PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 155, 252-258 (1977)

Actinomycin D Acts on an Intracellular Process to Inhibit Replication of Rhinovirus Type 14 (39784)

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Introducation. Multiplication of picornaviruses is generally considered to be resistant to the action of actinomycin D (Act D) (1). Exceptions to this statement may be found and, in fact, sensitivity or resistance to Act D depends on virus strain, host cell type, and physiologic condition of the cells (2). Where adequately studied, the replicative event(s) that is sensitive to Act D occurred early in the growth cycle of strains of poliovirus (3), mengovirus (4), and echovirus type 12 (5). There are no published data on the effect of Act D on replication or rhinoviruses, the largest subgroup within the picornaviruses. In three reports, rhinoviruses are mentioned as being sensitive (5, 6) and resistant (7) to Act D. In this report, data are presented showing that replication of rhinovirus type 14 (RV14) in HeLa cells is most sensitive to Act D early in the growth cycle, but that sensitivity extends into the exponential phase of the growth cycle. Adsorption, penetration, and uncoating of RV14 particles are not affected in cells treated with Act D. However, synthesis of viral RNA containing both single- and double-stranded regions is inhibited in cells incubated with Act D.

Materials and methods. Cells and viruses. Methods for culture of HeLa (rhino-HeLa) and prepartion for monlayer cell cultures were previously described, as well as methods for preparing RV14 stocks and plaque assay of RV14 (8). Methods for preparing stocks of mengovirus and plaque assay of mengovirus have been reported (9).

Inoculation of cells with virus. Cell culture growth fluids were removed, the cells were washed twice with minimal essential medium (MEM), and, unless noted, cells in all experiments were inoculated with 20 to 25 plaque-forming units (pfu) per cell of either virus. Zero time in all experiments was the instant of mixing virus with cells. After 2 hr for adsorption of virus at 34.5°, the inoculum was removed and the cell cultures were washed three times with MEM and incubated in virus growth medium (VGM) consisting of MEM with 1% heat inactivated fetal bovine serum and gentamycin (50 units/ml). Unless otherwise noted, all infected cell cultures were harvested at 14–16 hr post inoculation and the cell cultures were rapidly frozen (−70°) and thawed (37°) to release progeny virus.

Infectious center assay. The number of virus-producing cells per culture was measured as previously described (9). Briefly, cell cultures, incubated in MEM with 7% fetal bovine serum either with or without Act D, were washed and inoculated with RV14 as described above. After the 2-hr period for adsorption, the cells were washed three times with MEM and incubated with MEM containing sufficient anti-RV14 antibody to neutralize 99.5% of a suspension of RV14 containing 1×10^8 pfu/ml. After 10 min at 34.5°, the antibody-containing fluids were removed and the cells were then washed three times with MEM. The cells were scraped loose with a rubber policeman, counted, and 0.2 ml dilutions of cells were added to confluent monolayer cultures of HeLa cells. After 30 min at 34.5°, these cell cultures were overlaid with the nutrient–agar mixture used in the plaque assay for RV14.

Preparation and purification of ^{14}C-labeled RV14 particles. HeLa cell cultures were inoculated with RV14 and after the adsorption period the inoculum was removed and the cell cultures were incubated for an additional 14 hr in VGM containing 0.05 μCi/ml of [2-^{14}C]uridine. Infected cells were harvested and virus particles were purified by a method previously described, which included banding the virus particles in a CsCl gradient (6).

ISSN 0037-9727

Extraction of viral ribonucleic acids. Cell cultures inoculated with RV14 were harvested, washed twice with MEM, and the cytoplasmic fraction was extracted for nucleic acids with phenol and sodium dodecyl sulfate as previously described (6). Methods for measurement of trichloroacetic acid (TCA)-precipitable radioactivity in ribonuclease-sensitive and ribonuclease-resistant RNA were described earlier (6).

Chemicals. [5-^{3}H]Uridine and [2-^{14}C]-uridine were purchased from New England Nuclear Corp. (Boston, Mass.). Act D was a gift from W. B. Gall of Merck Sharpe and Dohme (Rahway, N. J.). Insulin was purchased from Sigma Chemical Co. (St. Louis, Mo.) and was stored at −90°. Fresh solutions of insulin at 400 μg/ml were prepared in MEM for each experiment.

Results. RV14 yields from HeLa cell cultures incubated in presence of Act D. The effect of various concentrations of Act D on yields of RV14 in HeLa cells is shown in Table I. Two experiments were selected to show the variation in effect of Act D on virus yields. At 5 μg/ml, Act D reduced virus yields by 24 to 54% of the yields obtained from control cell cultures. Addition of Act D at 5 μg/ml reduced virus yields when added to infected cell cultures at all times tested between 0 and 7 hr postinoculation (pi; Fig. 1); the latter time is in the exponential phase of virus replication (7) (see Fig. 2). Although the period between 0 and 4 hr pi is most sensitive to the effect of Act D, inhibition of some process(es) required for production of virus resulted even when Act D was added during the exponential phase of virus replication. Subsequent experiments were carried out with Act D at 5 μg/ml.

Increasing the length of time of incubation of HeLa cells with Act D prior to challenge with RV14 resulted in an increasingly larger reduction in virus yields (Table II). In the experiments, Act D was added to the cell culture fluids and the cultures were then incubated for various periods of time prior to inoculation with virus. At 5 min prior to zero time, the Act D-containing cell culture fluids were removed, the cells were washed twice with MEM to remove Act D, and the virus was inoculated as described in Materi-

TABLE I. EFFECT OF ACT D ON RV14 YIELDS IN HeLa CELL CULTURES.[a]

Concentration of Act D (μg/ml)	Experiment 1		Experiment 2	
	Virus yield (pfu/cell)	C (%)	Virus yield (pfu/cell)	C (%)
5	122	54	55	24
2	142	63	57	25
1	225	100	130	58
None	225	100	225	100

[a] Act D was added to VGM at 2 hr pi.

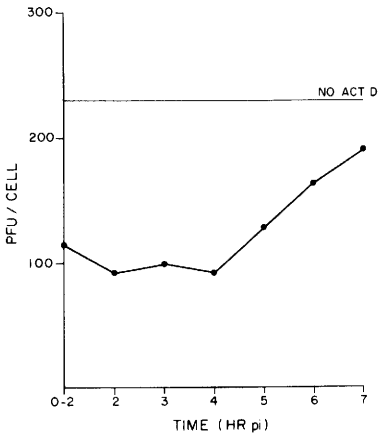

FIG. 1. Effect of time of addition (●) of Act D on yields of RV14 in HeLa cells.

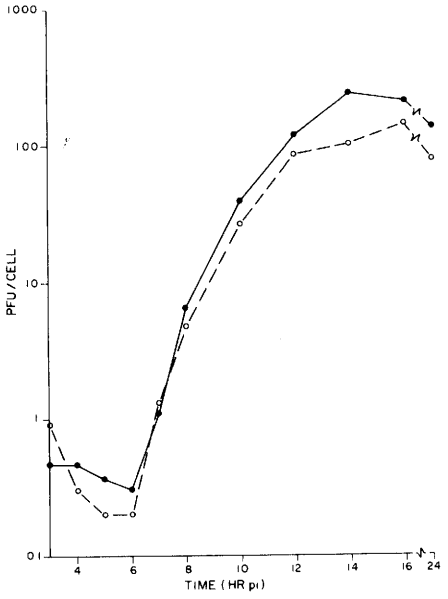

FIG. 2. Growth curves of RV in HeLa cells in absence (●——●) and presence (O---O) of Act D.

TABLE II. EFFECT OF LENGTH OF TIME OF INCUBATION OF HeLa CELLS WITH Act D PRIOR TO INOCULATION WITH RV14 OR MENGOVIRUS ON VIRUS YIELDS.

Experiment number	Time of Act D addition[a]	Yield of RV14 (%C)[b]	Yield of mengovirus (%C)[b]
1	−6	5	88
	−4	18	84
	−2	28	95
	None	100	100
2	−6	20	64
	None	100	100
3	−12	4	28
	None	100	100

[a] Act D at 5 μg/ml was added to growth fluids in which the cells were incubated at the times indicated prior to washing and inoculation of virus.

[b] Virus yields (pfu/cell) for RV14 and mengovirus were 353 and 2435, 191 and 1445, and 254 and 4693 in experiments 1, 2, and 3, respectively.

als and methods. That these cell cultures were capable of producing virus is shown by their capacity for synthesis of nearly full yields of mengovirus under all conditions examined, except incubation of cells with Act D for 12 hr prior to inoculation with virus. Clearly, these results show that the replication of RV14 is some 3- to 17-fold more sensitive to the action of Act D than is the replication of mengovirus. Cooper (10) had previously shown that insulin could reverse the antiviral action of Act D on replication of poliovirus type 1 in human amnion cells. In data not presented, it was found that insulin at 0.4 or 4.0 units/ml almost completely reversed the inhibitory effect of Act D on replication of RV14. At these two concentrations, insulin alone had little effect on replication of RV14.

Temporal aspects of the effect of Act D on replication of RV14 in HeLa cells. The effect of Act D on temporal aspects of the growth cycle of RV14 in HeLa cells is shown in Fig. 2. Act D was added to the VGM at 2 hr pi. Although the final yield was reduced by 32% in Act D-treated cells, all temporal aspects of the growth curves of RV14 were similar in untreated cells or in cells treated with Act D at 2 hr pi.

In order to more clearly define the time at which the Act D-sensitive event(s) occurs during replication of RV14 in HeLa cells, cells were incubated with Act D for 6 hr prior to removal of the antibiotic by washing and inoculation of virus to effect a greater sensitivity of replication of the virus to the antibiotic. Growth of mengovirus under these conditions was measured to assess the competency of the cells for replication of another picornavirus. Growth curves are presented (Fig. 3) for these two viruses in HeLa cells incubated with Act D under two conditions: Cells were incubated for 6 hr prior to inoculations of virus or with Act D added to the VGM at 2 hr pi. In this experiment, a 2-hr extension in the latent period of the growth cycle of RV14 was observed in cells treated with Act D for 6 hr prior to inoculation of virus, compared to cells incubated with Act D from 2 hr pi. The yield of RV14 from Act D-pretreated cell cultures was only 6% of the yield obtained from infected cell cultures incubated with Act D from 2 hr pi. In contrast, the yield of mengovirus was slightly greater and the growth cycle was completed earlier in cells treated with Act D prior to challenge compared to cell cultures given Act D at 2 hr pi, a result found several times in this study. Thus, the replicative events affected by Act D are not uniformly directed toward all picornaviruses. In the case of RV14, the Act D-

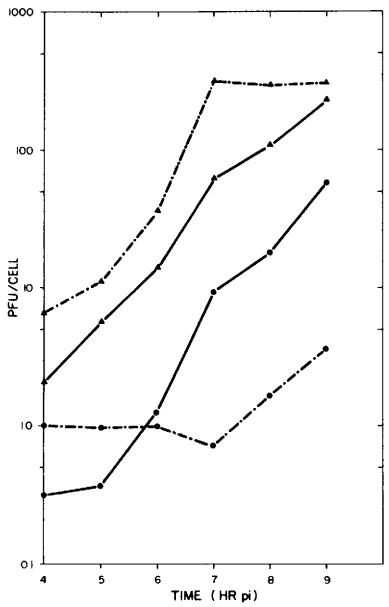

FIG. 3. Yields of RV14 (●) and mengovirus (▲) HeLa cells. Cells exposed to Act D for 6 hr prior to challenge (●–●–●–) or exposed to Act D at 0 time (——).

sensitive event can be detected early in the growth cycle.

The number of cells in control or Act D-treated (−6 hr prior to inoculation with virus) cultures that participated in replication of RV14 was determined by an infectious center assay (see Materials and methods for details). The results, presented in Table III, show that similar numbers of cells in each culture participated in replication of virus whether they had been incubated with Act D or not prior to infection. In experiment 1, 18%, and in experiment 2, approximately 8% of cells in either culture could act as infectious centers, respectivley. However, RV14-producing cells in Act D-treated cultures produced only 18 to 21% of the yield produced in cells from control cultures. Yields of mengovirus from Act D-treated cells in experiments 1 and 2 were 101 and 100% of control values, respectively.

Effect of Act D on early replicative events in the growth cycle of RV14. Attachment of RV to Act D-treated cell cultures was assessed in two experiments. Control cell cultures and cell cultures incubated with Act D for 6 hr prior to challenge were inoculated with approximately 40 or 16 pfu/cell in two experiments. After 2 hr for adsorption at 34.5°, the challenge inocula were removed and assayed for unadsorbed virus. Heat-inactivation studies of virus held for 2 hr at 34.5° revealed no loss in infectivity. The results of two experiments showed that 83 to 89% of infectious virus was removed from the challenge inocula in these experiments, regardless of whether the cells had been incubated with Act D or not. Adsorption of virions and perhaps some inactivation of virions by cell proteases were, therefore, similar in both types of cell cultures.

Uncoating of RV14 virus particles in control cell cultures and cell cultures treated with Act D for 6 hr prior to challenge was examined using purified [^{14}C]uridine-labeled virus (1.4×10^4 pfu/cpm). After challenge of control and Act D-treated cells with virus for 2 hr at 34.5°, the inocula were removed, the cells were washed three times with MEM, and VGM was added. All cultures were returned to the 34.5° incubator. At 5 hr pi, the cell cultures were harvested by centrifugation at 350*g* and 4° and, after freezing and thawing twice, the entire contents of the two cell pellets were layered onto separate preformed CsCl gradients (1.2 to 1.4 g/ml) and centrifuged to equilibrium (33,000 rpm in an SW50L rotor for 12 hr at 4°) in two separate gradients. The dispersion of counts in ^{14}C across these gradients is shown in one composite figure (Fig. 4). Under conditions of centrifugation in this experiment, free RNA and RNA associated with small amount of protein would pellet at the bottom of the tube (12). A larger proportion (threefold) of the virion RNA pelleted at the bottom of the tube into which control cell lysate materials were centrifuged in comparison with the tube containing Act D-treated cell lysate materials. Counts banding in the region of intact virus particles (11) (fractions 4 to 6) are noted by the arrow at a density of 1.412–1.414 g/ml. Control cell cultures contained 17% of the total ^{14}C counts as intact virus particles, whereas Act D-treated cell cultures contained 7% of the total counts per minute as intact virus particles. Thus, virus particles are uncoated in Act D-treated cells. The majority of ^{14}C counts in both gradients, although a greater proportion in the gradients into which Act D-treated cell lysates were centrifuged, was located in a region (fractions 24 to 26) of density of 1.298 g/ml or less near the top of the gradients. Approximately 47 and 41% of the total counts

TABLE III. NUMBER OF VIRUS-YIELDING CELLS PER CULTURE IN NORMAL AND ACTINOMYCIN D-TREATED (−6 hr) HeLa CELL CULTURES INOCULATED WITH RV14.

Experiment number	Act D	Infected cells per culture ($\times 10^5$)[a]	Virus yield [pfu/ml ($\times 10^6$)]	PFU per cell	Yield (%C)
1	−	2.40	15.0	626	100
	+	2.30	3.0	130	21
2	−	2.25	37.0	1646	100
	+	2.30	6.7	292	18

[a] Total cells per culture for control and Act D-treated cells in experiments 1 and 2 were 1.33, 1.24, 3.03, and 2.88×10^6, respectively.

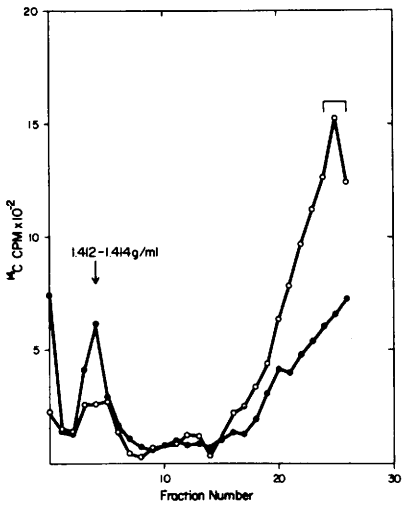

FIG. 4. Isopycnic centrifugation into CsCl gradients of cytoplasmic extracts taken from HeLa cells challenged with purified [^{14}C]uridine-labeled RV14. Materials from cells not exposed to Act D (●) or exposed to Act D 6 hr prior to infection (○).

in this region were recovered as TCA-precipitable RNA from control and Act D-treated cell cultures, respectively. Thus, nearly half of the input viral RNA associated with the low density material remained trichloroacid precipitable. Location of ^{14}C counts in the low density regions (<1.298 g/ml) of these gradients suggested that the RNA must either be attached to membranes or be of such small size that it cannot be banded in these gradients. To provide more information on the low density ^{3}H counts, a second experiment with purified [^{3}H]uridine-labeled RV14 was performed and similar results were obtained. Approximately 2.5-fold more ^{3}H counts in RNA pelleted in the tube into which control cell lysate was centrifuged in comparison with ^{3}H counts in RNA pelleted in the tube into which Act D-treated cell lysate was added. Counts banding in the region of intact virus particles represented 14 and 11% of total counts in control and Act D-treated cell cultures, respectively. Again, the majority of ^{3}H counts in both gradients was at a density of 1.290 g/ml or less and approximately one-half of the counts in each gradient were precipitable by TCA. Twice as many ^{3}H counts in this material were present in Act D-treated cell lysate material as in control cell lysate material. Aliquots of the ^{3}H-labeled materials banding at a density of 1.290 g/ml or less were mixed with deoxycholate to 1% and dialyzed against sterile 0.3 *M* STE buffer (0.03 *M* NaCl, 0.01 *M* Tris, pH 7.4, and 0.001 *M* EDTA) at 4° for 18 hr. The labeled materials were centrifuged into linear 5 to 20% sucrose gradients and assayed for TCA-precipitable ^{3}H counts (6). Approximately 90% (data not shown) of the total TCA-precipitable ^{3}H counts (88 and 91% in control and Act D-treated cell cultures, respectively) had sedimentation coefficients of 6S or less and there were no discernable peaks of viral RNA in either gradient. Thus, the low density counts which originated as virion RNA represent small fragments of viral RNA.

Synthesis of RV14 RNA in cell cultures incubated with Act D prior to challenge. Cell cultures were incubated with Act D (5 μg/ml) for 6 hr prior to inoculation with 20 pfu/cell and a second set of cell cultures were inoculated with a virus inoculum containing 20 pfu/cell and Act D at 5 μg/ml. Two hours were permitted for adsorption of virus at 34.5°, the cell cultures were washed twice with MEM, and VGM was added. Uninoculated cell controls receiving Act D at −6 hr or at zero time relative one to time of inoculation of virus were included in these experiments. At 1-hr intervals, beginning at 3 hr pi, duplicate cell cultures of each type were incubated for 1 hr with 1 μCi/ml of [^{3}H]uridine and the cells were extracted for RNA with SDS and phenol. Trichloroacetic acid-insoluble counts per minute in total and ribonuclease-resistant RNA were measured in uninoculated cell control and in RV14-inoculated cell cultures, and counts per minute in ribonuclease-sensitive RNA were determined by the difference in counts per minute of total minus ribonuclease-resistant RNA. All curves presented in Fig. 5 were corrected for incorporation of counts per minute into ribonuclease-sensitive and ribonuclease-resistant RNA by subtraction of uninoculated cell values from virus-inoculated cell cultures at each time point. Viral RNA synthesis was detected between 5 and 6 hr pi in cells treated with Act D at zero time and 2 hr later, and between 7 and 8 hr pi in cells incubated with Act D for 6 hr

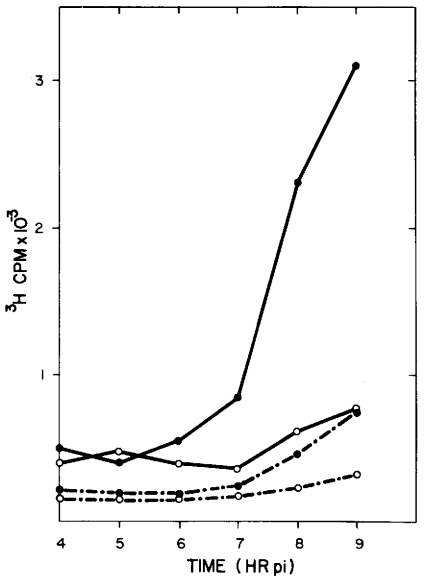

FIG. 5. Synthesis of acid-insoluble RNA in HeLa cells challenged with RV14 and incubated with Act D. RNA synthesized in infected cells incubated with Act D from 0 hr (●) or infected cells incubated with Act D for 6 hr prior to challenge with RV14 (○). RNase-sensitive RNA (——) or RNase-resistant RNA (●–●–).

prior to inoculation with RV14. RNase-resistant viral RNA accounted for 19 and 28% of the total RNA in cells incubated with Act D at 0 and −6 hr pi, respectively. In cells incubated with Act D for 6 hr prior to challenge, synthesis of RV14 RNA was inhibited by 72% compared to infected cells incubated with Act D from time zero. These data show that an increase in time of incubation of cells with Act D results in greater inhibition of synthesis of both ribonuclease-sensitive and ribonuclease-resistant RNA.

Discussion. Act D did not inhibit attachment, penetration, or uncoating of RV14 in HeLa cells. The process(es) affected by Act D is required for continued synthesis and/or maturation of virion components. The data are compatible with the hypothesis that the Act D-sensitive process(es) required for replication of RV14 is a host cell process for the following reasons: (i) the period of time over which the Act D-sensitive process occurs in infected cells is from 2 to 7 hr pi, a span of time that includes both latent and exponential phases of the growth curve (13); (ii) extension of the time of treatment of cells with Act D and subsequent removal of unbound Act D by washing prior to inoculation with RV14 resulted in increasingly greater reductions in yields of RV14 and this could be attributed to a reduction in yield of virus per a constant number of virus-producing cells; (iii) insulin was without appreciable effect on replication of RV14 but did reverse the inhibitory effect of Act D on replication of RV14; (iv) replication of RV14 was far more sensitive to Act D than mengovirus under conditions of pretreatment of HeLa cells prior to inoculation with these viruses.

The basic mechanisms of replication of both picornaviruses have been found to be similar in all aspects examined independently thus far (2, 7). Replication of rhinovirus RNA occurs via replicative intermediate (RI) structures which possess both single- and double-stranded regions (6, 14). Rapid turnover of RI structures occurs during the exponential phase of viral RNA synthesis and at least 90% of radiolabeled uridine is incorporated into single-stranded genomic RNA during 1-hr pulses in this period in RV14-infected KB (6) or HeLa (Gauntt, unpublished data) cells treated with Act D at zero time. Although recent evidence shows that Act D enhances synthesis of replicative form double-stranded RNA in rhinovirus type 2-infected HeLa cells (15), the data herein show that synthesis of both single- and double-stranded RV14 RNA was inhibited severely, but proportionally to the same extent. A 2-hr delay in synthesis of viral RNA was effected by treatment of cells with Act D for 6 hr prior to inoculation with virus. As greater than 90% of double-stranded viral RNA is associated with RI structures in KB (6) and HeLa (Gauntt, unpublished data) cells, the results presented in this paper suggest that the Act D-induced reduction in yields of RV14 is in part due to an inhibition of synthesis of viral RI structures for which a cell process is required. This cell process is only minimally or not at all required by menogivurs in HeLa cells. Instability of rhinovirus RNA polymerase(s) (16, 17) may be the result of lability of a host factor whose synthesis is reduced in the presence of Act D.

Summary. Act D inhibited replication of

RV14 but not mengovirus in HeLa cells. Early processes such as attachment, penetration, and uncoating of RV14 were not affected by Act D. Pretreatment of cells with Act D and removal of the antibiotic prior to infection increased the antiviral effect of Act D on replication of RV14. Yields of RV14 per cell were reduced in Act D-pretreated cell cultures but the number of virus-producing cells was not reduced. Cells treated with Act D for 6 hr prior to inoculation of RV14 synthesized only 28% of the ribonuclease-sensitive and ribonuclease-resistant RV14 RNA which was synthesized by cells treated with Act D at the time of virus inoculation. As most ribonuclease-resistant RNA is associated with viral replicative-intermediate RNA structures in these cells, the results are compatible with the hypothesis that a cell process is required for synthesis of viral replicative-intermediate RNA and it is the cell process with which Act D interferes and contributes to the reduction in RV14 yields.

I thank David R. L. LaBadie for conscientious and excellent technical assistance. This study was funded in part by an American Heart Association, Texas Affiliate, Inc. grant and by an Institutional Research grant from the University of Texas Health Science Center at San Antonio. I thank Kendall O. Smith and Stephen J. Mattingly for constructive criticisms of the manuscript.

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Received September 7, 1976. P.S.E.B.M. 1977, Vol. 155.