NUTRITIVE REQUIREMENTS OF A SMALL PLAQUE MUTANT OF WESTERN EQUINE ENCEPHALITIS VIRUS*

LISE QUERSIN-THIRY

From the Institut Pasteur du Brabant, Brussels, Belgium

Received for publication May 5, 1961

A MUTANT of Western Equine Encephalitis (WEE) virus has been isolated, which forms very clearcut small plaques, allowing titrations as accurate as with the wild type. Its sensitivity to variations in conditions of the nucleic acid metabolism of the cell may make it a useful tool to study some characteristics of this metabolism.

MATERIALS AND METHODS

Virus strains.—WEE virus was obtained from Dr. A. Sabin, in 1956 ; Newcastle Disease Virus (NDV) was isolated in Belgium by Dr. Florent, and the MEF1 virus was the classical poliomyelitis type II virus.

Culture Media.—Eagle's medium contained double concentration of amino acids and vitamins. Lac-yeast medium consisted of Hanks' solution 99·45 per cent, lactalbumin hydrolysate 0·5 per cent and yeast extract 0·05 per cent ; cells were grown in this medium plus 10 per cent calf serum until they were confluent monolayers.

Dialyzed serum was obtained by dialysing 250 ml. amounts of calf serum against 2 changes of 5 l. of phosphate buffered saline, for 48 hr. at 4°.

Structural analogues of nucleic acid precursors were commercially available, except for 5-fluorouracil, which was kindly provided by Hoffman-La Roche Laboratories.

Plaque assay.—Monolayers of chick embryo primary cultures (for the assay of NDV and WEE viruses) or monkey kidney primary cultures (for the assay of poliovirus) were grown in petri dishes 9 cm. in diameter. At infection 1 ml. of the appropriate virus dilution was placed on the plate and after an adsorption period of 1 hr. at 37°, an agar overlay was added. The optimal composition of this overlay was found to vary with the various viruses : 5·4 ml. of agar solution (1·3 per cent) were supplemented with (*a*) in the case of NDV and MEF1 virus, 1·8 ml. 4-fold concentrated Eagle medium (minus bicarbonate) + 1·2 ml. dialyzed calf serum + 0·05 ml. of a 7 per cent bicarbonate solution ; (*b*) in the case of the mutant WEE/S, 1·8 ml. 4-fold concentrated balanced salt solution (BSS) + 1·2 ml. non-dialyzed calf serum + 0·05 ml. of the same bicarbonate solution ; (*c*) in the case of the wild type WEE/L virus, the same as in (*b*), except that bicarbonate was omitted.

WEE, MEF1 and NDV plaques were read, respectively, after 40 hr., 3 and 4 days ; 1 ml. of neutral red 0·075 per cent, adjusted to isotonicity with a concentrated balanced salt solution, was poured on the agar, and the plaques read after 3 hr.

Anti WEE virus serum.—To 2 ml. of allantoic fluid infected with the wild type WEE virus, were added 2 ml. Arlacel A + Bayol F, and the mixture was injected subcutaneously into a rabbit ; 7 injections were performed at 3–4 days intervals, and the rabbit was bled a week after the last injection.

RESULTS

Isolation of a small plaque mutant (WEE/S).

When assayed for plaques on chicken fibroblasts, appropriate dilutions of allantoic fluids from chick embryos infected with WEE virus gave rise to large

* Supported by grants from the " Fonds de la Recherche Scientifique Médicale " and the " Centre d'Etudes Princesse Josephine-Charlotte ".

plaques (6 mm. diameter), quite homogeneous in size, appearing as soon as 36 hr. after inoculation of the monolayer. Similar plaques were obtained when the virus stock was prepared *in vitro*, by growth on chicken fibroblasts supplemented with Eagle's nutrient medium (with or without calf serum added), or with lac-yeast medium plus 10 per cent calf serum. It was however noticed that when no serum was added to the lac-yeast medium, the virus stocks thus grown gave rise to about 10 per cent small plaques (1 mm. diameter). These plaques were very clear-cut and not of the delayed type, the highest number being found at 36 hr. There were no plaques of intermediary size and for both small and large plaques, plaque morphology was found to be a hereditary character ; several plaques of each type were picked and were found to reproduce the same type of plaque on re-plating.

Purification was performed by 3 successive isolations and virus stocks were then prepared by growing the population of one plaque in chicken fibroblasts with one of four media (Eagle's or lac-yeast medium, with or without 10 per cent calf serum) ; these stocks were then assayed for plaques. Conditions of titration were such that 1 large plaque could be detected in a plate where 800 small plaques were present, while the existence of 1 small plaque was apparent in a population of 100 large plaques. Results obtained from virus stocks derived from large plaques confirmed those previously noted with the wild type population : a small percentage of small plaques were observed when growth had taken place in lac-yeast medium without serum, and none were detected (*i.e.* the proportion of small plaques was less than 0·01) in the case of growth in the 3 other media. On the other hand, virus stocks derived from small plaques and grown in lac-yeast or Eagle's medium contained a few large plaque-formers per 800 small plaque-formers ; this proportion increased by a factor of 3–5, when growth had taken place in media supplemented with 10 per cent serum.

As these facts indicated that serum was favourable to the selection of a few large plaque revertants, and that lac-yeast medium was more favourable than Eagle's medium, for the selection of a few small plaque mutants, it was decided to study the nutritive requirements of these two mutants.

The optimum conditions for plaque assay of the mutants were first investigated. It was found that plaque morphology and number of each type of plaques did not vary when the agar overlay (containing 15 per cent calf serum) was adjusted to isotonicity with any one of the three following media : Eagles, BSS or BSS + lactalbumin hydrolysate and yeast extract. In contrast, the optimal concentration of bicarbonate was not the same for both mutants.

Influence of bicarbonate concentration on plaque assay and growth of WEE/S and the wild type WEE/L viruses.

Appropriate dilutions of WEE/L and WEE/S viruses were plated on monolayers of chicken fibroblasts and the agar overlay was supplemented with BSS + 15 per cent calf serum ; to this were added various amounts of a 7 per cent bicarbonate solution, to reach final concentrations from 0 to 1·6 per cent. Results in Fig. 1 show that the number of large plaques of WEE/L virus progressively decreases with increasing concentrations of bicarbonate ; the higher figure was obtained when no bicarbonate was added (some CO_2 however was supplied under these conditions, as the atmosphere in the incubator is enriched with 5 per cent CO_2). With WEE/S virus, high concentrations of bicarbonate were much less

inhibitory ; there was an optimum concentration of 0·4 per cent and this concentration was chosen for routine assay of WEE/S virus ; from this experiment on, WEE/L virus was assayed without any bicarbonate added to the agar overlay.

The fact that concentrations of bicarbonate which are optimal for WEE/S are in contrast inhibitory to WEE/L virus, was confirmed in experiments where both viruses were grown separately in liquid medium (Eagle's minus bicarbonate), supplemented with various concentrations of bicarbonate. In this instance, in contrast to the preceding experiments, there was no external supply of CO_2, as the culture tubes were stoppered with rubber. About 1 infective virus particle per 10 cells was introduced into the tubes, and several cycles

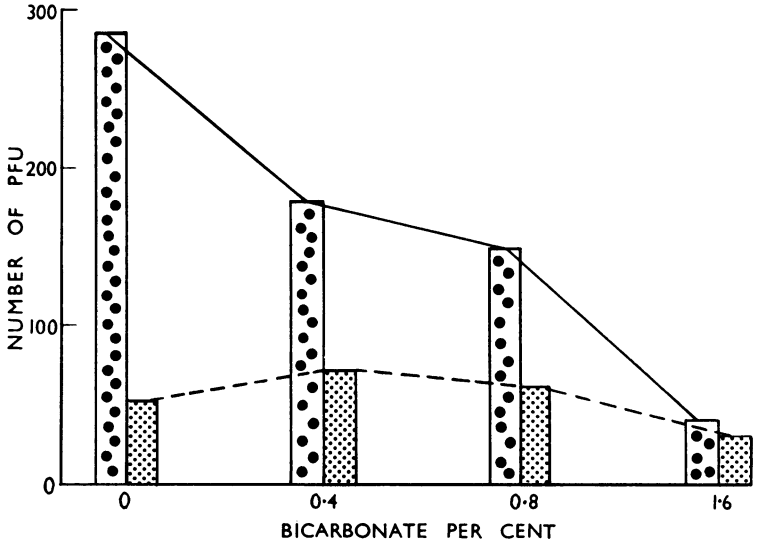


FIG. 1.—The influence of bicarbonate concentration in the agar overlay, on plaque assay of WEE/L (large dots) and of WEE/S (small dots).

of infection were allowed to proceed for 48 hr. ; cells and fluid were then submitted to 6 cycles of freezing and thawing, and the virus titres determined. As shown in Fig. 2 optimal bicarbonate concentrations were 0·4 per cent for growth of WEE/S, and 0·1 per cent for growth of WEE/L virus (concentration in normal Eagle medium is 0·275 per cent) ; when WEE/S mutant was grown in 0·1 per cent bicarbonate, large plaques appeared in the virus stock, in the proportion of 20 per cent. Bicarbonate concentrations as low as 0·05 per cent were insufficient, even for growth of WEE/L virus, either because the pH was unfavourable, or because synthesis of WEE/L, although inhibited by large doses of bicarbonate, was dependent on some bicarbonate supply.

Some other nutritive requirements of WEE/S and WEE/L viruses, when grown in the presence of optimal bicarbonate concentration.

Both viruses were grown in liquid media, under the same experimental conditions as described in the preceding paragraph ; bicarbonate concentration was

0·1 per cent for growth of WEE/L virus, and 0·4 per cent for WEE/S. Calf serum, when added, had been dialyzed.

Experiment A in Table I shows that addition of 10 per cent serum to the Eagle's medium increased the growth of WEE/L virus by a factor of 200 while growth of WEE/S was only stimulated by a factor of 2. On the other hand (expt B), lac-yeast medium was twice as favourable to the growth of WEE/S as to growth of WEE/L virus : compared with Eagle's medium, lac-yeast medium was a better nutrient for both viruses, but the increase in titre was 2·5 in the case of WEE/L and 5 in the case of WEE/S.

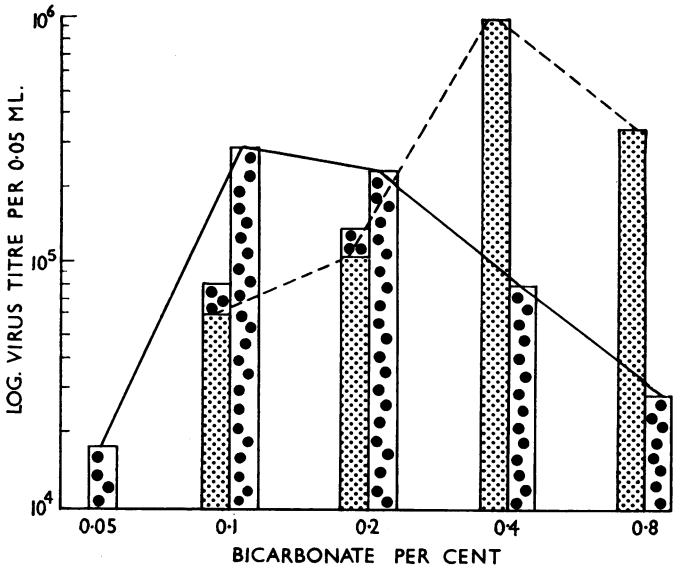


FIG. 2.—Growth of WEE/L (large dots) and of WEE/S (small dots) in liquid Eagle's medium added with various concentrations of bicarbonate. Virus titres in cells + fluid, after 48 hr. at 37°, as determined under optimal plaque assay conditions, for each type of mutant (no bicarbonate added, in the case of WEE/L ; bicarbonate 0·4 per cent in the case of WEE/S).

Eagle's media were then prepared, in which one amino acid or one vitamin was omitted. No obvious influence on the growth of the WEE mutants was observed, except in the case of the omission of folic acid : this omission unexpectedly increased the synthesis of WEE/L virus (factors of increase 2·5 and 1·6 respectively in the absence and presence of dialyzed serum), although it decreased growth of WEE/S by a factor of 3.

When amethopterin (0·001 mg. per ml.), was added to Eagle's medium minus folic acid to antagonize the folic function of the cells, growth of WEE/L was still stimulated (factor of increase 1·5), while growth of WEE/S virus was further depressed by a factor of 2.

When all the conditions favourable for the growth of WEE/L were met (*i.e.* Eagle's medium minus folic acid plus amethopterin plus serum, bicarbonate concentration 0·1 per cent), chicken fibroblasts fed with this medium and inoculated with the population of one small plaque gave rise to a virus stock which formed

TABLE I.—*Growth of WEE/L and WEE/S Viruses in Various Media. Preferential Stimulation of WEE/L Synthesis by Calf Serum, and of WEE/S by Lac-yeast Medium. The Importance of the Folic Acid Function for the Growth of WEE/S.*

	WEE/L	WEE/S
Titre in the control medium (Eagle's*)	$1{\cdot}12 \times 10^6$†	$2{\cdot}24 \times 10^7$
Ratio of titres obtained in various media		
EXPT A:		
Eagle's med. + 10 per cent serum‡ / Eagle's med.	200	2
EXPT B:		
Lac-Yeast med. + 10 per cent serum / Eagle's med. + 10 per cent serum	2·5	5
EXPT C:		
Eagle's med. minus folic acid / Eagle's med.	2·5	0·3
Eagle's med. minus folic acid + 10 per cent serum / Eagle's med. + 10 per cent serum	1·6	0·4
Eagle's med. minus folic acid + amethopterin / Eagle's med. minus folic acid	1·5	0·5

* Added with 0·1–0·4 per cent bicarbonate in the cases, respectively, of WEE/L and WEE/S virus.
† Number of PFU per ml., after several cycles of infection for 48 hr. at 37°.
‡ Dialyzed calf serum.

as much as 45 per cent large plaques in the absence of bicarbonate in the overlay (Fig. 3).

Folic acid plays a part in three functions of the cell: the synthesis of glycine, the methylation of uridylic acid into thymidilic acid, and the *de novo* synthesis of purines. A preliminary experiment indicated that addition of glycine (0·05 mg./ml.) to Eagle's medium minus folic acid plus amethopterin, did not restore the optimal synthesis of WEE/S virus. On the contrary, significant observations were made when attempts were made to remedy the defects of either the thymidine or the purine metabolism. This led to the following investigations.

Influence of normal and abnormal nucleic acid precursors on the growth of WEE/S and WEE/L viruses, and a comparison with the action on two other viruses (NDV and poliovirus)

WEE/S and L viruses were grown as above in Eagle's medium minus folic acid + amethopterin + calf serum, and the effect of adding one of the five purine and pyrimidine bases, or their nucleoside, at a concentration 0·25 mM, was studied; (guanine is poorly soluble and was partly in suspension when used). For comparison, two other viruses were also grown under the same conditions (NDV, on chicken fibroblasts, and poliovirus MEF1, on monkey kidney primary cultures).

Figures in Table II represent the ratio of virus titre in the presence of a nucleic acid precursor to the titre in the absence of this precursor. Most striking was the high increase of WEE/S virus titre in the presence of some of the precursors (factor of increase 53 with guanosine, 46 with cytidine, 41 with adenine, 30 with thymidine and 27 with cytosine). Factors of increase were much lower in the case of the 3 other viruses, and in no instance reached 3-fold. The high dependence of WEE/S virus on nucleic acid precursors for growth suggests its use as a sensitive

tool to detect, for instance, whether the cell utilizes a nucleoside more readily than the free base or vice versa. No generalization can be derived from Table II ; the nucleosides thymidine, cytidine and guanosine were more efficient growth

TABLE II.—*Growth of Four Viruses in Eagle Medium Minus Folic Acid + Calf Serum + Amethopterin, Supplemented with Various Normal or Abnormal Nucleic Acid Precursors, at a Concentration 0·25 mM*

	WEE/L*	WEE/S	NDV	MEF1
Adenine	0·6†	41	0·5	2
Adenosine	0·4	10	1	1
Guanine	1·2	15	0·8	1·3
8-Azaguanine	0·2	2·2	0·8	0·6
Guanosine	2	53	0·9	1·1
Cytosine	1·9	27	—	1·7
Cytidine	2·9	46	0·5	2·1
Uracil	0·5	3	0·5	1·6
Thiouracil	2·9	4	2·1	1·5
5-Fluorouracil	1·6	27	1	0·9
Uridine	0·25	0·7	0·8	1·2
Thymine	1	9	1·1	2
5-Bromouracil	1·7	7·7	—	2·3
Thymidine	1·9	30	0·2	1·7
5-Bromodeoxyuridine	2·2	3	1·5	2·1

Results are the average of 3 expts ; figures lacking in this table are those for which the mean deviation was too great.

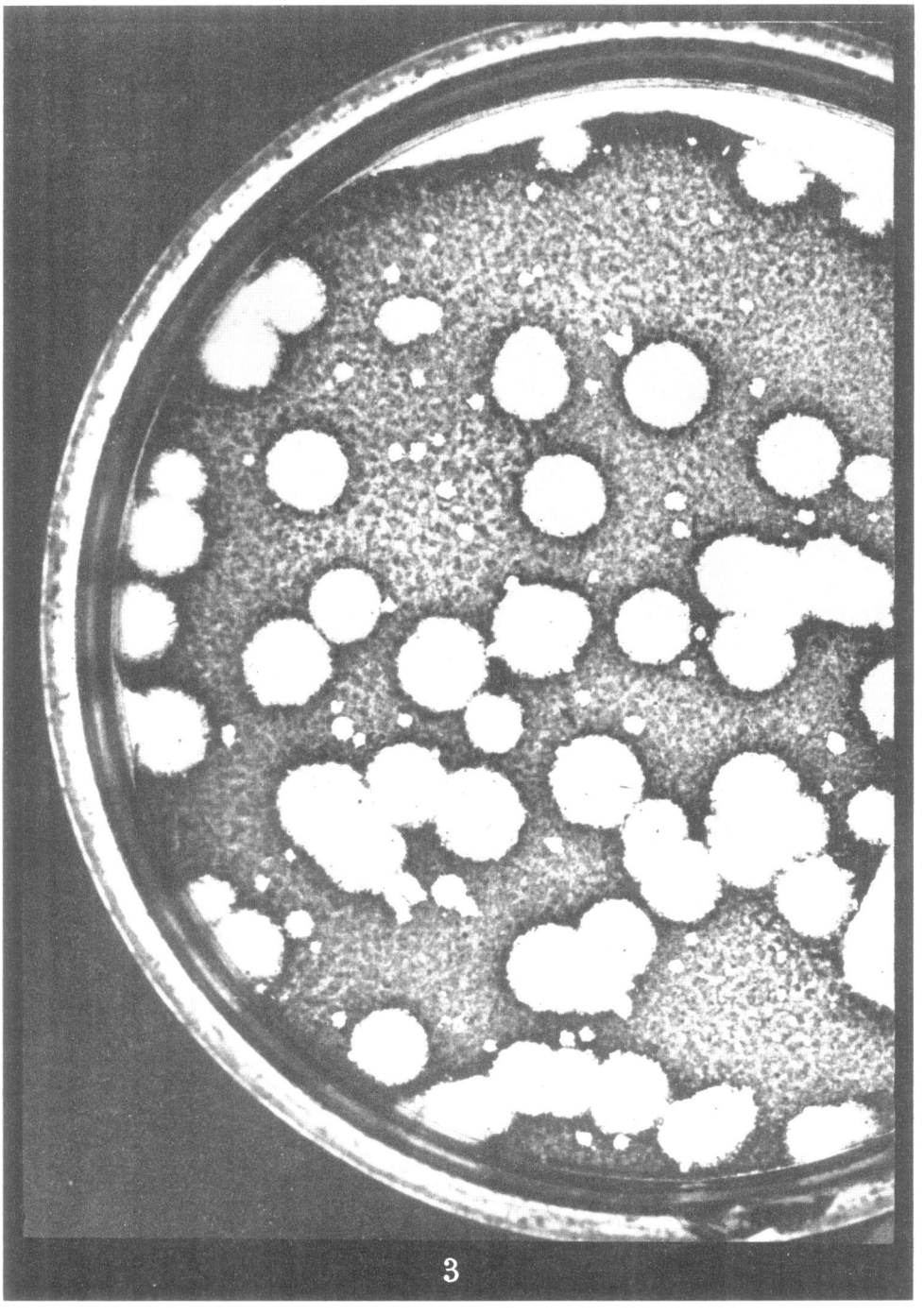
* Ratio of virus titre in the presence of one nucleic acid precursor, to the titre in control medium (without nucleic acid precursor added). Titres in control medium were respectively, per ml., $2{\cdot}48 \times 10^7$ for WEE/L virus ; $6{\cdot}5 \times 10^7$ for WEE/S virus ; $8{\cdot}52 \times 10^6$ for NDV and $2{\cdot}64 \times 10^6$ for the MEF1 strain.

factors for WEE/S virus than the free bases but, in contrast, adenine was more active than the corresponding nucleoside.

When cells were deprived of their capacity to synthesize thymidine, and purine bases, by the presence of amethopterin, structural analogues which have been described to be inhibitory for growth of plant and bacterial viruses (see discussion) were found to increase the synthesis of WEE/S virus : the factor of increase, which was 3 in the presence of uracil, was 4 with thiouracil and as high as 27 with 5-fluorouracil. An analogue of thymine, 5-bromouracil, had nearly the same activity as thymine itself (factors of increase respectively 7·7 and 9). In contrast, 5-bromodeoxyuridine, a deoxyribose product which is known to intervene exclusively in the metabolism of DNA, did not here act as thymidine as a growth factor for WEE/S virus : a slight increase of growth was found with the 4 viruses, in the presence of 5-bromodeoxyuridine (increase of titre from 1·5 to 3), but thymidine was 10 times more active than 5-bromodeoxyuridine towards WEE/S virus, and this was a specific effect, not found with the 3 other viruses. Thus, structural analogues of uracil and thymine, or of their nucleoside, were at least as good growth

EXPLANATION OF PLATE

FIG. 3.—A mixture of small and large plaques, obtained when the mutant WEE/S was grown and assayed under optimal conditions for the selection and expression of large plaque revertants.



Quersin-Thiry.

factors as the normal products themselves, for WEE/S virus, provided that, in the case of the nucleoside, the analogue was of the ribose and not of the deoxyribose type.

Only one structural analogue of purine bases was studied; 8-azaguanine (0·25 mM) was shown (Table II) to be inhibitory for growth of WEE/L virus, and slightly so for NDV and poliovirus, although it increased the formation of WEE/S virus by a factor of 2·2. This rather low increase might be the result of two oppo-

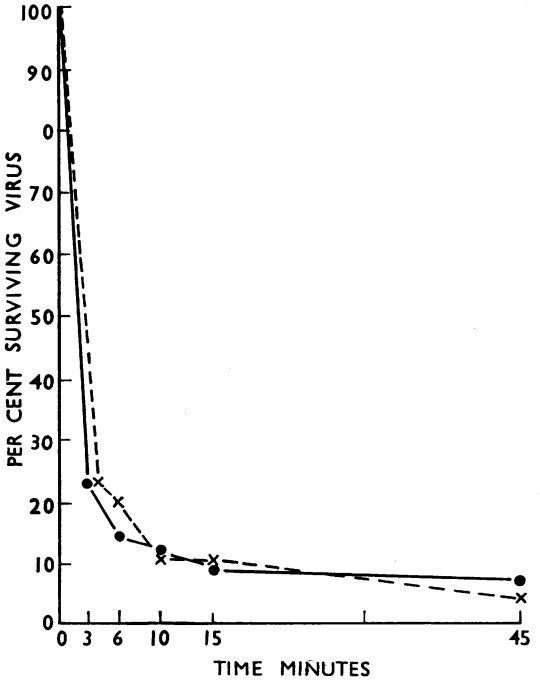


FIG. 4.—Kinetics of the neutralization of WEE/S (———) and of WEE/L virus (– – – –) by anti WEE/L serum. One volume of prewarmed antiserum, diluted 1/30, was added to one volume of prewarmed appropriate dilution of virus. At various times, aliquots were diluted 1/200 in ice cold lac-yeast medium. One ml. of this dilution were plated on each of three monolayers of chicken fibroblasts. The number of PFU, in the controls, was around 70 plaques.

sing effects of 8-azaguanine on WEE/S: a guanine-like growth promoting effect, specific for WEE/S, and an inhibitory action, as observed with the other viruses. In favour of this hypothesis was the finding that WEE/S was not a mutant resistant to 8-azaguanine: when grown in the presence of folic acid, and in absence of amethopterin, WEE/S was inhibited by 8-azaguanine just as WEE/L was (both titres were decreased ten times, as compared with controls, without 8-azaguanine).

When the experiments reported in Table II were repeated, with the only difference that folic acid (0·004 mg./ml.) was present, instead of amethopterin, it was observed that under these conditions, not a single nucleic acid precursor, normal or abnormal, increased the growth of any of the viruses tested; on the other hand, most of them decreased the growth of the viruses, by a factor which ranged from 5–1, an effect which was more accentuated for WEE/S virus.

The synthesis of WEE/L and WEE/S viruses, in cells pretreated with some of the growth factors

Experiments were performed under conditions of a single cycle of viral infection. Culture tubes of chicken fibroblasts were first treated for 6 hr. either with Eagle's medium minus folic acid plus amethopterin (minimal medium), or this medium supplemented with 10 per cent serum, with or without 0·25 mM guanosine or thymidine ; these media were renewed after 3 hr. at 37°. At time 6 hr., the cultures were washed twice and inoculated with 5 virus PFU per cell in 0·3 ml. of minimal medium ; 1 hr. later, non-adsorbed virus was eliminated by washing and 1 ml. minimal medium added. Virus growth was left to proceed for 10 hr. at 37°, after which period cells + fluid were submitted to 6 cycles of freezing and thawing. This series of culture tubes was compared to another series, in which serum, supplemented or not with guanosine or thymidine, was present during adsorption and synthesis of the viruses and not during the pre-treatment period.

TABLE III.—*Growth of WEE/L, WEE/S and NDV, under Conditions of One Cycle of Infection, in the Presence of Calf Serum (alone or with Guanosine or Thymidine), or in Cells Pretreated for 6 hr. with these Ingredients*

Eagle Medium Minus Folic Acid + Amethopterin supplemented with ↓	WEE/L		WEE/S		NDV	
	Before virus	With virus	Before virus	With virus	Before virus	With virus
Serum	1×*	102×	1·5×	10×	0·8×	1·2×
Serum + guanosine	1·5×†	1·8×	11×	3×	0·7×	0·9×
Serum + thymidine	0·25×	1×	2·2×	1·3×	0·9×	0·8×

* Along the first row, the figures relate to the modification of titre, as compared to medium without serum.

† Along the second and the third row, the influence of guanosine or thymidine is emphasized and the figures represent the modifications of titres, as compared with medium plus serum.

As shown in Table III, calf serum increased the growth of WEE/L virus by a factor of 102, when added at the same time as the virus but had no influence when used for pre-treatment of the cells. As already noted the enhancing activity of serum on WEE/S mutant growth was lower than on WEE/L (factor of increase 10) ; with this virus too, the influence of serum depended on its presence during virus synthesis.

A supplement of guanosine, added to the medium plus serum, had small effect on WEE/L growth, but increased the formation of WEE/S virus by a factor of 11, in the case of a pretreatment of the cells, and only by a factor of 3 when added at the same time as the virus. Thus, in contrast to the effect of serum, guanosine is more useful for virus synthesis when it has been stored by the cells first, or has had some effect on the cells. Results with thymidine tend to the same conclusion, although the increase was much lower under the present conditions of one cycle of infection than was found before.

The same experiments were repeated with NDV and MEF1 virus, and disclosed no significant influence of serum, guanosine or thymidine. This comparison indicated that dependence on calf serum for growth was a specific characteristic of both WEE/L and S viruses, the requirement being greater for the wild type

WEE/L ; in addition, the WEE/S mutant was characterized by a supplementary requirement for nucleic acid precursors.

Some other properties of WEE/S mutant

A rabbit antiserum against the wild type WEE virus was assayed against both WEE/L and WEE/S viruses ; the kinetics of the neutralization, a sensitive test (McBride, 1959), did not disclose antigenic difference between the two viruses (Fig. 4).

Thermostability of both viruses at 37° was then assayed. Prewarmed lac-yeast medium was added to each of the two viruses, to make 200 PFU per ml., and left at 37° for 2 hr. ; aliquots were plated at various times. No inactivation of WEE/L virus took place, but infectivity of WEE/S dropped to 66 per cent in 30 min., 50 per cent in 1 hr. and 32 per cent in 2 hr. There were indications too that WEE/S lost infectivity more rapidly than WEE/L, when kept frozen at −70°.

The LD_{50} of WEE/S virus for 10 days old chick embryos, inoculated by the allantoic route, was the same as that found with WEE/L virus ; allantoic fluids recovered from eggs inoculated with WEE/S virus, did not contain more than one or two large plaque-formers, per 800 small plaque-formers. Studies on the pathogenicity of WEE/S virus for mice have not been completed because of an intervening epidemic of ectromelia ; however it has been found that WEE/S virus killed mice by the intracerebral route, and that many small plaque-formers were recovered from the brains.

DISCUSSION

The fact that a few small plaque mutants can be detected in WEE virus stocks grown on chicken fibroblasts, in lac-yeast medium without serum, and the fact that virus stocks derived from small plaques are contaminated with large plaque-formers, if growth has taken place in lac-yeast or Eagle medium supplemented with 10 per cent serum, can best be explained by selective pressures exerted (*a*) by nucleic acid precursors (yeast extract in lac-yeast medium), in favour of a spontaneous small plaque mutant (WEE/S), and (*b*) by calf serum, in favour of large plaque-formers, revertants from WEE/S.

When plaque-purified WEE/S and WEE/L viruses were compared as to their requirements for growth, the addition of 10 per cent calf serum to the medium was found to increase the synthesis of WEE/L virus by a factor of 200, and the synthesis of WEE/S only by a factor of 2. On the other hand, several findings pointed to the importance of optimal conditions in nucleic acid metabolism of the cell, to ensure optimal synthesis of WEE/S mutant. First, the rather high requirement of this virus for bicarbonate—a requirement which cannot be met by controlling the pH with Tris buffer (Fefer, unpublished) ; secondly, a dependence of WEE/S on the folic acid metabolism, as proved by a 3-fold decrease in growth when folic acid was removed from the medium, and a further decrease when this medium was supplemented with amethopterin. The fact that amethopterin, in the absence of an external supply of folic acid, had some effect on the cellular metabolism is of some interest, as it indicates that primary cultures of chicken fibroblasts have at least some folic function of their own, although it has been

reported (Lieberman and Ove, 1960) that this function is poor in primary cultures of rabbit organs, as compared with established mammalian cell lines.

It is to be noted that WEE/L virus is also influenced by the conditions of nucleic acid metabolism : but in the opposite direction from WEE/S virus. Doses of bicarbonate which are optimal for WEE/S growth and plaque formation are inhibitory for WEE/L ; omission of folic acid increases WEE/L synthesis by a factor of 2·5, and a further increase of 1·5 is noted when amethopterin is added.

In the presence of folic acid and of optimal amounts of bicarbonate, the growth of WEE/S virus is not influenced by the addition of purine or pyrimidine bases, or of their nucleosides, to the nutrient medium. A need for these nucleic acid precursors becomes evident when the folic acid function is antagonized by amethopterin : in that case such precursors as guanosine, cytidine or adenine increase the growth of WEE/S by a factor around 50, whereas the factor of increase for WEE/L only ranges from 3–0·6.

The fact that adenine is more effective as a growth factor than adenosine, and cytidine more than cytosine, is consistent with published data (Broda, 1960) which indicate that adenine C14 is incorporated better into ribonucleic acid of rat liver, as the free base, than as the nucleoside,—while the contrary is true for cytosine. However, the same does not hold true for uracil, which was found here to have a low growth promoting action, and uridine none at all, although uridine is incorporated into rat liver ribonucleic acid better than uracil : either the growth promoting effect measured here does not reflect the degree of incorporation of precursors into the cell, or this incorporation is different for chicken fibroblasts cultures from that for rat liver.

The specific increase of growth of WEE/S, in the presence of nucleic acid precursors, may not be an indication that these precursors are directly incorporated into the virus ribonucleic acid : thymine and thymidine also increase the growth of WEE/S, although they are known to be incorporated only in the DNA. Thus, the effect of the precursors may be of a more indirect nature.

Some structural analogues, whose inhibitory actions have been reported in other conditions, can replace the normal nucleic acid precursors, as growth factors for WEE/S virus. (*a*) Thiouracil, an inhibitor of tobacco mosaic virus (Jeener, 1957) and of influenza virus synthesis (Amos and Vollmayer, 1958) was found here, in the presence of amethopterin, to display the same growth promoting activity as uracil. (*b*) 5-fluorouracil was even much more active than uracil. Although fluorouracil inhibits the growth of tobacco mosaic virus, it has been found to be utilized by tobacco leaf to make fully virulent particles, in which as much as one third of the uracil was replaced by fluorouracil (Gordon and Staehelin, 1958). Such an incorporation might take place with WEE/S virus too, but it is hard to explain why fluorouracil should be so much better utilized than uracil. The high growth promoting effect of fluorouracil on WEE/S virus is surprising anyway, in view of the fact that this analogue inhibits the growth of continuous cell lines, due to an interference with DNA metabolism, probably at the site of the thymidine synthetase ; in HEp/1 cells, but not in HeLa cells, there is a second site of inhibition, involving the RNA metabolism (Rich *et al.*, 1958, and 1960). No such data are available concerning primary cultures of chicken fibroblasts. (*c*) 5-bromouracil, an analogue of thymine, provoked the same increase of WEE/S growth as thymine did. Data are lacking too, concerning the way 5-bromouracil is utilized by chicken fibroblasts, but there are indications (Djordjevic and Szybalski, 1960)

that 5-bromouracil as the free base, and in contrast to 5-bromodeoxyuridine, is not incorporated into the DNA of a mammalian cell line, D98. These cells thus seem to behave differently from bacteria, in which 5-bromouracil C14 is freely incorporated into nucleic acids, partly as the abnormal base, partly as thymine, cytosine and uracil C14 (Wacker, Kirschfeld and Weinblum, 1960). As regards the influence of bromouracil on viruses, no effect has been reported on the synthesis of RNAviruses, while as much as 100 per cent of thymine DNA of bacteriophages can be replaced by 5-bromouracil, with a level of 11–13 per cent plaque morphology mutants (Litman and Pardee, 1960). (*d*) 8-azaguanine increased the growth of WEE/S virus by a factor of 2·2, although it was inhibitory for WEE/L, and has been found to inhibit the formation of vaccinia virus (Cogniaux-Le Clerc, 1959). This effect on a DNA virus must be reconciled with all other published data which point to a specific effect of azaguanine on the RNA metabolism, either of *Bacillus cereus* (Chantrenne and Devreux, 1958 ; Mandel and Markham, 1958), or of a mammalian cell line (Hagiwara, 1960). It could be that unnatural RNA is formed in azaguanine treated cells, with a consequent inhibition of the synthesis of viral protein, affecting both DNA and RNA viruses, and that, on the other hand, in cases where there is a marked need for nucleic acid precursors, (*e.g.* for the synthesis of WEE/S mutant) azaguanine could be used by the cell to make viral RNA. Thus, the final titre of WEE/S virus would result from the conflict between inhibition of viral protein formation and promotion of viral RNA synthesis : this would explain why azaguanine is much less active than guanine, as a growth promoting factor for WEE/S, in contrast to analogues of uracil and thymine which are at least as active as the normal base.

Under conditions of a single cycle of virus infection, it was found that enhancement of WEE/S growth by guanosine is not linked to the presence of guanosine in the medium, during virus synthesis, but rather to pretreatment of the cells by this nucleoside ; experiments actually in progress indicate that, to get an optimal virus growth promoting effect, the duration of treatment must be as long as 3–6 hr. Thus, it appears that guanosine needs time to prepare the cell for virus synthesis either through induction of the formation of some adaptive enzyme, or through enlargement of some purine-containing pool. Indications in favour of the existence, in L cells, of adaptive enzymes involved in the conversion of guanine to adenine, have been given by McFall and Magasanik (1960) ; they found on the other hand that incorporation of guanine and adenine C14 into the nucleotide pool is very rapid, a fact not consistent with the long lag observed here between addition of guanosine and the effect on WEE/S virus synthesis : if this effect were to depend on some storage of guanosine, then the incorporation would be related to some remote step of the RNA metabolism, concerning the microsomes, for instance.

SUMMARY

A small plaque mutant of WEE virus has been isolated, with a marked dependence on nucleic acid metabolism for growth. As compared to the wild type, this mutant was found to be more dependent on bicarbonate and folic acid content of the medium ; in the presence of amethopterin, its growth was greatly increased by the presence of some purine and pyrimidine bases, or of their nucleoside ; some structural analogues could replace the normal nucleic acid precursors, as growth factors.

Under conditions of one cycle of infection, the growth promoting effect of guanosine was only found when cells had been pretreated with this nucleoside, before addition of the virus.

In contrast to NDV and poliovirus (MEF1 strain), the wild type WEE/L virus was found to be highly dependent on the presence of calf serum for growth ; this property was less accentuated in the mutant WEE/S.

No antigenic differences could be discovered between the 2 WEE variants. WEE/S virus was pathogenic for mice and chick embryos, and bred true *in vivo*. This mutant was more thermolabile than the wild type.

The excellent technical assistance of H. van Haelen and L. Tack is acknowledged.

REFERENCES

AMOS, H. AND VOLLMAYER, E.—(1958) *Virology*, **6**, 337.
BRODA, E.—(1960) In ' Radioactive isotopes in biochemistry '. Groningen (Elsevier), table 11, p. 261.
CHANTRENNE, H. AND DEVREUX, S.—(1958) *Nature, Lond.*, **181**, 1737.
COGNIAUX-LE CLERC, J.—(1959) In ' Groupes de Contact, Fonds Recherche Scientif. Médicale '. Louvain (Wouters), p. 440.
DJORDJEVIC, B. AND SZYBALSKI, W.—(1960) *J. exp. Med.*, **112**, 509.
GORDON, M. P. AND STAEHELIN, M.—(1958) *J. Amer. chem. Soc.*, **80**, 2340.
HAGIWARA, P. J.—(1960) Xth International Congress of Cellular Biology, Paris, (L'Expansion Scientific Française), p. 108.
JEENER, R.—(1957) *Biochim. et biophys. Acta*, **23**, 351.
LIEBERMAN, I. AND OVE, P.—(1960) *J. biol. Chem.*, **235**, 1119.
LITMAN, R. M. AND PARDEE, A. B.—(1960) *Biochim. biophys. Acta*, **42**, 117, 131.
MCBRIDE, W. D.—(1959) *Virology*, **7**, 45.
MCFALL, E. and MAGASANIK, B.—(1960) *J. biol. Chem.*, **235**, 2103.
MANDEL, H. G. AND MARKHAM, R.—(1958) *Fed. Proc.*, **17**, 268.
RICH, M. A., BOLAFFI, J. L., KNOLL, J. E., CHEONY, L. AND EIDINOFF, M. L.—(1958) *Canc. Res.*, **18**, 730.
Idem, SASLAW, L. AND EIDINOFF, M. L.—(1960) *Proc. Soc. exp. Biol. N.Y.*, **103**, 791.
WACKER, A., KIRSCHFELD, S. AND WEINBLUM, D.—(1960) *J. molec. Biol.*, **2**, 72.