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*THE DRUG-REQUIRING PHASE IN THE GROWTH OF DRUG-DEPENDENT ENTEROVIRUSES**

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It has been shown recently that the intriguing phenomenon of drug dependence occurs not only in bacteria, but also in viruses.[1-4] Variants of enteroviruses have been isolated which require for their multiplication 2-(α-hydroxybenzyl)-benzimidazole (HBB) or guanidine, two chemical compounds which specifically inhibit the reproduction of members of this group of viruses.[5-11] Dependence is a property of the genetic material of the virus: infective RNA extracted from drug-dependent virus requires the drug to induce production of dependent virus.[1, 10]

It should be emphasized that the single cycle growth characteristics of drug-dependent virus growing in the presence of the drug at optimal concentration are similar to those of the drug-sensitive parent virus growing in the absence of the compound.[10] Furthermore, the drug concentrations required for maximal growth of dependent mutants are strikingly similar to those causing marked inhibition of sensitive parent viruses.[10] These results suggest that dependence and sensitivity involve opposite drug effects at the same site of action. Some evidence in support of this view was obtained in experiments on the effects of HBB and guanidine on the production of poliovirus-induced RNA polymerase.[12] The compounds prevented the appearance of virus-induced RNA polymerase activity in cells infected with drug-sensitive virus; but, on the other hand, guanidine was required for the appearance of this enzyme activity in cells infected with guanidine-dependent virus.

The kinetic and biochemical experiments reported in the present communication show that the HBB- or guanidine-requiring phase in the growth of dependent virus corresponds to the drug-sensitive phase in the growth of sensitive virus; the compounds are required for the replication of the RNA of dependent virus, whereas they prevent the replication of the RNA of sensitive virus.

Materials and Methods.—Coxsackie A9 virus (Woods strain) and its HBB-dependent variant were grown in primary cultures of rhesus monkey kidney cells;[10, 13] poliovirus (Brunhilde strain) and its guanidine-dependent variant, kindly made available by Dr. Nada Ledinko, The Public Health Research Laboratories of The City of New York, were grown in HeLa cells in suspension.[4, 12] Infective virus

was assayed by the plaque technique in monkey kidney cell cultures.[14] The HBB-dependent Coxsackie A9 virus was assayed in the presence of 22 μg/ml HBB, and the guanidine-dependent poliovirus in the presence of 100 μg/ml guanidine hydrochloride. All growth curve experiments were carried out at 37°C in a constant temperature room.

Growth curve experiments with Coxsackie A9 virus were carried out as described previously.[13] Synthesis of viral RNA was measured by extraction and assay of infective viral RNA or by incorporation of uridine-H^3 into RNA of actinomycin-treated cells.[13, 15]

Experiments with poliovirus in HeLa cells were performed as follows: HeLa cells, 10^6 cells/ml, were suspended in serum-free Eagle's spinner medium[16] with 4 mM glutamine, and incubated for $1^1/_2$ hr in the presence of actinomycin D (5 μg/ml). The cells were sedimented in the centrifuge and infected in a small volume (8×10^7 cells in 2 ml) with concentrated poliovirus at a multiplicity of 20 infective units of virus per cell. After 15 min adsorption at room temperature, the cells were washed once, resuspended to the original density in warm spinner medium containing 5 μg/ml of actinomycin D and 1 μc/ml of uridine-H^3 (specific activity 0.5 μc/μg), and incubation at 37°C was begun (zero time). At intervals, 10 ml samples were chilled in $N/4$ perchloric acid for determination of uridine incorporation into RNA,[13] and simultaneously 0.5 ml samples were frozen in an alcohol-dry ice bath for determination of virus infectivity. For addition or removal of compound, aliquots of cell suspension were centrifuged in the constant temperature room at 37°C and resuspended in the desired medium.

2-(α-Hydroxybenzyl)-benzimidazole was obtained through the kindness of Dr. Karl Folkers of Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

Results.—The HBB-requiring phase in the growth cycle of HBB-dependent Coxsackie A9 virus: The time course of the HBB-requiring phase in the multiplication of HBB-dependent mutant virus was compared with that of the HBB-sensitive phase in the reproduction of HBB-sensitive parent virus. First, the time of onset of the critical phase was determined. Figure 1 shows the development of HBB-dependent Coxsackie A9 virus in a single cycle of multiplication when the compound was added 1, 2, 3, or 4 hr after virus inoculation. The latent period was 4 hr in control cultures which contained HBB throughout the multiplication cycle. There followed a rapid increase in virus, and maximum yields were reached 8 hr after virus inoculation. When HBB was added either 1 or 2 hr after virus inoculation, the course of virus production was indistinguishable from that in control cultures. When HBB was added 3 or 4 hr after the virus, the latent period was 5 or $5^1/_2$ hr, thus being extended 1 or $1^1/_2$ hr beyond normal. These experiments demonstrate that HBB is not required during the first half of the latent period; the HBB-requiring phase begins in the second half of the latent period.

Corresponding experiments with the HBB-sensitive parent virus are shown in Figure 2. In this case, HBB was present initially, but was then removed at various times after virus inoculation. The latent period was $3^1/_2$ hr, and maximum yields were reached at about 8 hr in control cultures, which did not contain HBB at any time. There was no substantial difference between the growth of virus in control cultures and the curves obtained when HBB was removed 1 hr or $1^3/_4$ hr after virus

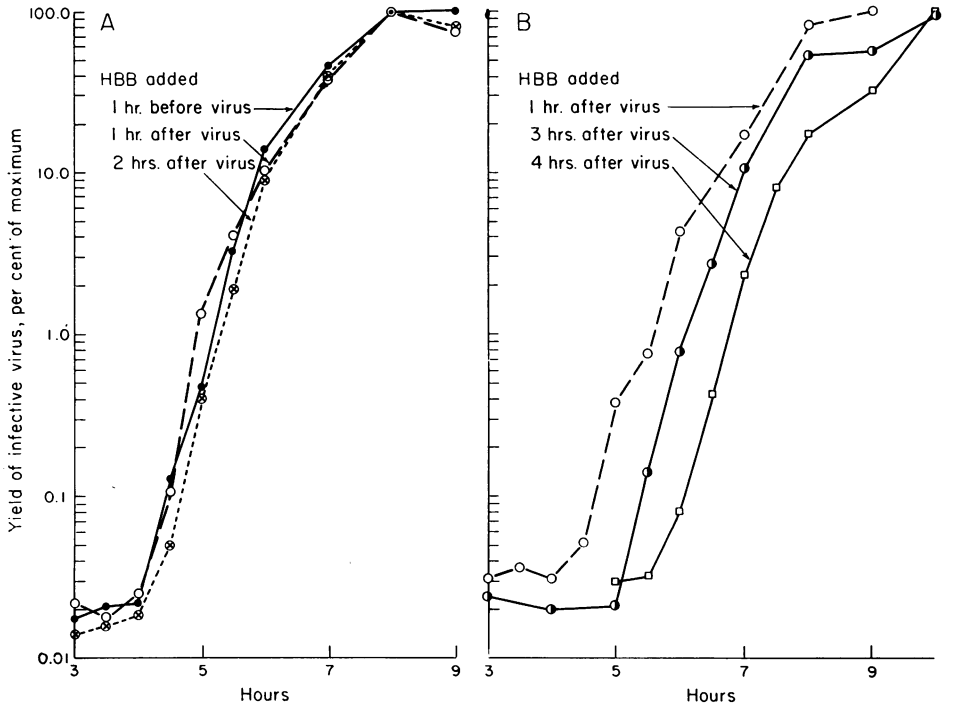

FIG. 1.—Time course of multiplication of HBB-dependent Coxsackie A9 virus when HBB was added 1, 2, 3, or 4 hr following virus inoculation.

inoculation. However, virus reproduction was significantly delayed when cultures were treated with HBB for 3 hr: the latent period was prolonged $1^1/_2$ hr beyond normal. These results show that during the growth cycle of the HBB-sensitive parent virus, the HBB-inhibitable phase begins in the second half of the latent period.

Next, the duration of the HBB-requiring or the HBB-sensitive phase was investigated. In the experiment performed with HBB-dependent virus (Fig. 3, left), HBB was present from the beginning, but was removed $5^1/_2$ hr after virus inoculation, i.e., during the exponential increase in

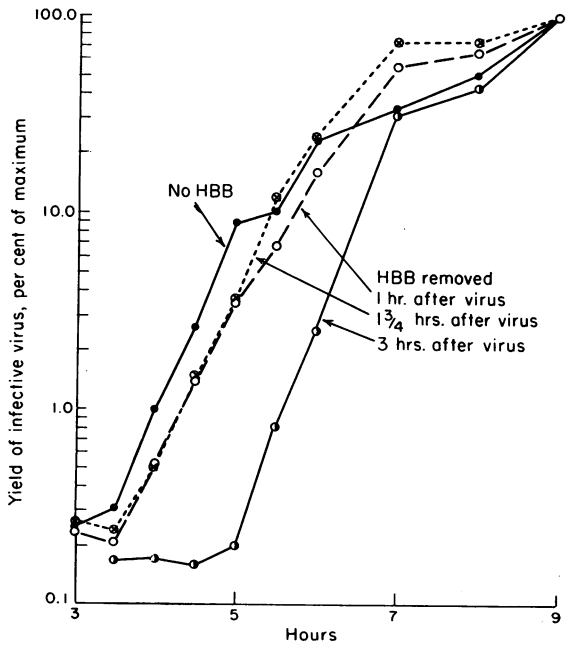

FIG. 2.—Time course of multiplication of HBB-sensitive Coxsackie A9 virus with HBB (49 μg/ml) present for 1, $1^3/_4$, or 3 hr following virus inoculation.

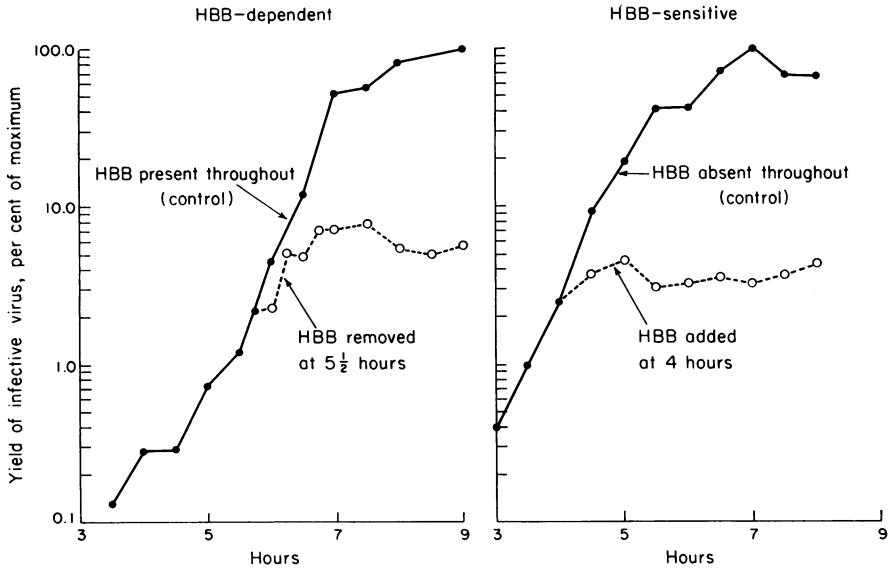

FIG. 3.—Effect of removal or addition of HBB on the multiplication of HBB-dependent or HBB-sensitive Coxsackie A9 virus during the exponential increase phase in virus. HBB-dependent virus was grown in the presence of 22 μg/ml HBB.[10] 49 μg/ml HBB was added to the sensitive strain.

virus. Within 30–45 min after removal of compound the production of virus came to a complete halt. Thus, the HBB-requiring phase extends from the beginning of the second half of the latent period well into the exponential increase phase.

Analogous findings were obtained with the HBB-sensitive parent (Fig. 3, right). When HBB was added 4 hr after virus inoculation, virus reproduction stopped within 15 min. A similar result was obtained when HBB was added to the cultures $4^1/_2$ hr after virus inoculation. Thus, as with the HBB-requiring phase in the multiplication of HBB-dependent virus, the HBB-sensitive phase in the reproduction of the drug-sensitive virus also extends from the beginning of the second half of the latent period well into the exponential increase phase.

Replication of RNA of HBB-dependent Coxsackie A9 virus: Requirement of HBB: It was reported previously that HBB inhibits the synthesis of the viral RNA of HBB-sensitive Coxsackie A9 virus.[13, 15] Table 1 shows that HBB was required

TABLE 1

EFFECT OF HBB ON THE SYNTHESIS OF INFECTIVE RNA OF HBB-DEPENDENT COXSACKIE A9 VIRUS

Compound	8-hr yield, multiple of 2-hr value	
	RNA	Virus
HBB, 22 μg/ml	76	188
None	0.93	0.7

for the synthesis of the infective viral RNA of HBB-dependent virus. Further evidence in support of this conclusion was obtained in experiments on the incorporation of uridine-H^3 into actinomycin-treated infected cells under conditions where cellular DNA-dependent RNA synthesis is inhibited more than 90 per cent.[15] Figure 4 shows that infection of actinomycin-treated cells with HBB-dependent

virus caused a marked increase in the uptake of uridine-H^3 into acid-insoluble form, provided that HBB was present in the culture. The first significant uptake of label in HBB-treated infected cultures occurred 4–5 hr after virus inoculation, i.e., at the time when virus maturation first becomes demonstrable. This is consistent with previous findings that enterovirus RNA is synthesized at the time of virus assembly, or only a short time earlier.[17, 13, 15]

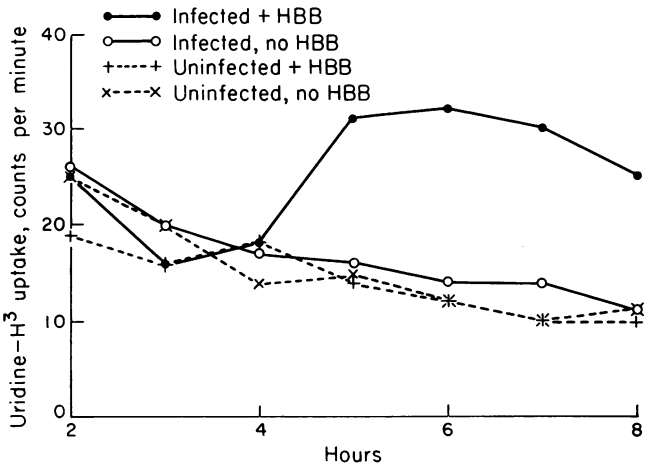

FIG. 4.—Effect of HBB on virus-induced RNA synthesis in actinomycin-treated monkey kidney cells infected with HBB-dependent Coxsackie A9 virus. Actinomycin D (1 μg/ml) was present 2 hr prior to virus inoculation and throughout the viral multiplication cycle. At intervals, cells were exposed to 1.0 μc uridine-H^3 for 15 min, and uptake into acid-insoluble form was measured.[15]

These experiments demonstrate that the RNA of HBB-dependent virus requires HBB for replication; that of the sensitive virus cannot replicate in the presence of the compound.[13, 15]

The data presented in Figure 4 require one further comment. The uptake of uridine was consistently lower in cells infected with HBB-dependent virus compared with that in cells infected with HBB-sensitive virus. This is probably related to the fact that actinomycin, 1 μg/ml, reduces the yield of HBB-dependent virus about 4-fold, but does not significantly affect the yield of HBB-sensitive parent virus.

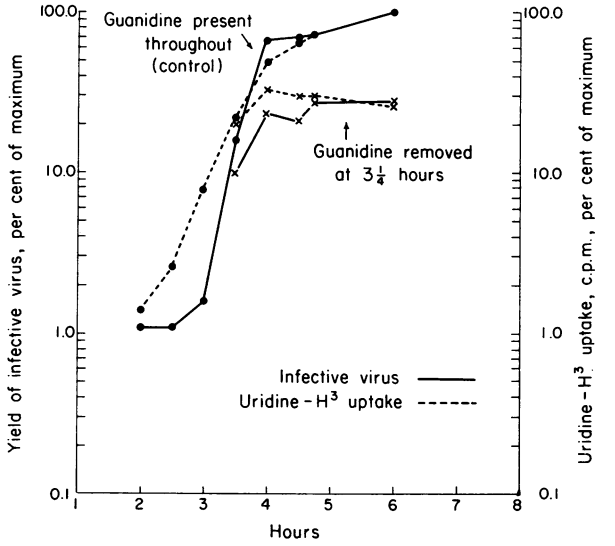

FIG. 5.—Effect of removal of guanidine on the synthesis of the viral RNA, and on the growth of guanidine-dependent poliovirus 1. Actinomycin D (5 μg/ml) was present $1^1/_2$ hr prior to virus inoculation and throughout the viral multiplication cycle. Viral RNA synthesis was measured by incorporation of uridine-H^3 into RNA.[13]

Replication of RNA of guanidine-dependent poliovirus 1: Requirement of guanidine: The guanidine-dependent mutant of poliovirus 1 and the guanidine-sensitive parent virus were studied in experiments which were in several respects similar to those described for Coxsackie A9 virus and its HBB-dependent variant. Figure 5 records the single cycle growth curve of guanidine-dependent poliovirus, and the

course of synthesis of its RNA as measured by incorporation of uridine-H^3. As can be seen, when guanidine was removed at $3^1/_4$ hr, i.e., during the exponential increase phase, virus multiplication and viral RNA synthesis came to a complete halt. These results are similar to those obtained with HBB-dependent Coxsackie A9 virus, but in addition they show directly that the replication of viral RNA requires the continued presence of guanidine.

In an experiment with the guanidine-sensitive parent virus, the compound was added to the cultures at $2^2/_3$ or $3^1/_4$ hr, i.e., during the exponential increase phase. Figure 6 shows that, 15 to 30 min later, virus multiplication and viral RNA syn-

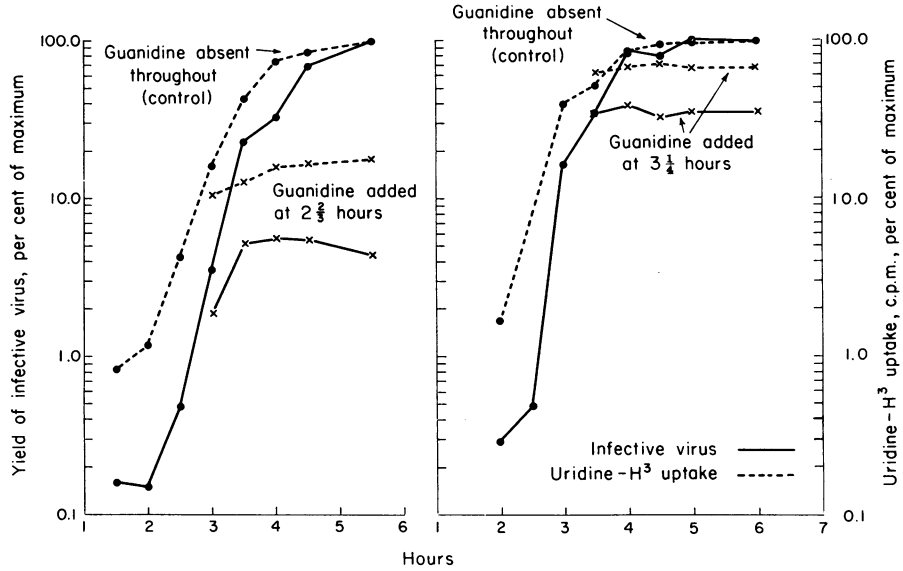

FIG. 6.—Effect of addition of guanidine (150 μg/ml) on the synthesis of the viral RNA and on the growth of guanidine-sensitive poliovirus 1. Viral RNA synthesis was measured as described in Fig. 5.

thesis became completely inhibited. It is important to note that this occurred even when the compound was added late, i.e., at $3^1/_4$ hr, when about 25 per cent of the total virus and an even larger proportion of the viral RNA had already been formed.

An experiment similar to that described in Figure 6 was carried out with HBB. The compound, at 68 μg/ml, was added to the cultures at $2^2/_3$ or $3^1/_4$ hr. Like guanidine, HBB inhibited both viral RNA synthesis and virus growth. However, the inhibition was incomplete in that some replication of viral RNA and infective virus occurred. It is known that polioviruses, and particularly some poliovirus 1 strains, are not very sensitive to the action of HBB.[5]

Discussion.—We have shown in the present study that the time course of the HBB-requiring phase in the growth cycle of HBB-dependent Coxsackie A9 virus is strikingly similar to that of the HBB-sensitive phase in the reproduction of the HBB-sensitive parent virus. In each case, the critical period begins toward the end of the first half of the latent period and continues well into the exponential increase phase of virus growth. Our results furthermore show that the compound

is necessary for the replication of the RNA of the dependent mutant, whereas it inhibits RNA synthesis of the sensitive parent virus. It thus appears that the critical processes in HBB dependence and HBB sensitivity are functionally and biochemcally analogous. There is evidence from previous work[8, 18] that in the growth cycle of drug-sensitive enteroviruses a guanidine-sensitive process begins very shortly after infection, i.e., 1–2 hr before the onset of the HBB-sensitive phase. In the present study with the guanidine-dependent mutant of poliovirus and its drug-sensitive parent, a kinetic analysis of guanidine requirement or sensitivity during the early phases of viral growth was not carried out. It was shown, however, that the guanidine-requiring or -sensitive phase in the reproductive cycle of poliovirus 1 extends through the exponential increase phase in virus. Furthermore, it was shown that the presence of guanidine during the exponential phase is necessary for RNA synthesis of the dependent mutant, but is inhibitory for RNA synthesis of the sensitive parent virus. These results too are compatible with the hypothesis that the drug-dependent and drug-sensitive processes are biochemically analogous.

Further experimental support for this hypothesis comes from our recent findings concerning the appearance in poliovirus-infected HeLa cells of an RNA polymerase activity not demonstrable in uninfected cells.[12] The appearance of the RNA polymerase activity was inhibited by HBB and guanidine in cells infected with drug-sensitive virus, but required guanidine when cells were infected with the guanidine-requiring mutant. All of the results obtained support the hypothesis that HBB and guanidine have direct effects on the synthesis of virus-induced RNA polymerase, and thereby affect the replication of viral RNA. At the same time they strongly support the inference that the virus-induced RNA polymerase is in fact the enzyme system responsible for the synthesis of viral RNA. The nature of the early process in enterovirus growth, which is sensitive to guanidine but not to HBB, is not clear.

It has been shown with the aid of two inhibitors of protein synthesis, *p*-fluorophenylalanine and puromycin, that formation of poliovirus RNA is dependent on continuing protein synthesis.[19] If virus-induced RNA polymerase is indeed the protein on which the synthesis of viral RNA depends, then it follows that the synthesis of this enzyme continues after completion of the latent period in the multiplication cycle of the virus, and that its functional life is short. These conclusions have been experimentally verified in the case of the virus-induced RNA polymerase demonstrable in poliovirus-infected HeLa cells.[12] The experimental evidence obtained in our laboratory contradicts the notion that the protein on which the synthesis of viral RNA depends is nonenzymatic, as suggested by Wecker.[20]

The genetic analysis of the drug-sensitive, drug-resistant, and drug-dependent states of enteroviruses is as yet too incomplete to permit definite conclusions as to whether these phenotypes are controlled by a single gene occurring in several allelic states. However, the kinetic and biochemical results so far obtained with the mutant strains are compatible with this idea.

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[1] Eggers, H. J., and I. Tamm, in *8th International Congress for Microbiology* (Montreal, 1962)

Abstracts, p. 85.

[2] Eggers, H. J., *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 27 (1962) 309.

[3] Loddo, B., W. Ferrari, A. Spanedda, and G. Brotzu, *Experientia*, **18**, 518 (1962).

[4] Ledinko, N., *Cold Spring Harbor Symposia on Quantitative Biology*, vol. 27 (1962) 309.

[5] Eggers, H. J., and I. Tamm, *J. Exptl. Med.*, **113**, 657 (1961).

[6] Eggers, H. J., and I. Tamm, *Virology*, **13**, 545 (1961).

[7] Rightsel, W. A., J. R. Dice, R. J. McAlpine, E. A. Timm, I. W. McLean, Jr., G. J. Dixon, and F. M. Schabel, Jr., *Science*, **134**, 558 (1961).

[8] Crowther, D., and J. L. Melnick, *Virology*, **15**, 65 (1961).

[9] Loddo, B., *Boll. Soc. It. Biol. Sper.*, **38**, 8 (1962).

[10] Eggers, H. J., and I. Tamm, *Virology*, **20**, 62 (1963).

[11] Loddo, B., S. Muntoni, A. Spanedda, G. Brotzu, and W. Ferrari, *Nature*, **197**, 315 (1963).

[12] Baltimore, D., H. J. Eggers, R. M. Franklin, and I. Tamm, these PROCEEDINGS, **49**, 843 (1963).

[13] Eggers, H. J., and I. Tamm, *Virology*, **18**, 426 (1962).

[14] Choppin, P. W., and H. J. Eggers, *Virology*, **18**, 470 (1962).

[15] Eggers, H. J., and I. Tamm, *Nature*, **197**, 1327 (1963).

[16] Eagle, H., *Science*, **130**, 432 (1959).

[17] Darnell, J. E., Jr., and H. Eagle, *Adv. Virus Res.*, **7**, 1 (1960).

[18] Eggers, H. J., and I. Tamm, in manuscript (1963).

[19] Scharff, M. D., M. M. Thoren, N. F. McElvain, and L. Levintow, *Biochem. Biophys. Res. Comm.*, **10**, 127 (1963).

[20] Wecker, E., *Nature*, **197**, 1277 (1963).

THE EINSTEIN GENERALIZED RIEMANNIAN GEOMETRY

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1. In the fourth edition of *The Meaning of Relativity*, 1953, Einstein introduced the nonsymmetric covariant metric tensor g_{ij} of the space-time continuum of four dimensions and the contravariant tensor g^{ij} by the equations on page 135

$$g_{ih}g^{ih} = 1,\ g_{hj}g^{hj} = 1,\ g^{ih}g_{il} = 0,\ g_{hj}g^{lj} = 0, \tag{1}$$

where i and j are summed from 1 to 4, h is not summed, and h and l are different.

On page 137 of the fifth edition, he introduces the equation

$$g_{ik,l} - g_{sk}\Gamma_{il}{}^{s} - g_{is}\Gamma_{lk}{}^{s} = 0,$$

which, with change of indexes, is

$$g_{ij,k} = g_{hj}\Gamma_{ik}{}^{h} + g_{ih}\Gamma_{kj}{}^{h}. \tag{2}$$

Each of the terms in the right-hand member of this equation stands for the sum of terms as h takes values 1 to 4. In what follows, when in a term there is the same upper and lower index, the term stands for the sum of four terms as the index takes the values 1 to 4. In the left-hand member of equation (2), $g_{ij,k}$ is the derivative of g_{ij} with respect of x^{k}. This notation is used in what follows.

2. When equation (2) is multiplied by g^{ih} and summed for i, in accordance with equation (1), the result is