+	+	+	+	+

Key: + = Patent

Discussion

The results of the preliminary experiments showed that Minimum Essential Medium, in combination with any other medium investigated in this study is ideal for the *in vitro* cultivation of trypanosomes. In like manner, suitability of MEM for the *in vitro* cultivation of trypanosomes was reported[4, 5 and 15]. These observations form the basis for the selection of MEM as a base medium for modification in the present study, in the hope that an improved and a more reliable medium will emerge than those previously used.

The superiority of ME-99 over others was manifested when the growth rate of parasites in the medium was compared to that of other combinations. Whereas a maximum parasite count of not more than 2×10^4 with *T. vivax* was recorded in all the other media combinations as was clearly demonstrated in Table 3, a 2×10^6 maximum parasite count was obtained in ME-99 with *T. vivax*, an organism previously shown to be very difficult to culture[14]. Although infectivity of *T. b. gambiense* and *T. vivax* was lost after between 3 and 4 months in culture in the other media, in ME-99 the infectivity was retained until 9th month when the experiment was terminated. Good growth with SDM-79 medium had been reported[2]; this improvement was attributed to a number of additives contained in the medium. In the present study, similar additives with the inclusion of cysteine, were incorporated in the ME-99 medium. It is a probable that the presence of these additives accounted for the good result obtained. More recently, it had been shown that the additives were necessary and essential for trypanosome growth[17]. There is a possibility that some of the additives such as the amino acids chemically react with the parasite metabolic waste and as a result of this, some constituents were reduced in the modified ME-99. Some scientists showed that the involvement of cysteine may possible stimulate the progressive growth and provide retention of the infectivity of the parasite for longer periods as observed in this study[5].

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