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Amino Acids Requirements for Reproduction of Arboviruses[1] (39050)

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Since the work of Eagle and Habel (1), several reports have been published demonstrating that maturation of animal viruses in culture cells apparently depends upon quantities of particular amino acids in medium (2–8). As to arboviruses, however, similar descriptions are scanty, except for a study on the effect of proline for the growth of Japanese encephalitis virus (9). In the present paper, we will describe the amino acid requirements for the reproduction of group A chikungunya (CHIK) and group B dengue type 1 (DEN-1) viruses. Since it has already been shown that low ionic strength of the culture medium significantly inhibited maturation of these viruses (10, 11), we investigated the effect of amino acids with regard to the ionic strength effect.

Materials and methods. Viruses and cell cultures. We used CHIK African strain and DEN-1 Mochizuki strain viruses which had been cultivated in BHK-21 cells or propagated in mouse brains. The plaque forming units (PFU) were determined by BHK-21 cell monolayers through procedures described elsewhere (11). The culture medium for cell growth consisted of Eagle's minimum essential medium (MEM) containing 5% heat-inactivated calf serum supplemented with 60 γ/ml kanamycin. After inoculation of the virus to test the amino acids requirement, the calf serum was replaced by 0.4% bovine plasma albumin (Fraction V, Armour). In the "requirement tests", individual amino acids were deleted one by one from the medium. For the electron microscopic observation, the cells were treated with the procedures described elsewhere (11, 12). The specimens were examined with a JEM-100C electron microscope at direct magnification × 20,000–40,000.

Incorporation tests. Cells were incubated with normal or deprived medium containing 1 μCi/ml ^{3}H-uridine (Commissariat a l'Energie Atomique) or 0.2 μCi/ml ^{14}C-amino acid mixtures (New England Nuclear Corporation) in the presence or absence of actinomycin D (Schwarz Bio-Re-

TABLE I. YIELD OF CHIK AND DEN-1 VIRUSES IN BHK-21 CELL CULTURES FED WITH NORMAL OR AMINO ACID DEPRIVED MEDIUM.

Culture medium[a]	CHIK[b]	DEN-1[b]
Normal	100[c]	100[c]
deleted of:		
Arginine	41	51
Cystine	15	5.4
Glutamine	0.7	17
Histidine	69	41
Isoleucine	85	124
Leucine	83	121
Lysine	73	91
Methionine	40	102
Phenylalanine	98	39
Threonine	94	76
Tryptophane	56	61
Tyrosine	96	78
Valine	109	64
Amino acid free	0.05	5.7

[a] BHK cells infected with CHIK or DEN-1 viruses were incubated at 37° in medium from which individual amino acids were omitted.

[b] Infected culture medium was harvested at 16 hr of incubation in case of CHIK virus, and at 72 hr in case of DEN-1 virus.

[c] Virus titers (PFU/ml) were determined by using BHK-21 monolayer cells grown under methyl cellulose overlay medium in a 60 mm Petri dish and expressed as percentages of that of "normal medium" sample, the PFU'S of which were in the range of 10^7–10^8/ml of CHIK and 10^6–10^7/ml for DEN-1.

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search Inc.). In the latter cases, the constituents of the medium were adjusted as to contain the original concentrations of amino acids in Eagle's MEM. After the beginning of incubation, the cell cultures were washed with Hanks' balanced salt solution containing 0.2% bovine plasma albumin and scraped with a rubberpoliceman at various times. The cells, resuspended in 1 ml reticulocyte standard buffer (0.01 *M* KCl, 0.0015 *M* $MgSO_4$, 0.01 *M* Tris) (pH 7.4), were subjected to freeze–thawing three times and homogenized in a Dounce homogenizer. After being centrifuged at 3000 rpm for 15 min, the supernatant was treated with 5% trichloracetic acid. The acid-insoluble fraction was taken in a vial by adding toluene (containing PPO and POPOP) to count radioactivity in a Packard scintillation counter.

Hemadsorption tests. After the culture medium was removed, the cell monolayer was washed three times with virus adjusting diluent (VAD) plus borate buffer saline (BBS; pH 6.0–6.2) (13). One-half ml of 0.5% goose erythrocyte suspension in VAD-BBS was added to the cells which were then incubated at 37° for 60 min, receiving gentle rocking at 15 min intervals. After unadsorbed erythrocytes were washed out with VAD-BBS (pH 6.0), the cultures were observed under a phase contrast microscope (Nikon type MD).

Results. Table I shows the effects of the amino acid deprivation on CHIK and DEN-1 yields. It indicates that the deprivation of glutamine greatly inhibited the yields of CHIK virus, and similarly cystine as to DEN-1. Essentially the same results were obtained in other series of experiments in

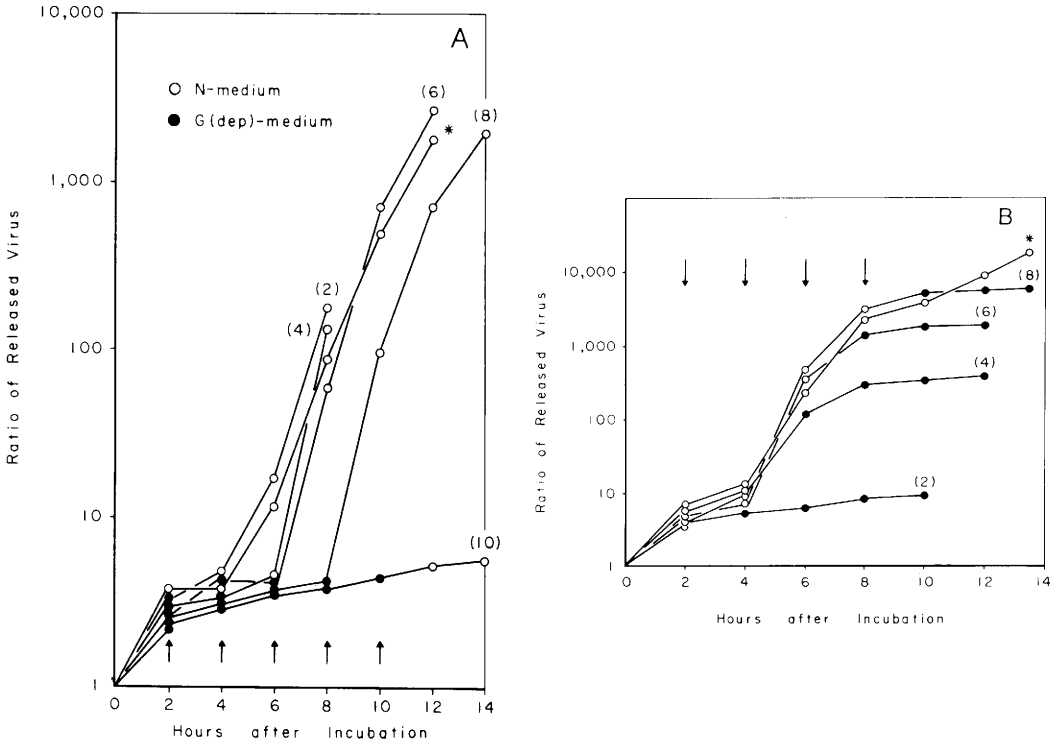
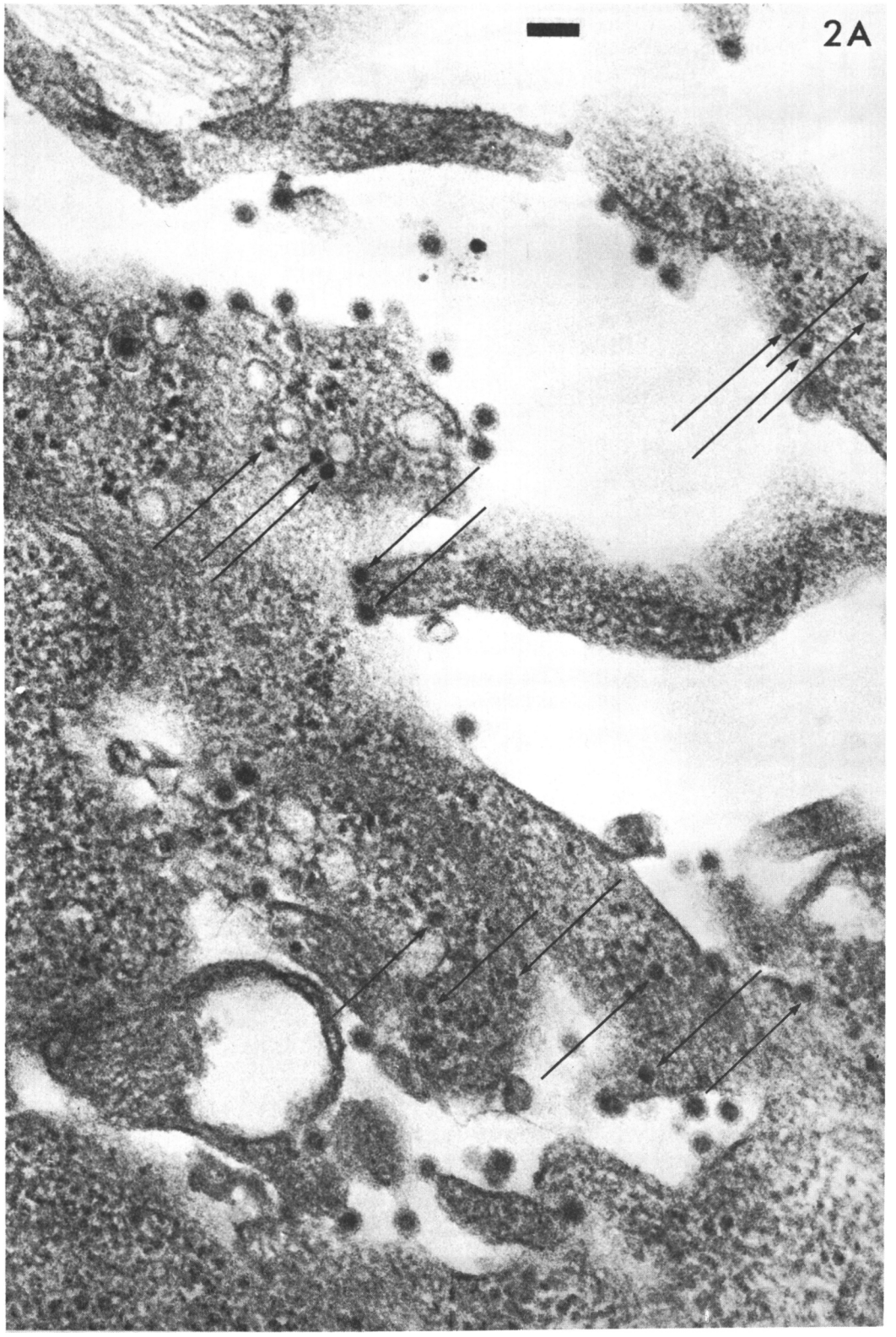


FIG. 1. (A) Reversible effect of glutamine on the production of CHIK virus. BHK cells were inoculated with CHIK virus and incubated at 37° for 90 min to complete the cell-virus adsorption. After being washed once with Hanks' balanced salt solution, the cultures were further incubated at 37° either (A) in G(dep)-medium which was replaced by N-medium at times indicated (arrows), or (B) in N-medium which was similarly replaced by G(dep)-medium. At intervals indicated in the abscissa, 0.2 ml of culture medium was withdrawn to determine the viral infectivity. Symbols: ○——○, N-medium; ●——●, G(dep)-medium; *, continuously fed with N-medium. Number in parenthesis indicates time of medium replacement.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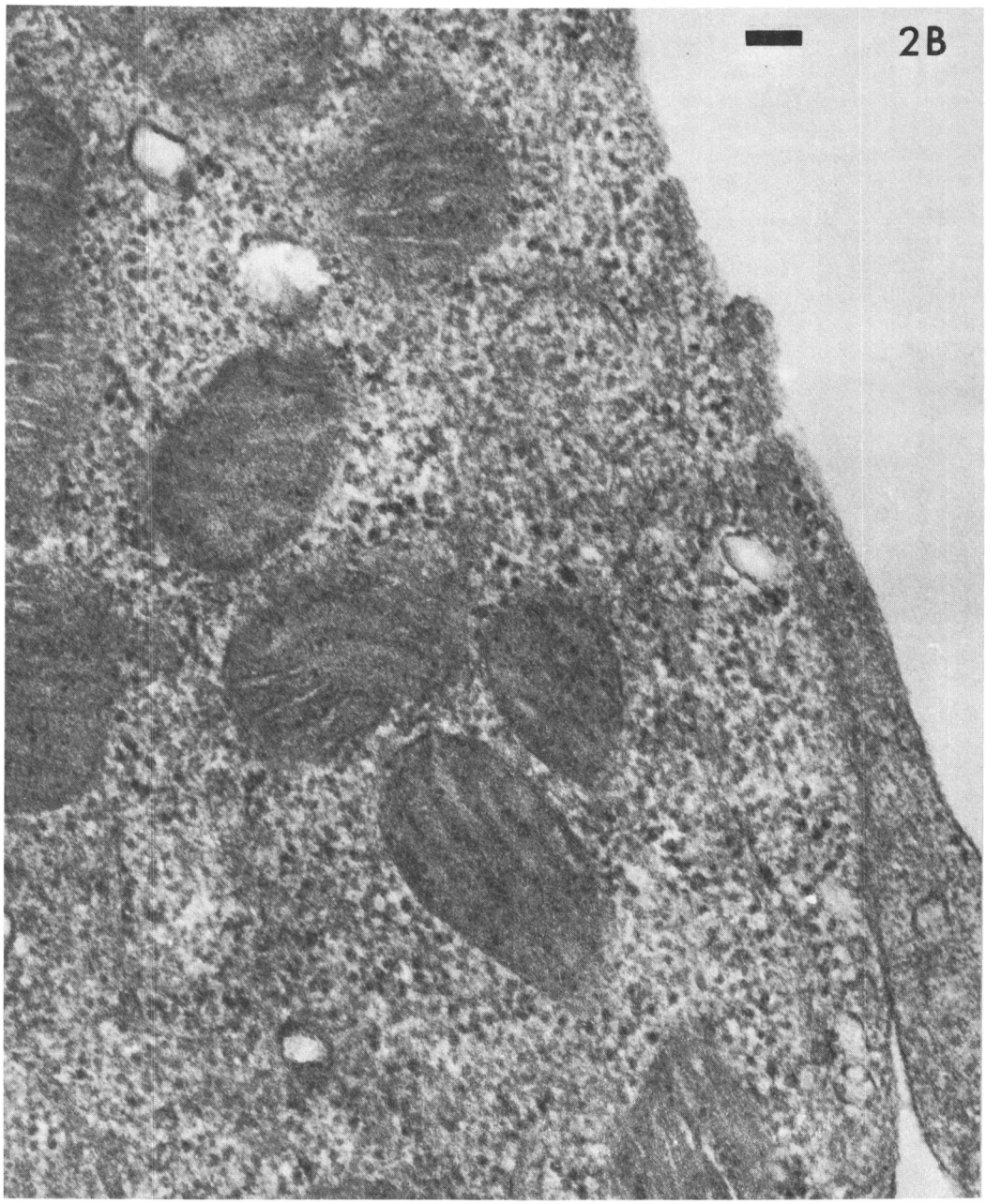

FIG. 2. (A) Electron micrograph of a BHK cell cultivated with N-medium (A) or with G(dep)-medium (B) for 16 hr after infection with CHIK virus. In (A), numerous viral cores (arrows) and budding particles across the cellular membrane can be seen. In (B), the same structures are absent. Bar = 100 nm. Magnification ×80,000.

which the cells were starved for 16 hr prior to the virus inoculation.

Further experiments were carried out using CHIK virus and glutamine-deprived [G(dep)]-medium to investigate some details of the phenomena. The infected cultures were first incubated with the G(dep)-medium which later was replaced by normal (N) medium. At intervals thereafter, the virus titers of the medium were assayed. It was noted in these experiments that the reduction of virus titers in the G(dep)-cultures was

restored within 2–4 hr after being put in the N-medium (Fig. 1A). When the medium was changed from N to G(dep), the virus production was also suppressed within 2–4 hr (Fig. 1B). It was shown, at the same time, that such reversibility was revealed unequivocally during the earlier periods of virus multiplication, i.e., 2–6 hr after the infection of the virus; in the later stages, i.e., about 10 hr after infection, the restoration or suppression of the virus multiplication by changing the media was not clear.

Although the incorporation of ^{3}H-uridine into cultures fed with G(dep)-medium were reduced from one-half to one-fifth of those in N-medium, the magnitudes of the change were almost the same in both infected and uninfected cultures. The same statements could be made as to the incorporation of ^{14}C-amino acid mixtures into G(dep)-cultures. These data seemed to indicate that the deprivation of glutamine did not directly affect the RNA and protein syntheses related to the maturation of the virus in the cells.

Hemadsorption tests indicated that erythrocytes adsorbed by cells fed with G(dep)-medium were significantly fewer than those by control *N*-cultured cells. Sixteen hr after the CHIK virus infection, BHK cells incubated in *N*-medium revealed a number of electron dense spherical particles, about 30–40 nm in diameter, which were regarded to be identical with the "core" structures described in (11). We could observe the "budding process" of these particles (Fig. 2A). However, the pictures of the cells infected with CHIK and incubated in G(dep)-medium were different, i.e., few "core" structures were revealed, and no budding particles adjacent to the cell surface membrane were found (Fig. 2B).

These findings suggested that the glutamine deprivation affected the assembly of the viral components prior to the appearance of the viral "core", preventing the formation of complete virus particles. This is well compatible with the results of incorporation experiments and hemadsorption tests described in the preceding paragraphs.

The effects of glutamine and ionic strength on CHIK-infected cells which were cultivated with normal ionic strength or low ionic strength medium containing various concentrations of glutamine were also studied and the virus yields under these conditions studied are summarized in Table II. It is clear that the ionic strength effect was not influenced by the absence or presence of glutamine. Apparently, the glutamine could not compensate the ion's action upon the production of viruses.

Discussion and summary. In the present experiments, it was shown that the maturation of CHIK and DEN-1 viruses was markedly suppressed by the deprivation of glutamine and cystine, respectively. The difference of amino acid requirements may reflect a difference in the maturation process of the two kinds of viruses. Evidence have been presented, indicating that the maturation sites of group A and B arboviruses were characteristically different from each other (11, 14).

Some details of the mechanism(s) of the phenomena were investigated, especially those dealing with CHIK virus and glutamine. The inhibitory effect of deprivation was reversible; the virus yields were restored by putting the cells from deprived medium into normal medium and vice versa. Such restoration and suppression were particularly clear during the earlier stages of the virus infection. In the radioisotope incorporation experiments, it was shown that the deprivation was not directly related to the viral RNA or protein synthesis in the

TABLE II. YIELD OF CHIK VIRUS IN NORMAL OR LOW IONIC STRENGTH MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF GLUTAMINE.

Medium[a]	Conc of glutamine added (mg/ml)	Virus yield[b]
N-medium	290	100
	29	33
	2.9	0.6
	0	0.4
L-medium	290	0.034
	2900	0.031
	14,500	0.147

[a] BHK cells infected with CHIK virus were incubated for 16 hr in normal (N) or low (L) ionic strength medium (ionic strength 0.15 and 0.09, respectively) containing various concentrations of glutamine. Original content of glutamine in Eagle's MEM: 290 mg/ml.

[b] Percentage of PFU titers obtained.

infected cells. In the electron microscopic examinations, the cells infected with virus and cultured with deprived medium did not reveal the "core particles" inevitably seen in the same cultures fed with normal medium. This finding suggests that the deficiency influences the step(s) prior to the appearance of the core particles. This may be compatible with the above-mentioned data indicating that the reversibility of the deficiency effect was more clearly demonstrated during the earlier stages of the virus infection than in the later stages.

It can be postulated therefore that the amino acid deprivation (L-glutamine in the case of CHIK virus) is possibly involved at certain stages for the assembly of viral structures to produce complete virions. Since, however, it was clearly shown that the glutamine did not compensate the low ionic strength effect already described, the viral maturations stages affected by amino acid deficiency and low ionic strength are probably different from each other.

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1. Eagle, H., and Habel, K., J. Exp. Med. **104,** 271 (1956).
2. Rouse, H. C., and Schlesinger, R. W., Virology **33,** 513 (1957).
3. Ito, Y., Okazaki, H., Sakuma, S., Honma, M., and Ishida, N., Virology **39,** 277 (1969).
4. Medvedkina, O. A., Maslova, S. V., and Shirman, G. A., Arch. ges. Virusforsch. **37,** 191 (1972).
5. Iinuma, M., Maeno, K., and Matsumoto, T., Virology **51,** 205 (1973).
6. Romano, N., and Scarlata, G., Arch. ges. Virusforsch. **43,** 359 (1973).
7. Ito, Y., Kimura, Y., Nagata, I., and Kunni, A., J. Virol. **13,** 557 (1974).
8. Mikami, T., Onuma, M., and Hayashi, T. T. A., J. Gen. Virol. **22,** 115 (1974).
9. Igarashi, A., Sasao, F., and Fukai, K., Biken J. **17,** 39 (1974).
10. Waite, M. R. F., and Pfefferkorn, E. R., J. Virol. **5,** 60 (1970).
11. Matsumura, T., Shiraki, K., Hotta, S., and Sashikata, T., Proc. Soc. Exp. Biol. Med. **141,** 599 (1972).
12. Matsumura, T., Stollar, V., and Schlesinger, R. W., Virology **46,** 344 (1971).
13. Clarke, D. H., and Casals, J., Amer. J. Trop. Med. Hyg. **7,** 561 (1958).
14. Murphy, F. A., Harrison, A. K., Gary, G. W., Whitfield, S. G., and Forrester, F. T., Lab. Invest. **19,** 652 (1968).

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