I. TRANSFER OF DNA FROM PARENT TO PROGENY CELL

II. A STUDY OF THE POSSIBLE ROLE OF DNA IN VIRAL RNA SYNTHESIS DURING THE MULTIPLICATION OF AN ANIMAL VIRUS

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PREFACE

The original purpose of this work was to determine whether interaction with cellular DNA was a prerequisite to the replication of the RNA of RNA-containing animal viruses. In the course of these investigations, it became desirable to incorporate the thymine analog 5-bromouracil into the DNA of the host cells. After we had shown that this could be accomplished readily, Dr. Matthew Meselson suggested that the system be used to perform a DNA transfer experiment. We decided to follow up the suggestion, and the work was carried on simultaneously with the original virus investigation. Both projects led to firm conclusions.

To simplify the presentation of the results, this thesis has been divided into two parts, dealing with the DNA transfer experiment, and the DNA-RNA interaction work respectively. Cross references between the parts are given in terms of section (Introduction, Methods, Results, Discussion) and where applicable, subdivision within the section.

ABSTRACT

This work considered two approaches to the general problem of information transfer between molecules.

The first approach dealt with one aspect of the mechanism of deoxyribosenucleic acid (DNA) reproduction. In 1953 Watson and Crick proposed a structure of DNA which more recent work has essentially confirmed. The key postulate of the Watson-Crick structure is that DNA consists of two complementary chains of which either one may serve as a template for the formation of the other. This structure suggested three general models for DNA reproduction. In this work a test was devised to distinguish among these models by labeling cellular DNA with the thymine analog 5-bromouracil for one and/or two cell divisions. The results indicate that the semi-conservative model for DNA replication originally proposed by Watson and Crick is valid for mammalian DNA.

The second approach considers the problem of whether in RNA-containing animal viruses the RNA is able to reproduce itself without interaction with cellular DNA. Various methods were used which either prevented the synthesis of DNA or led to the synthesis of abnormal DNA during the growth of the RNA-containing virus. These experiments led to the conclusion that viral RNA is able to multiply without interaction with newly synthesized DNA and probably without interacting with pre-existing cellular DNA.

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PART I

TRANSFER OF DNA FROM PARENT TO PROGENY CELL

INTRODUCTION

Starting with the suggestion by Muller, in 1926, that genes might duplicate autocatalytically, increasing attention has been given to the problem of their chemical nature. Several lines of evidence have lead to the conclusion that the key component of the gene is deoxyribose nucleic acid (DNA) (1). The first evidence suggested that DNA was associated with the gene: 60% of the dry mass of a sperm head is DNA (2); within somatic cells, DNA is confined to the chromosomes, and within the polytene chromosomes of Drosophila to the bands (3) which have been shown by cyto-genetic analysis to contain the genes (4). Similarly, even as the number of chromosomes is constant in all cells of an organism (5), so is their cellular DNA content, and in the cases when the number of chromosomes in a cell increases (polyploidy) or decreases (aneuploidy) its DNA varies proportionally (5).

The discovery that transformation is mediated by DNA is more direct evidence of the genetic importance of DNA (6). When cells of certain strains of bacteria are exposed to pure DNA of related strains, the recipient cell and its descendants can permanently acquire certain genotypic characters of the donor. In addition, the DNA of the transformed strain can pass the acquired characteristic on to still other strains (7). Lerman and Tolmach (8) have shown

that the donated DNA enters the recipient cell, and furthermore, that those bacterial strains which do not show transformation incorporate little, if any, DNA. Therefore, the unique property of transformable species lies in their permeability characteristics, rather than properties of their DNA or chromosomes.

Another direct line of evidence for the genetic function of DNA is the demonstration that reproduction of bacteriophage involves virtually complete separation of DNA from protein, only the DNA being required for production of progeny phage (9). Furthermore, Hershey and Melchen (10) have shown that, after a short lag period, phage DNA can reproduce in the absence of protein synthesis, and that this DNA is later incorporated into viable phage particles. Finally, there is the demonstration by Garen and Skaar (11), that the bacterial chromosome transferred from donor to recipient strain (12) contains DNA, and that as the genes enter, a comparable amount of DNA also enters the cell.

In the course of the reported investigations, it became clear that DNA must be able to direct its own duplication, a vital property of any hereditary material. Watson and Crick in 1953 (13), suggested a structure for DNA. Their key postulate is that DNA is a plectonemic double helix made up of two complementary chains, each consisting of nitrogenous bases connected by a sugar-phosphate backbone. This postulate has remained virtually unchallenged, although the

details of the structure have been refined in several ways (14,15). The complementarity is introduced by the selection rule that adenine pairs with, and only with, thymine; and guanine, with, and only with, cytosine. However, analogs of the bases involving substitutions which do not interfere with their hydrogen bonding properties (see Fig. 1) can replace the normal ones. Examples are hydroxymethylcytosine (HMC) and 5-methylcytosine (MC) among the naturally occurring bases, and 5-bromouracil (5BU) and 5-iodouracil (16) among the purely synthetic analogs.

Two objections have been raised against the universality of the complementarity feature of this structure. Bendich and his co-workers (17) have repeatedly pointed out that when they fractionate calf thymus DNA they recover fractions with adenine : thymine, and guanine : cytosine ratios widely different from unity. Although in recent discussions of the subject, this discrepancy from the expected 1:1 ratios has been mentioned, it is generally not considered to be of primary importance (15). A possible explanation is that these fractions represent single stranded DNA, a form known to occur in certain phage (16). A more subtle objection is based on the distribution of the 5 bases which occur in some DNA's. Sinsheimer (15) has shown that the 5-methylcytosine (MC) in calf thymus DNA is distributed differently from the cytosine, a result not explained by the simple complementarity rule which predicts that MC or cytosine would

be equally likely to pair with guanine. This observation implies that some mechanism in addition to complementarity determines the base sequence.

On the basis of their structure for DNA, Watson and Crick suggested a mechanism by which it might duplicate (13). They postulated that the two chains separate, each then serving as a template for the formation of its complement. This replication results in two new helices, each identical to the original. Any mistakes in this copying process, i.e. adenine pairing with cytosine, would constitute a mutation.

Since the Watson-Crick structure was postulated, a number of other mechanisms for DNA duplication have been proposed. Delbrück (19) suggested that separation of the original chains and replication occur simultaneously, and that as each half turn separates the old chains break and the lower terminals of the breaks are joined to the free ends of the new chains; under this mechanism the double helix would be unwound turn by turn and at no time would any appreciable length of the strand have to rotate. Watson (20) suggested that one of the chains of the duplex could be digested away enzymatically before replication. The remaining chain could then serve as a template for the synthesis of one or more other chains not necessarily intertwined with it. These chains would then serve as templates for the synthesis of their own complementary chains for "maturation" into "resting" duplexes. Block (21) has proposed a mechanism

whereby the parental duplex as a whole serves as the template for the production of a daughter duplex. He imagines that the bases of the original chains rotate 180° around their glycosidic bonds, while the backbones are held rigidly in position by their attachment to a histone. The bases now would form a template on which a new duplex structure might form. Several other schemes are outlined by Delbrück and Stent (22).

All of the proposed models may be divided into three general classes (22): conservative, semi-conservative, and dispersive (Fig. 2). Conservative models have the property that the original parental strands remain intact and do not separate. Bloch's scheme is typical of these. Models in which the DNA is duplicated through a protein or RNA intermediary would also fall in this class (22). Semi-conservative models have the property that the two parental strands separate, but each individual strand remains intact. This is the original Watson-Crick hypothesis and the mechanism of duplication suggested most directly by their structure. Dispersive models, of which Delbrück's is typical, have the property that the parental material is more or less equally distributed among the four daughter strands. Any model in which the original DNA is broken down and then reused in the synthesis of daughter duplexes would also fall into this class.

The problem of distinguishing between these models has been vigorously attacked in the past few years by experiments following the transfer of label from parental DNA to

progeny DNA. This work has been done primarily with phage and with bacteria. The phage work has been equivocal (22,23), chiefly because the progeny have not been examined after only one or two divisions of the DNA have taken place. Since the progeny which were examined represented an unknown number of divisions, the observed distribution might have been affected by recombination among the DNA molecules. Although there is reason to believe that this recombination is not fragmenting, i.e. a physical recombination between two strands of DNA is not involved (24,25,26), the mechanism of recombination has not yet been unequivocally determined (22). The most clearcut work in this field has been done by Levinthal, using P^{32} labeled phage and autoradiography. Recently, however (27,28), some doubt has been cast on his discovery, that there is a "big piece" of the phage genome, half of which is conserved after one cycle of growth, but is not reduced further upon a second cycle. First, Stent et al. (29) have shown that if such a big piece exists, it is probably not associated with the phage genes. They infected bacteria with one highly radioactive phage and 10 cold phage differing from the minority parent by four markers. Progeny particles of the genotype of the minority parent, were not subject to radioactive decay at an appreciable rate, as would be expected if the conserved piece contained the phage genes. Secondly, recent work has indicated that the "big piece" itself might be an artifact of P^{32} labeling (30,31).

An experiment designed to determine the distribution of DNA after transfer from parental to daughter nuclei in bacteria was carried out by Fuerst and Stent (32). They compared the rate at which progeny of bacteria originally heavily labeled with P^{32} died after growth for a defined number of divisions in nonradioactive medium. After the first division, the rate of inactivation was lower in a manner consistent with equipartition of radioactive atoms between the daughter nuclei. After further divisions, however, some cells began to appear in the culture which were killed at a rate inconsistent with continued equipartition of the original P^{32}, but completely resistant cells did not appear even after 4 generations. As Stent points out (22), these results are compatible with semiconservative replication, if the absence of P^{32} stable cells is attributed to random segregation of different "chromosomes." They are also compatible with dispersive replication if heterogeneity in growth rate of the population is assumed, thus permitting some cells to dilute out the label faster than others.

Plaut and Mazia (33), using autoradiography with C^{14} label, studied the distribution of DNA between chromosomes in Crepis roots. They found results compatible with conservative duplication. Taylor, on the other hand, working with Vicia and Bellevalia and using H^3 label, finds results which seem to indicate clearly that the mechanism is semiconservative(34). Neither study throws much direct light on the problem of DNA

replication, however, since the structure of the chromosome is virtually unknown (35,36) and by assuming different structures and distribution of DNA molecules, the results can be made to fit any model.

The density gradient technique developed by Meselson, Stahl, and Vinograd (31) permits one to examine directly species of DNA molecules of different density. By labeling the parental DNA so that it will have a density different from that of the progeny DNA, it is possible to distinguish among the three models of replication. Using this technique with bacteria pre-labeled with N^{15} (heàvy nitrogen) and then grown in N^{14} medium, Meselson and Stahl (37) have followed the transfer of label in DNA over the course of many divisions and found that at the first division the label was divided equally between the daughter molecules and that after subsequent divisions a constant number of half-labeled molecules were found. This is the result predicted by the semiconservative model. It is in contradiction to the other two.

In the present work, the density gradient method has been used to investigate the transfer of 5-bromouracil (5BU) from parental DNA to progeny DNA in HeLa cells (human cancer cells). The use of the thymine analog 5BU as the label has the advantage of making the density difference between normal and half-labeled DNA about four times greater than in the original Meselson and Stahl experiments. It will be shown that HeLa DNA (like bacterial DNA) multiplies by a semiconservative mechanism.

MATERIALS AND METHODS

1. Media and Culture Conditions.

The cells used in the following experiments were derived from a clonal line of HeLa S3, stem 1, originally isolated from a human carcinoma of the cervix by Gey (38), and later cloned by Puck (39) who designated it S3, and then recloned by Vogt (40) who designated it stem 1. The cells, under optimum conditions, have a division time of 17-20 hours.

Cells were grown in 50 mm petri dishes using 5 ml of modified Eagle's medium (41), as described in Temin and Rubin (42), with 15% horse and 15% human serum. Stock lines of cells were routinely transferred by trypsinization (40) every 3-4 days and new stock plates seeded with 2-3 x 10^5 cells. Cultures were kept in a humidified (98%) incubator at 37-38°C. The CO_2 concentration in the incubator was automatically regulated to maintain a pH within the medium of about 7.2. Media changes were usually made every two days, the entire volume being replaced.

2. Labeling Procedure.

Healthy cultures containing 1-3 x 10^6 cells/petri dish were trypsinized for 10 minutes, pelleted, diluted with TD .05M tris buffer in isotonic sodium and potassium chloride and counted in a hemocytometer. 5 x 10^4 - 3 x 10^5 cells were then transferred into 5 ml of medium supplemented with a suitable amount of 5-bromodeoxyuridine (BDU). In some cases,

aminopterin was also added to the medium to eliminate endogenous thymidylic acid formation (Thesis: part II, sec DII-1). BDU was obtained from Cyclo Chemical Co. of Los Angeles, and aminopterin through the courtesy of the Lederle Research Laboratories, Pearl River, New York.

3. Cell Growth Studies.

Growth rates under different conditions were determined by the following method. Two circles, about 5 mm in diameter were drawn on the bottom of the petri dish and then 5×10^4 - 1.5×10^5 cells in 5 ml of medium were placed on the plate. The cells within the circles were counted using the 50X power of an inverted microscope 12-24 hours after plating and on successive days thereafter. A four- to six-fold increase of the cells could usually be followed. In general, two plates were used for each determination, the counts from both circles on each plate being pooled. This method does not disturb the cells during the counting process and at low cell concentration is probably more accurate than trypsinization followed by hemocytometer counts. Working at low cell densities minimized overcrowding and the consequent slowing down of cell multiplication.

4. Preparation of DNA.

Three methods of preparing DNA were used during this work. The density distribution of the DNA was found to be independent of the method of isolation. In all methods

2-30 x 10^6 cells were removed by trypsinization, centrifuged, and the supernatant discarded.

In the first, or duponol method, as modified from Simmons et al. (43), the cells were suspended in 1-2 ml 0.9% NaCl - 0.01M Na citrate, ground with 20 strokes in an iced TenBrock glass grinder and centrifuged for 30 minutes at 4°C in a clinical centrifuge at 2,000 rpm. This procedure was repeated 3 times, using the sediment (containing the isolated nuclei) each time. Soluble RNA and microsomes are lost in the supernatant. The sediment was then taken up in 8 ml of a 0.9% saline, 10^{-3} versene solution, ground once more, and then enough duponol (sodium lauryl sulfate) added to make a final concentration of 0.45%. The resulting gel was stirred for 3 hours at room temperature (duponol precipitates in the cold), the NaCl concentration brought to 1M, and the mixture centrifuged at 13,000 rpm for 30 minutes in a Spinco preparative centrifuge. The DNA was precipitated by adding two volumes of cold ethanol and the precipitate redissolved in 0.9% NaCl + 10^{-3}M versene.

The second, or direct, method has the advantages of speed, efficiency, gentleness of treatment, and economy of material. Cells were removed and ground once in the TenBrock as previously described. The mixture was spun at 4°C in a Serval centrifuge, brought to 10,000 rpm and then allowed to stop without braking. The sediment was suspended in 0.1-0.3 ml citrate saline, and a suitable aliquot placed in

a 0.45% duponol solution in concentrated CsCl for subsequent running in the analytical ultracentrifuge (see below). Later work demonstrated that grinding may be replaced by freezing and thawing the cells 3 times by placing them in a -60°C deep freeze and thawing in a 37° water bath and that it was not necessary to add duponol to the centrifuge cell. By either variation of the direct method it was possible to have the preparation in the ultracentrifuge within 1-1/2 hours after removing the cells from the plates. Therefore, there was little chance for DNAase to act since cells were either kept below 4° C or in concentrated CsCl the entire time, or for artifacts of preparation to occur. The disadvantage is that little material is removed, and as a result there is considerable UV absorbing material other than DNA in the centrifuge cell. This makes accurate density measurements (see below) difficult.

This difficulty was eliminated by the third, or phenol, method which confirmed results obtained by the direct method. The phenol extraction was modified from Kirby (44). Ten to thirty million cells were removed as above and immediately suspended in 1.5 ml fresh neutral (pH 6.8-7.2) trichloro-acetate and 1.5 ml purified phenol. The cells were ground with a mechanically driven Teflon grinder, transferred to a centrifuge tube, and spun at 10,000 rpm for one minute at 4°C in the Serval centrifuge. The aqueous phase was removed, an equal volume of phenol added, the mixture shaken by hand

for one minute and centrifuged as above. The aqueous phase was extracted several times with ether, and the nucleic acid precipitated with two volumes of cold ethanol. The precipitate was dissolved in 1 ml 0.04M NaCl and reprecipitated with cold ethanol. After redissolving, the absorption spectrum was determined and a suitable aliquot run in the ultracentrifuge. Yields were of the order of 13-18 gamma nucleic acid/million cells. By precipitating quickly, i.e. saving only the material which came down in the first 30 seconds, a partial separation of the DNA from the RNA was achieved. This method has the advantage of yielding nearly pure, undegraded nucleic acid, the rapid treatment of the cells with phenol effectively stopping DNAase action. It has the disadvantage of requiring more cells than the direct method.

4. Measurement of Amount of Incorporation of 5BU into DNA.

The amount of 5BU incorporation into DNA was evaluated by determining the density of a sample of DNA using the density gradient centrifugation method developed by Meselson (45) and Meselson, Stahl and Vinograd (31).

From 2-5 gamma of DNA were placed in 1 ml of purified CsCl (optical density at 260 less than 0.1). The density of the solution was adjusted to about 1.75, and 0.7 ml of this solution was placed in the ultracentrifuge cell and centrifuged for about 15 hours at 44,770 rpm. Pictures were taken every 2 hours to show the approach of the bands of DNA to the final position. Meselson (46) points out that 15 hours is probably

not sufficient to bring the band to its true equilibrium position. However, pictures taken every hour showing the approach of the bands to equilibrium indicate that there is no detectable change in position between 12 and 14 hours, and that therefore the approximation is sufficient for our purposes.

The density of a band may be calculated in two ways. A fair approximation can be obtained from the initial density of the CsCl solution (ρ_o) and the distance of the band from the center of the fluid column. ρ_o may be assumed to be the density of the solution at the center of the fluid column (47). Since the distance of the band from this point may be measured, and the change of density with distance within the cell is known (31), the density of the band can be computed. The accuracy of this method depends on how accurately ρ_o is known and how accurately the band center can be determined, which depends on the density distribution of the material. Using this method, it was shown that normal HeLa DNA has a density of approximately 1.700. A more convenient method is to run a marker DNA of given density with each sample. The resulting picture may then be traced with a densitometer (the Joyce Loebl Double Recording Beam Microdensitometer was used), and the distance between peaks measured directly. Since the necessary conversion factors are known (45) this distance may be translated into a density difference. In the experiments described here, normal HeLa DNA is taken

to have a density of 1.700, and other densities given relative to this standard. One unit is defined as equal to 1 mm on the trace and approximately equals $2/3 \times 10^{-3}$ density units. The results will be given in terms of this parameter, assuming normal DNA to be at zero units.

RESULTS

Under the standard conditions used in these experiments the number of HeLa cells doubles every 17-24 hours. This value is comparable to that found in other laboratories (48). Vogt (40), using microdrops, has shown that no division takes place for at least the first 12 hours after plating, although most of the cells become attached to the glass. Therefore, the "zero" count of normal cells was made 12 hours after plating.

1. Normal Cells.

DNA was prepared from normal, exponentially growing cultures by each of the three methods, and banded in the density gradient. Figures 3, 4, and 6 (left band only) show photometer tracings of the resulting band. They are all identical and decidely skewed towards the heavy side. A similar result is found with DNA isolated from calf thymus (31).

2. Cells One Division in BDU.

Two methods were used to demonstrate the effect of one division in BDU on cellular DNA.

In the first, cells were plated at a concentration of 1.5×10^5 per plate into BDU containing medium. In early experiments 20 gamma/ml was used, later experiments employed 12 gamma. Both types gave similar results. Counts made 12 and 24 hours after plating show that both control and treated

cells had approximately doubled in that interval (Table 1). The cells were immediately removed for assay of incorporation by the direct method. Figure 5 shows a photometer trace of an experiment of this type. Its key features are common to DNA from cells which have divided once in the presence of BDU and independent of mode of isolation of DNA. First, there is no trace of any heavier or lighter band. (The "band" 85 units from the DNA is an artifact cf: Fig. 6). Second, the band is relatively symmetrical and sharp compared to the normal one. Figure 6 is the same preparation with marker DNA added and permits ready comparison of the differences between the two bands. The density of the substituted band varies over a narrow range from one experiment to another (48-57 units). The origin of this variation is not known; however, it might be a function of the amount of thymine incorporated into DNA either because of thymine or its derivatives present in the serum in the growth medium or because of incomplete inhibition of thymidylic acid synthesis by BDU. Both factors may lead to variable incorporation in different experiments. The amount of thymine normally occurring in horse or human serum is not known (49) but as little as 5 gamma/ml would probably be enough to significantly depress incorporation of BDU (50).

The second method used the observation that cells in the presence of the folic acid analog aminopterin and BDU (A+B) appear to divide only once (Thesis: part II, sec R4). Cells in the presence of aminopterin alone do not divide (Thesis: part II, sec R1). Aminopterin among other things prevents the

TABLE 1

Growth for 36 hours in 20γ/ml BDU

Hours after plating	Experiment A		Experiment B	
	12	36	12	36
Control	77*	175	155*	332
	195	369	135	286
	58	107	118	229
Total	330	651	408	847
BDU	130	267	162	390
	127	230	129	294
	120	298	86	155
Total	377	795	377	839

*Number of cells/2 circles/plate. Cells were plated into normal medium and medium containing 20γ/ml BDU. First count made 12 hours, second count 36 hours after plating.

transfer of the methyl group to deoxyuridine (51), this block effectively eliminates thymidylic acid synthesis in the HeLa cells (Thesis: part II, sec DII-1). In an attempt to limit the action of aminopterin to blocking thymidylic acid synthesis, adenosine and glycine were added to the medium (52). Under these conditions, maximal BDU incorporation is expected to occur. A total of 4×10^5 cells were placed into each of several plates containing 5 gamma aminopterin/ml, 20 gamma BDU/ml, 40 gamma adenosine/ml, and 40 gamma glycine/ml. For counting purposes 1×10^5 cells were plated into an identical medium. DNA was prepared by direct method from cells of first group 4 days after plating, at which time control cells had approximately doubled (Table 2)(see Thesis: part II, sec R4 for further data on the apparent doubling of A+B cells). A single, sharp band of density 1.735 was found. This indicates that one division in BDU alone suffices for approximately maximum incorporation.

3. Cells Two Divisions in BDU.

Preliminary experiments indicated that whereas the first division in 20 gamma BDU/ml occurs within a short, well defined time interval (about 36 hours after plating) it takes several days for the number of cells to double again. With such asynchrony in growth, it was impossible to attain a population of cells all of which had divided exactly twice in the presence of BDU. Therefore a time was found during which an appreciable

TABLE 2

Growth of Cells in Aminopterin + BDU

Days after plating	1	2	3	4	5	6
	138*	238	-	300	-	287
	126	189	-	270	-	263
Total	264			579		

*Number of cells/2 circles/plate. Cells were plated in usual manner into medium containing 5γ/ml aminopterin, 20γ/ml BDU, 40γ/ml adenosine, and 40γ/ml glycine. First count made about 15 hours after plating.

fraction of the cells had divided twice but none could have divided a third time. Fifty to fifty-five hours proved to be adequate for this purpose. At this time, no normal DNA was ever present. It is crucial for the interpretation of these results to know precisely the relative densities of the bands appearing at these times. In some of the experiments described below, an aliquot of control cells (grown at the same time) were added to the test cells and the isolation of DNA carried out on the pooled cells. The results were the same as when independently isolated DNA was added as a marker.

The major difficulty in interpreting this experiment was the presence of UV-absorbing material near the heavy end of the ultracentrifuge cell which obscured the position of any band in this region. Figure 7 shows that differential absorption of the CsCl itself was approximately linear. More important was the presence of RNA in DNA preparations made by the direct method, and possibly fragments of DNA from <u>in vivo</u> degradation of half heavy DNA. (The base line was generally lower for cells isolated at 36 hours than for cells isolated at 50 hours). The first factor could be eliminated by isolating the DNA via the phenol method; the second, by taking the sample as soon after the first division as practical.

Figures 8, 9, and 10 show photometry traces of DNA bands formed after two divisions from cells prepared by three different methods. Normal DNA was added as a marker in each

case. The DNA of Fig. 8 was isolated by the phenol method from cells grown 55 hours in the presence of 20 gamma/ml BDU. A preliminary run (Fig. 8) in the ultracentrifuge showed that no normal material was present, then 3 gamma of normal DNA was added as a marker (Fig. 8). Figure 9 shows DNA from cells grown 51 hours in the presence of 12 gamma/ml BDU. The cells were fluid changed 24 hours after plating with extreme care being taken to keep them at 37° to reduce to a minimum any disturbance of their metabolism. The DNA was isolated by the direct method with about 3 gamma of normal HeLa DNA added as a marker. This picture is superior to most obtained by this method as regards the small slope of the base line, cf: Fig. 7 (CsCl base line). The difference in distance between normal and half substituted DNA in Figures 8 and 9 are not caused by the different amounts of BDU used, since DNA from cells grown in 8 gamma/ml BDU under the same conditions had a density of 66 units. The cells of Fig. 10 were similarly grown for 51 hours in the presence of 12 gamma/ml BDU and fluid changed at 24 hours. The cells were mixed with an aliquot of normal cells grown at the same time at a ratio of 4:1 and the DNA isolated by the phenol method. Seven gamma of the yield was run in the ultracentrifuge. The broken line shows the same run after absorption due to the CsCl base line is subtracted.

DISCUSSION

Figure 2, showing three general models for duplication of DNA, indicates that each of these models predicts a specific distribution of parental DNA among the progeny. The conservative model implies that after the first division two types of DNA will be found, parental and new. The other two models predict that only "hybrid" DNA should be present. Figure 5 shows that after one division in BDU only new DNA is found. Since the conservative model predicts equal quantities of each type, it is unequivocally excluded.

It remains to distinguish between the semi-conservative and dispersive models. Figure 2 shows that after the second division the semi-conservative model predicts two bands, one hybrid and one fully substituted, while the dispersive one predicts a single 3/4 substituted band. Cultures containing only cells which had divided twice in the presence of BDU could not be obtained, as discussed in the section on results; therefore, the dispersive model cannot be excluded on the basis of the presence of two substituted bands as it could by Meselson and Stahl (37). It can, however, be excluded for other reasons. To show this, we must consider the past history of the heavy DNA, found from cells kept 51 hours in BDU.

The first possibility is that this DNA results from two divisions in BDU. Under the semi-conservative mechanism the band should have a density corresponding to 106 units or twice

the density of the hybrid band (Fig. 12). Under the dispersive model the density should be 80 units larger than normal (53 + 53/2). The density of the observed band is 100 units.* The slight discrepancy between the observed and expected value under the semi-conservative model, can be explained by selection of DNA molecules of lower incorporation at the second division as discussed later. The much greater discrepancy between the predicted value under the dispersive model and the observed one would have to be explained by a very strong selection of heavily incorporated molecules. In view of the demonstrated detrimental effect of incorporation of 5BU into DNA (Thesis: part II, sec R3), such an interpretation appears extremely unlikely.

The second possibility is that the DNA of the heavy band has divided more than twice. We proceed to exclude it: The occurrence of more than three divisions can be eliminated on the a priori grounds that insufficient time has elapsed between plating and harvesting the cells. The possibility of three divisions is ruled out as follows: As already reported, the DNA of cells collected 36 hours after plating gives rise to a single band. We interpret this to mean that all, or a large proportion of the molecules have divided once and only once;

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*The effect of the non-linearity of the CsCl gradient, and the compression of the CsCl solution on the relative positions of the bands was considered and found to be negligible. Both corrections would tend to equalize the distances between the bands.

a natural conclusion of a semi-conservative hypothesis. This single band is not a consequence of two dispersive divisions; if it were, the half substituted material would have a density of 35.3, and no material heavier than 71 units could be found.* Therefore, if the heavy DNA had undergone three divisions, two of them should have occurred between 36 and 51 hours: but this is impossible, since normal cells under the best of conditions have a division time of 17 hours. The conclusion that three divisions could not have occurred, is strengthened by the fact that no trace of a band between the hybrid and heavy ones was seen in any of the experiments with DNA from cells taken 48-55 hours after plating. Such a band would have been caused by DNA which had replicated twice. Multiple bands would be expected because of the asynchrony of the culture. These findings essentially rule out the dispersive model for DNA replication.

The relative lightness of the fully substituted band should be discussed further. We have assumed that the difference is due to selection of special molecules. They can arise by two methods. One is the presence of cells in the culture able to restrict incorporation of BDU, which would probably be the ones most likely to divide first; the other is heterogeneity in 5BU incorporation among molecules within

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*These numbers are derived as follows: 35.3 + 35.3/2 = 53, the observed density after the postulated two divisions; 71 = 2 x 35.3, or twice the half-substituted material.

individual cells. This is possibly related to their guanine-cytosine content (see below). The selection hypothesis is based on the observation that DNA synthesized in the presence of aminopterin and BDU and therefore maximally substituted is incapable of further multiplication (Thesis: part II, sec R1). In the absence of aminopterin, submaximal levels of incorporation are possible and heavily substituted molecules would tend to be selected against.

The presence of a distribution of guanine-cytosine content of DNA molecules is suggested by the striking asymmetry of the band formed by normal HeLa DNA. The band has a pronounced heavy tail, and this indicates that not all its molecules are of equal density. Rolfe and Meselson (53) have shown that the density of DNA increases with increasing guanine to adenine ratio. Accordingly, the heavy tail may consist of molecules rich in cytosine and poor in thymine.

The hybrid band, on the contrary, is nearly symmetric. This finding would be a consequence of the assumed guanine-cytosine distribution, since the molecules of the tail, which are rich in cytosine, can incorporate less 5BU than the others and therefore have a smaller increment in density. This difference in incorporation would tend to offset the original difference in density and reduce the tail in the half substituted band. After a second division in BDU, these molecules would be lighter than in the majority; if they tend to be the first molecules to divide, this would explain the observed

density of the heavy band.

It is necessary to point out, as did Meselson and Stahl (37), that the observed distribution of DNA between parent and progeny does not prove that the Watson-Crick model of duplication is correct. It is possible that the basic unit of DNA is two strands of DNA so attached to each other that they behave as one unit in the centrifuge, but as two in X-ray diffraction analysis. In other words, the conserved unit which is observed may not be a single strand but a whole molecule of DNA. On the other hand, the molecule may divide medially rather than lengthwise, and the upper and lower segments be conserved throughout future generations. Figure 12 shows these schemes.

One method by which these alternatives may be excluded is the following: If 5BU substituted DNA hydrolyses more readily than normal DNA (as some preliminary results seem to indicate), it may be possible to remove selectively the substituted chain of the hybrid. Then if the Watson-Crick model is correct, the remaining chain would be denatured and therefore have a higher density than normal DNA (37); if on the contrary the conserved unit is a double helix, the remaining chain would be of normal density.

PART II

A STUDY OF THE POSSIBLE ROLE OF DNA IN VIRAL RNA SYNTHESIS DURING THE MULTIPLICATION OF AN ANIMAL VIRUS

INTRODUCTION

This paper will consider the mechanism of replication of viral ribosenucleic acid (RNA) during the multiplication of RNA-containing animal viruses, and in particular whether interaction with DNA is required for this multiplication. This problem arises from a consideration of the quasi-universal role of DNA as a self-duplicating genetic material. One might suspect that direct interaction with deoxyribosenucleic acid (DNA) is necessary for viral RNA synthesis, since on the one hand there is considerable evidence (see Thesis: part I, sec 1 for detailed discussion) that except for the RNA-containing viruses, DNA is the genetic material of all organisms; and on the other hand Kornberg et al. (54,55) have shown that specific DNA can be produced in vitro from a DNA template, without RNA. In addition, the results of transfer experiments in bacterial (37) and human cells (Thesis: part I) indicate that no non-DNA-containing intermediate is involved in DNA synthesis.

In contrast to the autonomy of DNA replication (as regards high molecular intermediates) there is no evidence in non-viral systems that RNA can reproduce itself. Reports to the contrary by Plaut (57) and Brachet (58) have been refuted. Prescott (59) has shown that the net RNA synthesis in enucleated Amoeba reported by Plaut (57) was caused by bacteria

growing in the vacuoles. Brachet's early claims of RNA synthesis in enucleated Acetabularia (58) were refuted by Richter (60) while working in Brachet's own laboratory. Furthermore, Borsook (61) working with reticulocytes (which contain no nuclei) and Goldstein (62) with enucleated human amnion cells have shown that microsomes do not multiply in these anucleate cells.

This negative evidence suggests that in cells the RNA species, whose origin has been investigated so far, are synthesized on a DNA template. Other evidence shows that RNA is intimately associated with protein synthesis (63,64). For these reasons, the concept has developed that the major direction of information transfer in a cell is DNA → RNA → protein. In such a scheme, the DNA serves to transmit the genetic information from one generation to another. This information is transferred to microsomal RNA which then serves as a template for the formation of enzymatic and structural protein (64,65).

The existence of plant and animal viruses which contain RNA but no DNA throws doubt on the generality of this scheme. Gierer and Schramm (66) have been able to isolate RNA from Tobacco Mosaic Virus which can by itself initiate the formation of new virus particles. Recent work with poliovirus (67, 68,69) murine encephalomyocarditis virus (70) and western equine encephalomyelitis (71) has demonstrated that infectious RNA may also be isolated from animal viruses. This work demonstrates that RNA is the hereditary material of these

viruses. It does not exclude a possible role of new or pre-existing DNA in its synthesis. For example, the viral RNA may act as a template for the synthesis of a DNA intermediate which would multiply exponentially and finally synthesize new RNA molecules. Such a mechanism would preserve the position of DNA as the only macromolecule capable of reproducing itself from low molecular intermediates.

The mechanism of the reproduction of RNA viruses is of interest for several reasons. If viral RNA could reproduce itself and transfer its information into a form usable by the cell, there would be no reason to assume that some species of cellular RNA could not do the same. This consideration raises the possibility that an independent hereditary system could exist in cells in addition to the one controlled by DNA. Also, if RNA could reproduce itself, the question of why DNA is the genetic material of almost all organisms must be considered.

In this thesis it will be demonstrated that viral RNA can reproduce itself in the absence of any DNA synthesis, and in addition, it will be shown that the presence of pre-existing DNA is probably not needed for the production of new RNA. This work employed the RNA viruses poliovirus (72) and Newcastle Disease Virus (NDV) (73) and the DNA virus vaccinia (74) grown in human cells in tissue culture. Two major approaches were used in this work. In the first approach,

analogs of folic acid and of thymine were used to prevent the cell from synthesizing any new DNA during virus growth. In the second approach, 5-bromodeoxyuridine (BDU) was used to make the cell produce only abnormal (5-bromouracil substituted) DNA, which is known to be highly mutagenic for phage (89). The rationale of this approach was that if RNA → DNA → RNA is the proper mechanism for RNA synthesis, such abnormal DNA would turn out abnormal RNA. This in turn might be detected by a decrease in active virus yield due to lethal mutation, or by an increase in the rate of some recognizable non-lethal mutation.

MATERIALS AND METHODS

1. Cell Growth Studies

A description of the cell line employed, the methods of culture and transfer, media, and the means of determining growth rate have already been given (Thesis: part I, sec M).

2. Analogs

The following analogs were used in the experiments: thymine analogs including 5-bromodeoxyuridine (BDU), obtained from Cyclo Chemical Co. of Los Angeles; 5-bromouracil, obtained from the California Corporation for Biochemical Research of Los Angeles; and 5-fluorouracil, supplied through the courtesy of Hoffman LaRoche Inc., Nutley, New Jersey; and the folic acid analog, aminopterin (4-aminopteroylglutamic acid), supplied through the courtesy of the Lederle Medical Research Department of American Cyanimid Co.

3. Virus Stocks

The poliovirus stock used was derived from Type 1, Brunhilde (SC - 1949 pool) virus. The virus has been passed through about 20 monkey kidney tissue culture passages in this laboratory. The virus stocks were supernatant fluids from infected monolayer kidney cultures.

The L-Kansas 1948 strain of Newcastle Disease Virus (NDV) used was originally obtained from Dr. C. A. Brandly of the

University of Wisconsin and has since been kept in this laboratory by passage through chick fibroblast and lung cultures. The virus used in these experiments was isolated from a single plaque on chick fibroblasts and a stock grown on chick lung cells (73).

Several vaccinia virus strains were used in these experiments. Since all gave the same results, the specific strain used will not be specified. The earliest experiments used vaccinia virus W.R. obtained from the American Type Culture Foundation. Dr. Frank Fenner kindly supplied a stock of his hemorrhagic (red) pock forming R.P.U. strain (75) and of a white pock forming variant derived from it. Stock cultures of all strains were grown in eggs. The chorioallantoic membrane (CAM) of each egg was infected (see assay method) with 10^4-10^5 pock forming particles, the membrane was removed 2 days after infection, and ground as described by Fenner (75). The membranes were frozen at -20°C in a small mortar, ground at room temperature until they thawed, refrozen, reground, suspended in 1 ml TD per membrane, clarified by a low speed centrifugation, and stored in the deep freeze.

4. Infection and Sampling Procedures

All experiments were carried out by infecting, on the plate, cultures of 5×10^5 to 3×10^6 HeLa cells grown in 60 mm (small) petri dishes. Except for adsorption time, identical procedures were used for all viruses. Plates were washed

once with 2 ml of TD; then 0.2 ml of a stock virus diluted in TD was placed on the cells. Input multiplicity was adjusted to 3 to 5 plaque forming units per cell, as determined on chick fibroblasts for NDV and vaccinia virus and monkey kidney for poliovirus. Poliovirus and NDV infected cells were then incubated at 37° for 30 minutes, and vaccinia virus infected cells for 1 hour. After adsorption the unadsorbed virus was removed from the cell layer by washing it three times with 1.5 - 2 ml of TD. The infection medium (Eagle's for NDV, Eagles + 10% horse for poliovirus and Eagle's + 10% calf for vaccinia virus) plus the desired analog (see below), was put on the cells which were then returned to the incubator.

An estimate of the number of cells on the infected plate was obtained by carrying one, or more often, two control cultures through the above procedure, but exposing them to 0.2 ml TD instead of virus stock. The cells were removed by trypsin in the usual manner and counted in the hemacytometer. Since the differences between treated and untreated cultures, when present, were of the order of a factor of 10, the variation in the number of cells per plate--rarely more than a factor of 2 in any one experiment--was ignored.

Yields of poliovirus and NDV from infected cells were sampled by placing 0.1 ml of the infection medium in 0.9 ml of dilution medium (Eagle's + 0.1 ml 10% bovine serum albumen in tris (BSA) + 0.1 ml 2.8% HCO_3 solution) and then freezing the sample at -20°C. The virus titer of the frozen sample

remained constant over a period of several months. When the samples were taken, the HeLa cells had detached from the glass and disintegrated due to virus action; and the floating cell debris was included in the sample. Therefore, intracellular and extracellular virus were sampled simultaneously. However, most of the vaccinia virus infected cells remained on the glass up to 50 hours after infection. Furthermore, the cells contained 20-100 times more virus than the supernate. Therefore, the cells were removed from the glass with a rubber policeman, suspended in the 5 ml of infection medium, and a 1 ml aliquot of cells plus supernate removed and frozen. This procedure was sufficient to release most of the intracellular virus.

5. Assay Methods

Poliovirus was assayed on monolayer cultures of monkey kidney cells by the standard plaque technique of Dulbecco and Vogt (76). Plaques were counted at 3, 4, and 5 days. The methods of Vogt et al. (77) were used in the mutation studies.

NDV was assayed by a modification of the technique of Rubin (78). Primary cultures of 3×10^6 cells from 10 day old chick embryos were made on a 60 mm petri dish with 5 ml of Eagle's medium supplemented with 10% calf serum and 10% tryptose phosphate. They were used for virus assay after two days incubation at 37°C; at this time the cells formed a continuous monolayer. 0.2 ml of virus diluted in TD was adsorbed for 30 minutes at 37° in the CO_2 incubator; then a mixture of

2.5 ml overlay medium (Eagle's + 10% calf serum) and 2.5 ml 1.8% agar was placed on the cells. The plates were incubated for 2 days, stained for 24 hours with 10^{-4} solution of neutral red in Eagle's, and the plaques counted. Up to 100 plaques per plate could be scored accurately. The smaller plates were easier to handle than the 90 mm ones used by Rubin (78) and reduced the plating time by almost one-half.

Vaccinia virus was assayed by the plaque method and by pock formation on the chorioallantoic membrane (CAM), as first described by Burnet (79). Although a tissue culture assay for vaccinia virus had been described by Noyes (80), we found it convenient to develop our own. The tissue culture method has proven to be simpler and more reproducible than the CAM assay.

The plaque method used was essentially the same as that for NDV. Dilutions of virus were made in TD plus 0.2% BSA (81). The addition of BSA increased the plaque forming yield by approximately a factor of 5. After the virus was adsorbed on the chick fibroblast monolayer for one hour, the agar overlay medium was added. After 2 days incubation at 37°C the cells were stained for 24 hours as with NDV. Plaques were counted either by eye or with the low power of the dissecting microscope. Their characteristic morphology made them readily distinguishable under the microscope from occasional tears or gaps in the monolayer. Up to 100 plaques/plate could be reliably counted.

In all assays using the plaque method, 2 plates/dilution

were counted and the results expressed as the average of the two counts.

It was necessary to assay vaccinia virus on the CAM for mutational work. Vaccinia virus lesions on the CAM are of two general types: hemorrhagic and nonhemorrhagic (red and white). Downy (82) and later Fenner (75) have shown that the red pock mutates to the white form at a frequency varying from strain to strain, but of the order of 10^{-2} to 10^{-3}. On the CAM, one white pock may be distinguished among 50 red.

Numerous papers have been written refining the original Burnet technique for assaying vaccinia virus on the CAM. The method we have devised, adapted from several sources (81,83,84), has proven to be fast, technically simple, and virtually free from non-specific lesions.

Twelve day old chick embryos were candled and a triangular area approximately 6 mm on a side marked out just below the air sac. A small opening was made in the air sac and the edge of an emery disc fitted to a mechanical drill was used to abrade the boundaries of the triangle, reaching the egg shell membrane at only one point. The triangle of egg shell was removed and the egg shell membrane punctured. A drop of TD at 45° (83) was then placed on the membrane and gentle suction applied at the air sac opening until the CAM dropped. After the egg shell membrane was removed, the egg was ready for inoculation.

The eggs were inoculated with 0.1 ml of virus diluted in TD plus 0.2% BSA (81) and the opening covered with a small plastic band. Following incubation at 37°C in an upright position for 2 days, the membranes were removed by opening the egg at a point diagonally opposite from the original opening. The yolk and embryo were pulled from the egg, leaving the CAM behind. The membrane was removed with forceps, rinsed in TD, and the pocks counted and classified under the dissecting microscope.

The classification of single pocks (white or red) was confirmed as follows: A pock, plus as small an amount of membrane as possible, was removed and placed in a 1 ml centrifuge tube with 0.1 ml of buffer. This was frozen once and 0.9 ml buffer added to the tube. The sample was then diluted and assayed in eggs as described above. Each pock treated in this manner yielded 10^3 - 10^4 pfu.

Experiments with tritiated thymidine using standard labeling techniques (85) were carried out in collaboration with Dr. Clyde Goodheart. Cells were grown on coverslips and infected with poliovirus in the usual way. Medium was then placed on the cells along with the labeled thymidine, removed 4 hours later, and the cells fixed; autoradiographic film (85) was applied and exposed for 5 days. The slides were examined with a phase contrast microscope which permits ready identification of the grains caused by tritium decay.

RESULTS

The object of these experiments was to find out whether compounds which either inhibit DNA synthesis, or cause the synthesis of an abnormal DNA in HeLa cells, would also inhibit the multiplication of either RNA- or DNA-containing viruses, or produce an increase in their mutation frequency. The results show that in HeLa cells, DNA synthesis can be inhibited, that RNA-containing viruses can grow normally despite this inhibition, and that DNA-containing viruses cannot.

1. Effect of Aminopterin on HeLa Cells

These experiments were designed to 1) show that aminopterin inhibits division of HeLa cells, and 2) that this inhibition may be reversed by thymidine, indicating that aminopterin blocks thymidylic acid synthesis in these cells. The following procedure was followed in all cell growth experiments. Cultures were started by transferring 10^5 cells into each of a number of 60 mm petri dishes containing 5 ml of medium. The control dishes contained standard medium, the others were supplemented either with aminopterin or aminopterin plus presumed antagonists of it. The cells, unless stated otherwise, were kept in the presence of the indicated compounds from the moment of transfer. Cell multiplication was followed by counting the number of cells present within each of two circles drawn on the bottom of each petri dish on successive

days. With this method of cell counting, it was not possible to follow an increase in cell number greater than a factor of 4 to 6.

Figure 13 shows the results of typical experiments where HeLa cells were treated with aminopterin in the presence and absence of various suppressors of its action. It can be seen that aminopterin blocks cell division, and that the inhibition persists after the analog is removed. The inhibition is only partially reversed by thymidine. The incompleteness of this reversion can be attributed to the fact that aminopterin affects purine synthesis and one carbon transfers, in addition to thymidylic acid formation (86). The addition of adenosine, glycine, and thymidine to the medium (52) reversed the effects of aminopterin on HeLa cells for at least 2 divisions, but not indefinitely. The fact that cells can divide in the presence of aminopterin if thymidine is added, leads to the conclusion that aminopterin blocks thymidylic acid synthesis, and hence DNA synthesis in HeLa cells.

This conclusion was verified using autoradiographic techniques (85). 0.01 microcuries/ml of tritiated thymidine (concentration 0.06 gamma/ml) was added to normal cells and to cells pre-treated for 18 hours with 10 gamma/ml aminopterin and post-treated with the same amount during the labeling period. After 4 hours incubation, the cells were fixed and the film was applied. The film was exposed for 5 days, developed, and the cells scored for the presence of heavy, moderate, or light labeling (Table 3). (The labeling was too heavy for accurate

Table 3

Effects of Aminopterin on Incorporation of Tritiated Thymidine with Infected and Non-infected HeLa Cells

Treatment	Activity of label in μcuries/ml	Degree of Labeling: None or light ~0-100	Moderate ~100-1000	Heavy >1000	% heavy labeling
Control*	0.1	104[a]	29	76	36%
A*A	0.1	10	4	75	84%
AP*A	0.1	10	12	77	78%

HeLa cells were grown on cover slips for 3 to 4 days and then labeled for four hours with the indicated activity of tritiated thymidine in Eagle's medium. The concentration of the thymidine was 0.06 γ/ml in all cases. After labeling, the cells were fixed, and film applied and exposed for 5 days.

* = labeling with tritiated thymidine. Letters to the left of * represent pre-treatment with the analog for 24 hours. Letter to the right represents post-treatment for the entire labeling period.

A = 10 γ/ml aminopterin

P = infection with poliovirus in the standard manner

a = number of cells containing 0-100 grains/nucleus

determination of the amount of thymidine incorporated by each cell; the numbers given are crude approximations). Thirty-five percent of the normal and 80% of the treated cells were heavily labeled. This indicates that in the treated cells, DNA synthesis had ceased because of lack of thymidylic acid and that they started to make DNA as soon as it was provided. Furthermore, the results show that aminopterin blocks the synthesis, but not the incorporation of thymidylic acid into DNA. Only the 1/3 of the controls which were actively synthesizing DNA during the time of the pulse became heavily labeled (87).

2. Effects of 5-Fluorouracil on HeLa cells

Experiments similar to those with aminopterin were performed with 5-fluorouracil (5FU). These were also designed to show that the analog inhibits HeLa multiplication and that this inhibition may be reversed with thymidine. Typical results are summarized in Figure 14. Since the effect of the analog is partially suppressed by thymidine, but not by uridine, 5FU must prevent cell division primarily by blocking thymidylic acid synthesis. Unlike aminopterin, if 5FU is removed within 15 hours after plating, the cells resume an approximately normal growth rate. Cells kept in the presence of the analog for 3 days, however, will not resume synthesis when the analog is removed. The growth of established cells is halted within 24 hours after 5FU is added.

3. Effects of 5-Bromouracil and 5-Bromodeoxyuridine on HeLa Cells

Concentrations of 5-bromouracil (5BU) up to 160 gamma/ml had no effect on the growth rate or morphology of HeLa cells. It also did not inhibit the growth of vaccinia virus, nor could it reverse the aminopterin inhibition of HeLa cells.

5-Bromodeoxyuridine (BDU), however, profoundly affected the cells. Figure 15 gives the results of an experiment to determine the minimum level of BDU which inhibits cell division. Test plates were set up as described for the aminopterin experiments (first passage). To permit a longer observation period, the cells were trypsinized and replated after the fourth day and counting continued (second passage). The lowest concentration of BDU which inhibited cell division lies between 0.25 and 0.06 gamma/ml. At low concentrations of BDU the cells were inhibited for 2-3 divisions, but then resumed an approximately normal division rate. At higher concentrations, there was no recovery. Other experiments have shown that the first cell division, after the addition of 20 gamma/ml BDU is not delayed (Thesis: part I, sec R2). The difference, seen in the first passage, between control and test cultures at day 2, is an artifact caused by some cells having divided before the "zero" count was made.

Figure 15 shows that most cells in the presence of 20 gamma/ml BDU do not divide more than twice. However, clones of resistant cells usually appear in a culture left for many days in the presence of BDU with its medium changed every 3 days. In one experiment, 500 normal cells were plated into medium containing

20 gamma/ml BDU. After 22 days, 4 small clones had appeared, while control plates with no BDU showed 70 large clones at that time. Thirteen days later the clones in BDU were isolated and grown in the continued presence of BDU. The fastest growing culture of resistant cells had a division time of 2-3 days, well below the 17 to 24 hour division time of normal cells. All resistant cultures grew best under relatively crowded conditions (2×10^5 cells/plate or more). The resistant clones were tested for 5BU incorporation into their DNA by the density gradient method employing the "direct" method of DNA isolation (Thesis: part I, sec M). Three different resistant clones, tested after 20, 21, and 30 divisions had occurred in the presence of BDU over a period of 2, 3, and 4 months, had DNA of density 1.76, 1.75, and 1.77 respectively. The second clone was retested 2-1/2 months and 17 divisions later, at which time its density was approximately 1.74. Figure 16 shows a densitometer tracing of this last band, which is typical of those made by DNA from resistant cells. The standard deviations of DNA from resistant cells were 2-3 times larger than that of normal HeLa DNA. The results obtained from resistant cells after prolonged growth in BDU may be compared with a density of normal cells of about 1.74 after one, and 1.78 after two divisions in BDU. The BDU tolerant cells were heterogeneous in size, with 10% giants and most of the others somewhat larger than normal. Examination of uncrowded plates of resistant cells showed that probably some of them were not able to grow, since generally subclones

of growing cells emerged from a background of large, non-dividing ones.

4. Effects of Combined Treatments: Aminopterin plus BDU (A+B)

In an attempt to increase incorporation of 5BU into DNA, cells were simultaneously treated with aminopterin, which inhibits endogenous thymidylic acid synthesis, and BDU. Under these conditions the cell count increased to a maximum, and then declined. The maximum increase (average of two plates) varied from 1.6 to 2.3 and was reached within 3 to 5 days after plating. The presence of a maximum is not due to loss of cells from the glass, as indicated by the absence of cells in the supernate. Cells treated with aminopterin, BDU, adenosine and glycine (ABAdGl) also divided only once. Cell division did not resume when the cells were returned to normal medium. Figure 17 shows a typical experiment illustrating these points. A curve showing the growth of cells treated with aminopterin plus thymidine, adenosine, and glycine (ATAdGl) is added for comparison. Examination of the curves indicates that it is the incorporation of 5BU into the DNA which prevents the cells from dividing more than once. Nevertheless, A+B cells incorporated only as much 5BU as cells which have divided once in the presence of BDU alone (Thesis: part I, sec R2).

The apparent failure of A+B cells to divide more than once was investigated by autoradiographic techniques. Cells grown 3-1/2 days in A+B medium supplemented with adenosine and

glycine (ABAdGl) were exposed for 4 hours to 0.1 microcurie/ml of tritiated thymidine. Control cells, grown in the absence of the analog, were simultaneously labeled with 0.002 microcurie/ml tritiated thymidine. The concentration of the nucleoside was 0.06 gamma/ml in both cases. Table 6 (line A) gives the grain distribution for 70 control cells. Table 6 (line B) shows the distribution of grains in the nuclei of 77 ABAdGl cells; the average number of grains/nucleus containing less than 10 grains is 1.2. The expected background on slides of this type is 1-2 grains/nucleus (88). The grain counts, after correcting for the activity of the thymidine with which the cells were labeled, demonstrate that the treated cells incorporated at least 1000 times less thymidine than the controls.

5. Effect of the Analogs on Growth of NDV

We have shown that aminopterin and 5FU inhibit thymidylic acid synthesis in HeLa cells and that BDU is incorporated into the DNA of HeLa cells. We next demonstrate that these compounds have no effect on the replication of RNA-containing viruses.

Cells were treated with different analogs before and/or after infection with NDV as described in Methods. Table 4 summarizes the results of several experiments with the yield in each case normalized to control value of 1.0 plaque-forming unit (pfu) per cell. None of the treatments influenced the final yield of the virus, as can be seen by examining yields

Table 4

Summary of Results with NDV

Treatment	Time in hours	Yield in pfu/cell
N	2.5	.04
AN	2.5	.03
FN	2.5	.05
ABN	2.5	.14
N	24	1.0
AN	24	1.3
NA	24	.4
ANA	24	1.0
FN	24	1.4
FNF	24	1.3
NF	24	.96
ABN	24	3.1
ABNB	24	2.3
NB	24	.81
ABNAB	21	2.0

Plates containing 5×10^5 to 3×10^6 HeLa cells were infected with 2-5 pfu of NDV per cell, and the virus removed at the indicated times after infection.

N = infection with NDV. Letters to the left of N represent pre-treatment with the analog for the 24 hours prior to infection, except for AB which was for the 4 days prior to infection. Letters to the right represent post-treatment for the entire infection period

A = 10 γ/ml aminopterin

F = 5 γ/ml 5-fluorouracil

B = 20 γ/ml 5-bromodeoxyuridine

from cells treated in different ways. Since the yields were low, it was demonstrated that replication actually occurred by taking samples of cells and supernates at 2.5 and 24 hours after infection, using the method described for vaccinia virus. The increase in virus titer over the period demonstrated that virus multiplication took place. In a similar experiment, two HeLa monolayers were infected with about 30 plaque-forming units of NDV. Following infection, one was covered with Eagle's medium, the other with Eagle's plus 10 gamma/ml aminopterin, and the medium titered after 3 days. The final yield was 3×10^6 pfu in the control culture and 10^6 pfu in the treated culture. This shows that the yield obtained in the other experiments was not the result of eclipse and reconstitution of the original infecting virus.

6. Effect of the Analogs on the Growth of Poliovirus

HeLa cells, treated before and/or after infection with different analogs, were infected with poliovirus as described under Methods. The results of several experiments are summarized in Table 5. All values have been normalized to a control yield of 120 pfu/cell. The results show that none of the treatments significantly inhibited the growth of the virus. Figure 18 shows a growth curve of poliovirus in untreated cells, and in cells pre- and post-treated with 10 gamma/ml aminopterin. The cells were infected as described under Methods and removed

Table 5

Summary of Results with Poliovirus

Treatment	Time in hours	Yield in pfu/cell
P	20	120
PB	20	110
FBP	20	155
FBPB	20	125
AP	20	75
PA	20	110
APA	20	140
ABP	20	75
ABPB	20	95
ABPAB	20	95
FP	41	101
PF	41	116
FPF	41	97
PFB	41	90

The infection procedure, exposures to the drug, and definitions are the same as in Table 4 with the exception that poliovirus (P) instead of NDV was used for the infection.

from the plates at the indicated times using the method described for vaccinia virus. No influence on the drug on the length of the latent period or on the final yield was observed.

The normal growth of poliovirus in the presence of aminopterin or 5-fluorouracil suggested that synthesis of new DNA is not required for its replication. A direct test of this hypothesis was made possible by the already described observation that most cells after one division in A+B medium do not synthesize new DNA (Table 6, line B).

Cells grown in A+B medium for 3-1/2 days were infected with poliovirus in the usual manner. Forty-five minutes after infection, tritiated thymidine was added to the medium. The cells were fixed at 4 hours, that is, the end of the latent period of the virus (Fig. 18). Table 6 (line C) shows the detailed distribution of grains/nucleus for 117 infected cells. It is seen that there are two classes: "unlabeled" cells with less than 10 grains/ nucleus, and "labeled" cells with greater than 10 grains/nucleus. There is a sharp discontinuity between the two classes indicated by the absence of any cells with 10-24 grains/nucleus. The average number of grains/nucleus in cells with less than 10 grains/nucleus is 1.3. The expected background under these conditions is 1-2 grains/nucleus (88). In addition to these detailed counts, about 550 cells from the same experiment were classified according to whether they had less than 5, 5-10, or greater than 10 grains nucleus.

TABLE 6

Effect of Aminopterin + BDU on the Incorporation of Tritiated Thymidine in both Infected and Non-infected Cells

Column	Treatment	Activity in μcuries	Number of Grains per Nucleus 0-6	7-20	21-40	41-60	61-80	81-100	Total Cells	Av	Av >10
A	control *	0.002	32[a]	4	16	8	5	5	70	26	50

			Number of Grains per Nucleus 0	1	2	3	4	5-9	10-24	25	Total Cells	Av <10
B	ABAdGl*ABAdGl	0.1	32	21	7	8	7	0	0	2	77	1.2
C	ABP*	0.1	35	37	22	8	7	4	0	4	117	1.3

			Number of Grains per Nucleus 5	5-10	25-35	35	Total Cells	Pooled % >10
D	ABP*	0.1	524	18	6	1	549	1.7

HeLa cells were grown on cover slips for 3-4 days, and then labeled for four hours with the indicated activity of tritiated thymidine in Eagle's medium. The concentration of the thymidine was 0.06 γ/ml in all cases. After labeling, the cells were fixed, and film applied and exposed for 5 days. * = labeling with tritiated thymidine. Letters to the left of * represent pre-treatment with the analog. Letters to the right represent post-treatment for the entire labeling period. A = 10 γ/ml aminopterin; B = 20 γ/ml 5-bromodeoxyuridine; Ad = 40 γ/ml adenosine; Gl = 40 γ/ml glycine; P = infection with poliovirus in the standard manner (see Methods). ABAdGl and AB cells were pre-treated with the analogs for 3½ days before labeling.

a = number of cells containing 0-6 grains/nucleus.

Pooled counts of all infected A+B cells showed that 1.7% were labeled. The data, after correcting for the activity of the thymidine with which the cells were labeled, indicates that "unlabeled" cells incorporate at least 1000 times less, and the "labeled" cells 50 times less thymidine than the controls. The labeled fraction of the cells probably represents those cells which have not yet completed their first division in the presence of the analogs, and thus still contain some DNA capable of replicating. That not all A+B treated cells have divided until 3-5 days after plating, was already shown in Fig. 17.

Two lines of evidence show that the total virus yield cannot have come from those few A+B treated cells which were capable of making some DNA: In the first, the number of cells in an A+B culture which was capable of yielding virus was determined. Cells were infected in the usual way; the plates were washed to remove unadsorbed virus, the cells removed with trypsin, and counted. They were then placed in TD and 0.2 ml of a suitable dilution was mixed with 0.5 ml of agar overlay medium and poured on the plates. This procedure restricted the cells to a region close to the monolayer and insured prompt plaque formation by any virus particles which they released. The rest of the cells were freeze-thawed three times to release cell associated virus, and then assayed in the usual manner. Both A+B and control cells showed about 20% infectious centers and 4 times as

many plaques as the freeze-thawed cells. This result implies that the same number of cells in treated and control cultures can yield progeny virus. The second is the fact that A+B and control cells both yield almost equal numbers of pfu. The conclusion that poliovirus growth occurs in cells in which synthesis of new DNA is absent, is thus justified.

Since 5BU is known to have a strong mutagenic action on phage (89), a test was made to determine if BDU had a similar effect on poliovirus. Such an effect would be expected if interaction with DNA occurred. To test this possibility, cells grown for 10 days in the presence of 20 gamma/ml BDU and cells grown 5 days in the presence of A+B were infected with the d mutant of poliovirus (77). The frequency of mutations to the d^+ genotype was then determined by assaying the yield on monkey kidney plates under alkaline and acid agar. Both types of virus form plaques under alkaline agar, but only the d^+ under acid agar. In both experiments, the proportion of d^+ was approximately 2×10^{-5} for control and test cells (Table 7). This result supports the hypothesis that endogenous DNA is not involved in the replication of poliovirus.

Table 7

Summary of Mutation Experiments with Poliovirus

Treatment	(1) Virus titer on alkaline agar pfu/ml	(2) Virus titer on acid agar pfu/ml	(3) Proportion of d^+ reversion (2):(1)
Control	1.4×10^6	2.5×10 (21)	1.8×10^{-5}
A+B	5.4×10^6	9.8×10 (26)	1.8×10^{-5}
Control	2.4×10^5	4.8 (10)	2.3×10^{-5}
B	8×10^5	1.30×10 (8)	1.6×10^{-5}

Cells were grown in A+B medium for 5 days, and B medium for 10 days before being infected as described in Methods. The virus yield was assayed under acid and alkaline agar according to the method of Vogt *et al.* (77). Numbers in parentheses are the actual number of plaques counted.

A = 10 γ/ml aminopterin

B = 20 γ/ml 5-bromodeoxyuridine

7. Growth of Vaccinia Virus in the Presence of the Various Analogs

Synthesis of poliovirus and NDV is not affected when cellular DNA is substituted with 5BU, or when cellular thymidylic acid synthesis is presumably halted by the use of 5FU and aminopterin. On the contrary, these analogs strikingly inhibited the replication of the DNA-containing virus, vaccinia.

Preliminary experiments showed that the tissue culture and CAM assay gave quantitatively identical results. Accordingly, the plaque assay was used for all but the mutation experiments.

Cells grown in the presence of the various analogs were infected with vaccinia virus as described in Methods. Table 8 summarizes the results of several experiments demonstrating the effect of the different analogs on vaccinia virus growth. In particular, it shows that treating cells with aminopterin before infection, after infection, or both, all reduce the virus yield from 70 to about 0.1 pfu/cell. This inhibition is partially reversible by thymidine, and more completely reversible by a combination of thymidine, adenosine, and glycine. This work therefore confirms the conclusion that aminopterin blocks thymidylic acid synthesis in HeLa cells. The yield of vaccinia virus in aminopterin-treated cells in any one experiment (0.1 pfu/cell) was independent of the yield of the untreated controls in that

Table 8

Summary of Results with Vaccinia Virus

Treatment	Time in hours	Yield in pfu/cell
V	26	73.0
VA	26	.1
AV	26	.1
AVA	26	.15
VAT	26	1.4
VATAG1	26	12.0
AVT	26	.1
AVTAdG1	26	4.0
V	28	20.0
VF	28	.4
FV	28	20.0
FVF	28	.4
VFT	28	15.0
V	28	95
BV	28	33
VB	28	9
BVB	28	1
FBV	28	30
VBT	28	30
VB120T	28	70
V	24	10.0
V20B	24	.05
V4B	24	.3
V2B	24	1.0
V1B	24	4.1
V.5B	24	9.0
V.1B	24	7.5

Plates containing 5×10^5 to 3×10^6 HeLa cells were infected with 2-5 pfu vaccinia virus per cell, and cells plus supernate removed at the indicated times.

V = infeetion with vaccinia virus. Letters to the left of V represent pre-treatment with the analogs prior to infection.

Table 8 (contd.)

The time of pre-treatment was 5 days in the case of FB and B, and 24 hours for the others. Letters to the right of V represent post-treatment for the entire infection period. Numbers indicate concentration of the analog in γ/ml.

A = 10 γ/ml aminopterin

F = 5 γ/ml 5-fluorouracil

B = 20 γ/ml 5-bromodeoxyuridine

T = 20 γ/ml thymidine

Ad = 40 γ/ml adenosine

Gl = 40 γ/ml glycine

experiment. This finding suggested that the apparent yield from aminopterin-treated cells was due to elution of a proportion of the infecting virus. Figure 19 presents a growth curve of vaccinia virus in normal and aminopterin-treated HeLa cells which confirms this hypothesis. The controls show a typical one step growth curve (90) with a latent period of 7 hours, while the aminopterin-treated culture shows an initial decline followed by a leveling off. This decline might reflect late penetrating particles which have either entered the eclipse period and lost their infectivity, or became inactivated by some other mechanism. Four hours after infection, a strong cytopathogenic effect of the virus was noticeable in both control and treated cells. This phenomenon of the cytopathogenic effect cannot be explained at present, but appears worthy of further investigation.

5FU (Table 8) inhibits vaccinia virus growth in a manner similar to aminopterin, except that pre-treatment is ineffective, and that some virus production seems to occur in the presence of 5FU. The inhibition by 5FU was reversed by thymidine but not by uridine, verifying that 5FU blocks thymidylic acid synthesis.

BDU strongly inhibits vaccinia virus production at a concentration of 20 gamma/ml and retains some activity down to a level of one gamma/ml. Pre-treatment of cells with BDU has a slight influence on virus yield. The 6-fold excess of thymidine required to reverse 20 gamma/ml BDU, suggests

that BDU competes with thymidine in this system as it does in bacteria (50).

5BU is highly mutagenic for phage (89). It was of interest to test if a similar effect could be detected in vaccinia virus using the mutation from red to white pock forming particle on the CAM as a marker (see Methods for detailed description). The results obtained were equivocal. Preliminary reconstruction experiments demonstrated that white pocks may be detected in the presence of red pocks. In one experiment, 600 pocks of red pock-forming virus grown in the presence of 1 gamma/ml BDU were scored. Fifteen of those which appeared white (actually pale intermediate) were cut out, frozen, and then assayed. None of them was white. Similar experiments with virus grown in the presence of larger amounts of BDU also gave negative results, as did experiments scoring for the white to red mutation. In summary, out of a total of 3000-4000 red pocks examined, 2 white mutants were found. This mutation rate is slightly lower than that reported originally (10^{-3}) (82). Detection of mutants was hindered by the fact that on most membranes 40-60% of the pocks produced by white and red pock forming virus had an intermediate phenotype independent of the genotype of the initiating virus. The difficulty encountered in the recognition of the mutant type among an excess of the other type makes it impossible to assess the meaning of this negative result.

DISCUSSION

I. Preliminary: General Properties of Poliovirus, Newcastle Disease Virus and Vaccinia Virus

Before entering into an analysis of the results, the characteristics of the viruses employed will be briefly recalled.

The polioviruses are a distinct group consisting of three sereologically distinct types causing poliomyelitis in man and encephalomyelitis in mice (91). Poliovirus is a spherical particle about 28 millimicrons in diameter, consisting of about 75% protein and 25% RNA (72) and resistant to 20% ethyl ether. It is characterized by a narrow host range limited with few exceptions to certain tissues of man and the higher primates. The free viral RNA, however, can infect cells of other species, such as chicken embryo cells (69).

Newcastle Disease Virus (NDV) is a member of the myxovirus group including influenza and related viruses (92). It is a spherical particle, about 100 millimicrons in diameter, containing protein, phospholipid, and RNA, and is readily inactivated by treatment with 20% ethyl ether in the cold. The disease is endemic in chickens, but the virus can infect *in vitro* and *in vivo* a wide variety of avian and mammalian tissues (92).

These dissimilarities between poliovirus and NDV suggest

that the two viruses are unrelated, therefore the properties they have in common may be shared by many, and possibly all RNA-containing animal viruses.

Vaccinia virus is a member of the poxvirus group including among others, smallpox, cowpox, and ectromelia (93). It has a large quadrangular form with the longest diameter about 250 millimicrons. Vaccinia virus contains about 5% DNA (94) and this corresponds to about 2×10^{-16} grams DNA (95), the same as found in T2 (10).

II. Effects of Analogs on HeLa Cell Growth and on Virus Multiplication

The results obtained with the various analogs will now be analyzed and discussed in view of the known action of these inhibitors on other biological systems.

1. Effects of Aminopterin

Aminopterin is a close analog of folic acid. A number of lines of evidence show that folic acid and its derivatives are involved in thymine synthesis. Phear and Greenberg (96) studied the incorporation of C^{14} formate into thymidine in cell free systems from various mammalian sources. They found uracil deoxyriboside (UDR) to be the best substrate leading to thymidine, and tetrahydrofolic acid a necessary cofactor. Blakley (97) confirmed this finding using rabbit thymus, and Prusoff et al. with rabbit bone marrow and Ehrich ascites tumor cells (98). Friedkin and Roberts (100) confirmed that UDR

may serve as a thymidine precursor in rabbit bone marrow, and that a folic acid derivative is involved in this transformation. In a later paper, however, Friedkin and Kornberg (99) working with extracts of E. coli showed that UDR was converted first to deoxyuridylic acid (UDRP) and then to thymidylic acid with tetrahydrofolic acid as a necessary cofactor. Cohen et al. (108) have shown that the same reaction takes place in T^2 infected bacteria. For the sake of consistent terminology, we will assume that in mammalian cells, as in bacteria, DNA thymine is formed via the folic acid dependent methylation of UDRP to thymidylic acid.

There is extensive evidence that in bacteria and mammals, aminopterin blocks thymidylic acid synthesis. Bardos et al. (50) working with Lactobacillus leichmannii with folic acid limiting, found growth inhibition by aminopterin to be noncompetitively reversed by both thymine and thymidine, and competitively reversed by folic acid. Prusoff et al. (98) found that formate uptake into DNA was inhibited by aminopterin; and Friedkin and Roberts (99) that the methylation of UDR is extremely sensitive to aminopterin, while a 100 times greater dose fails to block the incorporation of thymidine into DNA. These results are consistent with the hypothesis that aminopterin blocks the folic acid requiring conversion of UDRP to thymidylic acid.

Besides the conversion of UDRP to thymidylic acid, folic acid and its derivatives are needed for the synthesis of

purines (86) and 5-methyl and 5-hydroxymethylcytosine (50). This perhaps explains why Hakala (52) has found that glycine and adenosine, in addition to thymidine are needed to reverse the toxicity of amethopterin (a close analog of aminopterin) in tissue cultures of sarcoma-180. Totter (86) has presented evidence for still other, less defined, functions of folic acid.

In the reported experiments, aminopterin was shown to have a profound and lasting effect on HeLa cells. Cells transferred into aminopterin-supplemented medium neither entered mitosis nor became "frozen" in any stage of it. When aminopterin was added to growing cultures, the growth rate was diminished at once; and growth stopped completely within 2 days. No later recovery was observed, either in the presence of the inhibitor or upon its removal. Unlike Hakala (52) we found that in HeLa cells, the rate limiting step is the thymidylic acid block, since addition of thymidine alone permits all of them to divide at least once. The additional functions for folic acid discussed by Totter (86) may explain why aminopterin is not permanently reversed in HeLa cells even when glycine and adenosine, as well as thymidine are added to the medium. Aminopterin does not prevent the incorporation of thymidine into HeLa cell DNA. Cells pre-treated with aminopterin for 24 hours incorporated approximately as much tritiated thymidine into their DNA as the normal controls, (Table 3). Aminopterin may form a lasting complex with the "folic acid enzyme" in HeLa cells since pre-treatment of cells

with aminopterin completely inhibited the subsequent multiplication of vaccinia virus.

The experiments with NDV and poliovirus demonstrate conclusively that aminopterin does not influence their reproduction. This has also been shown for encephalomyocarditis virus (EMC) grown on mouse embryo cells (101) and less rigorously, with influenza virus grown in the chicken embryo (102). Thus no requirement for new DNA synthesis during the multiplication of these viruses exists. The possibility that in the presence of aminopterin a thymineless DNA may satisfy a DNA requirement, is eliminated by the demonstration by Kornberg (55) that all 4 nucleotides are needed for DNA synthesis.

On the contrary, vaccinia virus multiplication is completely inhibited under the same conditions. Cells infected with vaccinia virus and then treated with aminopterin produced a final virus yield of one pfu for every 10 cells. The results of Fig. 19 show that this is probably a fraction of the infecting virus which was absorbed to cells but did not penetrate them.

In conclusion, the experiments with aminopterin show that RNA-containing viruses may reproduce in the absence of de novo DNA production. It does not exclude, however, that preexisting cellular DNA might be broken down, and the fragments used to produce whatever DNA may be needed for virus production.

2. Effects of 5-Fluorouracil

5-Fluorouracil (5FU) has been tested extensively in cancer chemotherapy (103,104,105,106). 5FU appears to inhibit cell multiplication by incorporation into cellular RNA with subsequent disruption of protein synthesis (105,107), and/or by being converted intracellularly to 5-fluorouracil deoxyriboside and then interfering with thymidylic acid and hence DNA synthesis (108).

5FU is incorporated into the RNA of a variety of tissues and organisms, including man (105,109). Horowitz et al. (107) have shown that when a uracil-requiring bacteria is given 5FU in the absence of added uracil, both protein and RNA synthesis occur; but that no beta-galactosidase is synthesized in induced cells, nor can induction take place in non-induced cells. In the absence of both 5FU and uracil, no net protein or RNA synthesis occurred. Cohen et al. (108) also demonstrated growth in uracil-requiring bacteria in the presence of 5FU. Furthermore, they showed that 5FU inhibition of normal bacteria is almost completely reversed by uracil and only partially by thymidine.

Cohen et al. (108) demonstrated that 5FU is converted intracellularly to 5-fluorouracil deoxyriboside (FUDRP), presumably via 5-fluorouracil riboside. FUDRP is a potent, irreversible inhibitor of thymidylate synthetase, which catalyses, in vitro, the conversion of UDRP to thymidylic acid. Cohen found that all three analogs, FU, FUR, and FUDR, completely

inhibited DNA synthesis in normal and T2 r^+ infected bacteria. Rich et al. (110) found that 5FU at the concentration of 1 gamma/ml and FUDR at a concentration of 0.01 gamma/ml completely inhibited growth of the H.Ep. line of human epithelial cells. Inhibition by FUDR was completely reversed by a four-fold excess of thymidine, but a hundred-fold excess failed to reverse 5FU inhibition, implying that in their cells 5FU inhibits uracil synthesis and/or is incorporated into RNA. Tracer studies support the conclusion that FUDR acts by blocking the de novo pathways of thymidylic acid synthesis. Danneberg et al. (111) found that in vivo incorporation of labeled formate into mouse DNA thymine was strongly inhibited by 5FU, but that the formate was incorporated normally into adenine and guanine. They also showed that neither uracil nor orotic acid is converted to thymidylic acid in the presence of 5FU. A similar inhibition by 5FU of formate incorporation into nucleic acid thymine but not adenine was demonstrated by Bosch et al. (109) using Ehrlich ascites cells. At higher levels 5FU inhibited the incorporation of uridylic acid into RNA. They demonstrated chemically the conversion of 5FU to FUDRP and also incorporation of 5FU into RNA. None of the fluorinated pyrimidines inhibited the incorporation of thymidine into DNA. Ackermann et al. (112) found that 0.5 gamma/ml 5FU stops HeLa cell division. This inhibition was reversed by thymidine. Chemical analyses of blocked cells showed that while the synthesis of cellular DNA was stopped, protein

and RNA synthesis continued. In time, giant cells were produced which no longer divided, even when returned to normal medium.

Our experiments showed that 5FU at 1 gamma/ml completely inhibits cell division and that this inhibition is reversed by thymidine. Unlike Ackermann et al. (112), we have not observed formation of giant cells. Cells treated with 5FU for 15 hours and then returned to normal medium resumed division, but those treated for 48 hours did not. The early reversibility may be due either to a true reversibility of the action of the analog, or to the fact that at 15 hours, its action, though irreversible, was not yet complete. The inhibition by 5FU after 48 hours may be irreversible either because of an irreversible reaction of 5FU with the enzyme, or because unbalanced growth occurred during the inhibition period. Since 5FU inhibition of HeLa cell growth was not reversed by uridine, but was by thymidine, its major action on these cells must be the inhibition of thymidylic acid synthesis.

5FU, like aminopterin, did not influence poliovirus, NDV, or EMC (101) multiplication. Vaccinia virus experiments, performed to confirm that 5FU prevented the synthesis of viral DNA, showed that pre-treatment of the cells with the analog did not affect virus yield, while post-treatment caused nearly complete inhibition. The lack of inhibition by pre-treatment cannot be due to the presence of an endogenous thymidylic acid pool, since in the experiments previously described, pre-

treatment with aminopterin completely inhibited virus growth. Pre-treatment may have been ineffective either because the enzyme-5FU combination is reversible, or because the virus induced the synthesis of new, unblocked, enzyme. It is known that the T-even phage induce the synthesis of thymidylate synthetase (108), among other enzymes (114); if vaccinia virus also does this, pre-treatment with 5FU would be expected to be ineffective. Under this hypothesis, pre-treatment with aminopterin would be effective as long as the postulated virus-induced enzyme is tetrahydrofolic acid dependent, providing the virus does not induce folic acid synthesis.

As in the case of aminopterin, the experiments which were performed do not exclude the possibility that breakdown products of cellular DNA could be used to produce whatever DNA is needed for virus synthesis.

Since 5FU is incorporated into RNA (105,107), it is surprising that it has no appreciable effect on either poliovirus or NDV multiplication. This may show that incorporation per se is not greatly inhibitory. Viral protein might be built in such a way as to be less dependent on these substitutions than some other cellular proteins (107). However, until incorporation of the analog into either poliovirus or NDV RNA has been demonstrated, further discussion of this phenomenon would be premature.

3. Effects of 5-Bromouracil and 5-Bromodeoxyuridine

a. 5BU

Incorporation of 5-bromouracil (5BU) into E. coli has been reported by Zamenhof et al. (115), who found that it is greatly enhanced by the presence of aminopterin, or the use of a thymine deficient strain. In the latter system, a maximum incorporation of 48% has been attained. Litmann (116) reported that incorporation into E. coli occurred in the presence of sulfanilamide but not in its absence. Kit et al. (117) found 5BU to be ineffective in reducing thymine incorporation into the DNA of several rat and mouse tumors, whereas BDU did this effectively.

b. BDU

1) Review of literature

5-Bromodeoxyuridine (BDU) is incorporated into DNA and to a certain extent inhibits DNA synthesis. Zamenhof (118) found that BDU inhibited the growth of some E. coli strains at a 20 times lower concentration (0.5 gamma/ml) than 5BU. Kit et al. (117) observed that BDU specifically inhibited the incorporation of C^{14} formate into the DNA-thymine of rat and mouse tumors, but had no effect on methylation, or purine and pyrimidine synthesis. They did not look for incorporation into DNA. Bardos et al. (50) demonstrated that bacterial inhibition by BDU is competitively reversed by both thymidine and thymine. Eidenoff et al. (119) found that about 45% of the thymine of H.Ep. cells (derived from a human cervical carcinoma) was replaced by BDU when the cells were grown in the presence of the analogs at a concentration of 50 gamma/ml, in the presence or absence of aminopterin.

The effect of incorporated 5BU on bacteria is not clear-cut. Zamenhof et al. reported that the degree of inhibition varied widely from strain to strain, and even among mutants of the same strain. In general, the degree of inhibition increased with the amount of incorporation of 5BU in DNA; however, maximum incorporation (48%) occurred in a thymine requiring mutant under conditions of almost no inhibition. Another strain showed altered colonies when substituted, but reverted to the original type when replaced in normal medium (120). In a recent paper, Zamenhof reported that BDU

induces mutations in bacteria at a frequency of 3×10^{-2} (121). Almost all bacteria whose growth is stopped by 5BU can resume division when returned to normal medium (120).

2) Cell studies

Our experiments demonstrate that BDU is incorporated into HeLa DNA without difficulty. This is shown by the fact that the first division of the cells in the presence of BDU was not delayed. Furthermore, the level of substitution after one division in the presence of BDU alone was equal to or greater than that in the presence of aminopterin plus BDU (Thesis: part I, sec R2). The inhibition of further multiplication for most 5BU substituted HeLa cells is thus due to the incorporation of 5BU into their DNA, rather than the mere presence of intracellular BDU.

Cells in A+B medium divided only once, and were essentially incapable of making more DNA, even after the analogs were removed (Table 6). A possible explanation of this finding is that each DNA molecule has certain sites which must remain unsubstituted if multiplication of the DNA is to take place, and which in the absence of aminopterin, preferentially incorporate endogenous thymidylic acid. The presence of 5BU in these sites could inhibit further DNA synthesis either directly, for instance by preventing the molecules from coming apart, or indirectly, by inhibiting the production of key enzymes. The number of these hypo-

thetical sites must be small compared to the total number of thymine molecules in DNA, since the amount of substitution is approximately the same whether or not aminopterin is present during the first division.

Whereas most cells divided only twice in the presence of 20 gamma/ml BDU, a small proportion of them divided further. Some of these rare cells were cloned and grown into a BDU resistant stock. These resistant cultures had three characteristics in common: 1) they incorporated 1/2 to 2/3 as much 5BU as the "fully substituted" DNA found after two divisions in BDU (Thesis: part I, sec R3), 2) they grew at a decidedly slower rate than normal cultures, and 3) their DNA formed a band in the CsCl gradient with a standard deviation 2-3 times greater than normal DNA.

The first two properties of the resistant cultures show that while considerable incorporation of 5BU <u>per se</u> is not lethal for these cells, substitution does cause these resistant cultures to have either an increased generation time, or an inviable fraction at each generation. The third property, the large standard deviation of the band formed by DNA from resistant cultures, might be a consequence of 1) density heterogeneity, caused by different levels of substitution either in different cells or among DNA molecules of the same cell, or 2) an average molecular weight about 5 times less than normal DNA (31). It is likely that cell to cell variation in degree of substitution exists. Such a variation

probably arises because these cells continue to synthesize and incorporate thymidylic acid into their DNA, in competition with the exogenous analog. The penetration of the BDU may vary in different cells for genetic* or physiological reasons; thus heterogeneity in the degree of substitution can be present at all times. Some degree of intracellular density heterogeneity may also exist. This could be caused by different guanine-cytosine ratios among the individual DNA molecules (Thesis: part I, sec D). However, the latter heterogeneity is probably not large enough to explain the observed results. Similarly, it is unlikely that the broadening of the band is due solely to a decrease in the molecular weight of the DNA; it does not seem reasonable that a cell containing DNA whose average molecular weight is 1/5 normal would still be viable. It is more likely that the broadening of the band is due to some combination of the above factors, rather than to any one of them.

The mechanism by which resistance manifests itself is not clear. It is possible that at low external BDU concentrations, some cells completely exclude the analog. Figure 15 shows that the lower the concentration of BDU in the medium, the higher the proportion of cells that can grow normally. In this range of BDU concentrations, after two or three divisions, the culture is growing at an approximately normal rate. We have shown that at higher concentrations, no cell

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*Vogt (40) has shown that genetic changes frequently occur in these aneuploid cells because of modification in karyotype and thus in gene balance.

can completely exclude the analog. Under these conditions various mutations, many of them lethal, might accumulate and lead to the death of a fraction of the cells at every division. The observation that crowded cultures survive better than sparse ones, suggests that cross feeding occurs. This finding indicates that many cells have become deficient in some growth factors and can only survive under crowded conditions when the missing factor can be supplied by the other cells.

Since the mechanism of BDU resistance is of considerable importance, two approaches for further investigation may be suggested: 1) Determination of the amount of 5BU substitution in the DNA of cells resistant to different concentrations of BDU at various times after their isolation, and 2) study of the rate of duplication of DNA of individual cells using tritiated thymidine and autoradiography (87). The former study would show whether cells resistant to high concentrations of BDU eventually exclude the analog, and the latter study would reveal whether the slow growth rate of substituted cells is caused by slow duplication of DNA, or by an abnormally long "interdivision" period.

3) Virus studies

5BU is strongly mutagenic for phage (89,122). Benzer and Freese (89) found that the enhancement of mutation frequency occurs mostly in certain "hot spots" within each cistron. It is thought that these mutations arise by a

tautomeric shift of the 5BU from the keto to the enol form, followed by improper pairing. This mechanism for point mutations was first suggested by Watson and Crick (13).

If DNA is an intermediate in the synthesis of viral RNA, hydrogen bonding between DNA and the bases of the new RNA is probably involved (123). Substitution of the DNA with 5BU would then be expected to produce mutations in the RNA by a mechanism similar to the Watson-Crick scheme for DNA. Therefore, if multiplication of an RNA virus involves interaction with 5BU substituted DNA, either an increase in the frequency of non-lethal, visible, mutations, or a decrease of the yield due to lethal mutations would be expected.

In our experiments, the effect of BDU on visible mutations was studied using the poliovirus d mutant system developed by Vogt et al. (77). In this system, one reverse mutant in the presence of 10^5 d infective particles can be detected. The experiments were carried out by growing poliovirus of the d genotype in cells pre-treated with either BDU or aminopterin plus BDU and determining the proportion of d^+ particles in the yield. In neither case was there an increase in mutation rate over background (Table 7). This result can be interpreted in different ways. The frequency of reversion to d^+ could have remained constant because viral RNA and cellular DNA do not interact. However, it is also possible that the result was accidental, since by studying the reversion of a given d to d^+, only mutations at a single

locus are scored. If this locus lacks RNA nucleotides, pairing, under the hypothesis, with thymine (or 5BU) of the DNA, an enhanced mutation rate would not be expected.

Unlike a visible mutation, however, inactivation can be produced by lethal mutations at any locus. To test for inhibition of multiplication of active virus, cells pre- and post-treated with A+B, which can make only substituted DNA whether by de novo or salvage pathways, were infected with either poliovirus or NDV (Table 4 and 5). The virus yield from these cells was normal. This fact speaks strongly against any mechanism for viral RNA multiplication which involves an interaction with cellular DNA.

This interpretation is supported by the observation that post-treatment of cells with BDU almost completely eliminated the production of viable vaccinia virus particles. Some inhibition of vaccinia virus multiplication was also found in cells pre-treated with BDU. This inhibition may be caused by incorporation of BDU derived from cellular DNA.

An attempt was made to test whether 5BU incorporation is mutagenic for vaccinia virus, as it is for phage (89). This was done by determining whether BDU can increase the frequency of a mutation affecting a morphological character. Downy et al. (82) and later Fenner (75) have shown that the particles of a vaccinia virus strain normally forming red pocks on the CAM mutate to a type giving white pocks with a frequency of 10^{-2} to 10^{-3} per virus generation. Tests were

performed to see whether virus grown in cells exposed to BDU showed an increase in mutation rate. The results were negative. Since there are a variety of mutations capable of giving a white phenotype (124), it is not likely that this negative result is due to the absence of 5BU hot spots in the mutating loci. As indicated in the experimental section, the negative results may be an artifact, due to a technical complication that could not be eliminated; namely the large proportion of pocks of intermediate phenotype which were formed whenever white or red pock forming particles were assayed. These could have easily obscured an increase in mutation rate of a factor of 10. One may also wonder whether the negative result may be correlated, in an unclear way, with the very high rate of spontaneous mutation at these loci.

4. Interaction of RNA with DNA, Quantitative Considerations and Conclusions

Several results have demonstrated that DNA synthesis is blocked in HeLa cells by treatment with either 5FU or aminopterin, and by a combination of aminopterin and BDU. The following two experiments indicate how complete this block is.

The first experiment is an autoradiographic determination of the amount of thymidine incorporated into cells pre-treated with A+B, and infected with poliovirus. These cells were placed in the presence of tritiated thymidine for

the entire latent period of virus growth. Autoradiographs of these cells were obtained as described in the experimental section. These autoradiographs showed that 98% of the cells were very weakly labeled and had an average of 1.3 grains per nucleus. A similar number of grains was found in 97% of uninfected A+B cells labeled in the same manner (Table 6). This grain density is approximately equivalent to that of background (88). If the grains represent incorporation of tritiated thymidine, 1.3 per nucleus would correspond to the synthesis of about 500 molecules of DNA of molecular weight 6×10^6. This calculation assumed that one H^3 disintegration in 10 resulted in a visible grain. Two percent of the cells had 20 times this number of grains. The question may be asked whether these thymidine incorporating cells are the source of the entire poliovirus yield. This question is answered by the experiments which showed that 20% of both control and A+B cells formed plaques when the infected cultures were plated for infected cells, and that the average yield per cell was almost exactly equal in both cultures. Thus all or most cells, certainly including many "unlabeled" ones, produced virus, and most likely in the usual amounts.

The second experiment is one showing that when vaccinia virus is grown in cells post-treated with aminopterin, no new virus is formed (Fig. 19). Although alternative explanations are possible, this implies that insufficient thymidylic acid is available to make even one infective particle/cell,

or about 20 molecules of DNA of molecular weight 6×10^6.

However, we cannot exclude that a smaller number of DNA molecules per cell was synthesized. We want to examine whether the synthesis of less than 20 DNA molecules could account for the synthesis of the viral RNA. Since the viral RNA appears to grow geometrically in the cell (125), the DNA template would be expected to grow in a similar manner. Therefore, the number of DNA molecules needed would be of the same order as the molecules of viral RNA produced--about 10,000 to 100,000 per cell for poliovirus (72).* This number of molecules is several orders of magnitude greater than the maximum amount of DNA that can be produced in the cells exposed to either aminopterin or to A+B.

We can now conclude that the described experiments make almost any use of DNA in the synthesis of the viral RNA unlikely. De novo synthesis of DNA was shown to be greatly inhibited, and the maximum possible synthesis of new DNA by the cell was found to be grossly inadequate when compared to the amount of RNA synthesis for which it should account. It is unlikely that synthesis of DNA occurs via salvage pathways since substituting the host DNA with 5BU has no effect on the yield or mutation rate of the virus.

If, therefore, RNA is able to multiply independently

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*Dulbecco (126) has shown that all poliovirus particles have very nearly the same density, and therefore they must all contain RNA.

of DNA, one may ask why DNA plays such a major role in heredity, and why there are two independent genetic systems in nature? One possible explanation of this state of affairs is that RNA was the original genetic material; then, in the course of time, DNA was synthesized. DNA has a selective advantage over the RNA because of its greater stability due to the presence of deoxyribose and to its two stranded structure. However, DNA did not become able to serve as a template for protein synthesis, and therefore could not displace RNA completely. The two systems now developed simultaneously, with the more stable DNA serving to make the less stable, and now more expendable RNA, while it served as the source of genetic information. The persistence of the independent genetic system formed by RNA could therefore be a remnant of the primeval situation.

CONCLUSION

The following major conclusions may be drawn from this work.

1. Aminopterin, 5-fluorouracil (5FU), and 5-bromodeoxyuridine (BDU) all inhibit DNA synthesis in the HeLa cell. Pre-treatment of cells with aminopterin plus BDU reduces the amount of DNA synthesis at least 1000 times.

2. The above compounds and combinations of them have no influence on the yield, and where tested, on the latent period or mutation rate of the RNA-containing viruses, poliovirus and NDV.

3. All of the compounds profoundly reduced the yield of vaccinia virus.

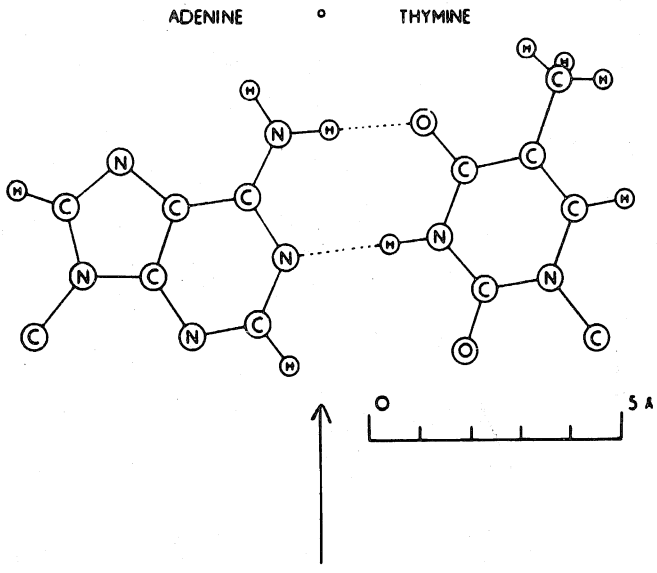
4. Since poliovirus and NDV are unrelated, we conclude that these findings may have general validity, and that all RNA-containing viruses may be able to grow in the presence of these analogs.

5. We finally conclude that RNA can multiply in the absence of interaction with newly synthesized DNA, and probably without interacting with pre-existing cellular DNA.

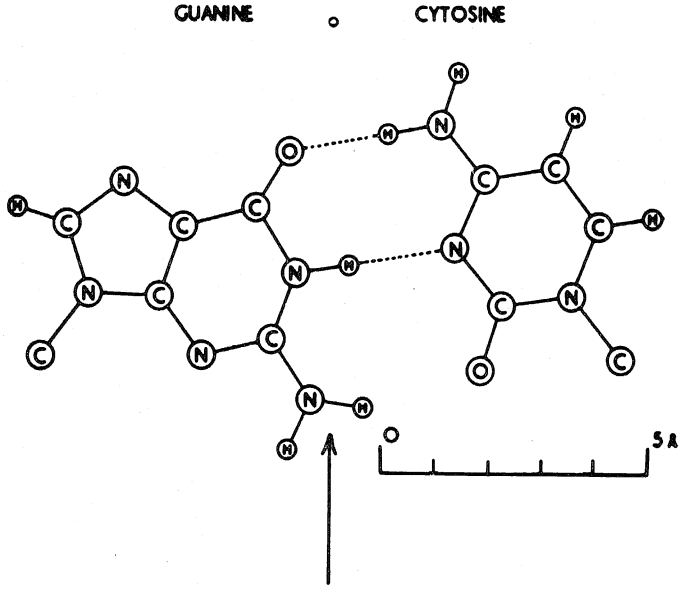
Figure 1

Hydrogen bonding between the bases of DNA as proposed by Watson and Crick. Note that neither the methyl group of thymine nor the 5 position of cytosine is involved in the hydrogen bonding.

Fig. 1



Pairing of adenine and thymine. Hydrogen bonds are shown dotted. One carbon atom of each sugar is shown.



Pairing of guanine and cytosine. Hydrogen bonds are shown dotted. One carbon atom of each sugar is shown.

Figure 2

Models of DNA replication. Predictions for the distribution of parental material between the daughter duplexes for the first and second division under the

(a) Conservative

(b) Semi-conservative, and

(c) Dispersive models of replication.

Fig.2

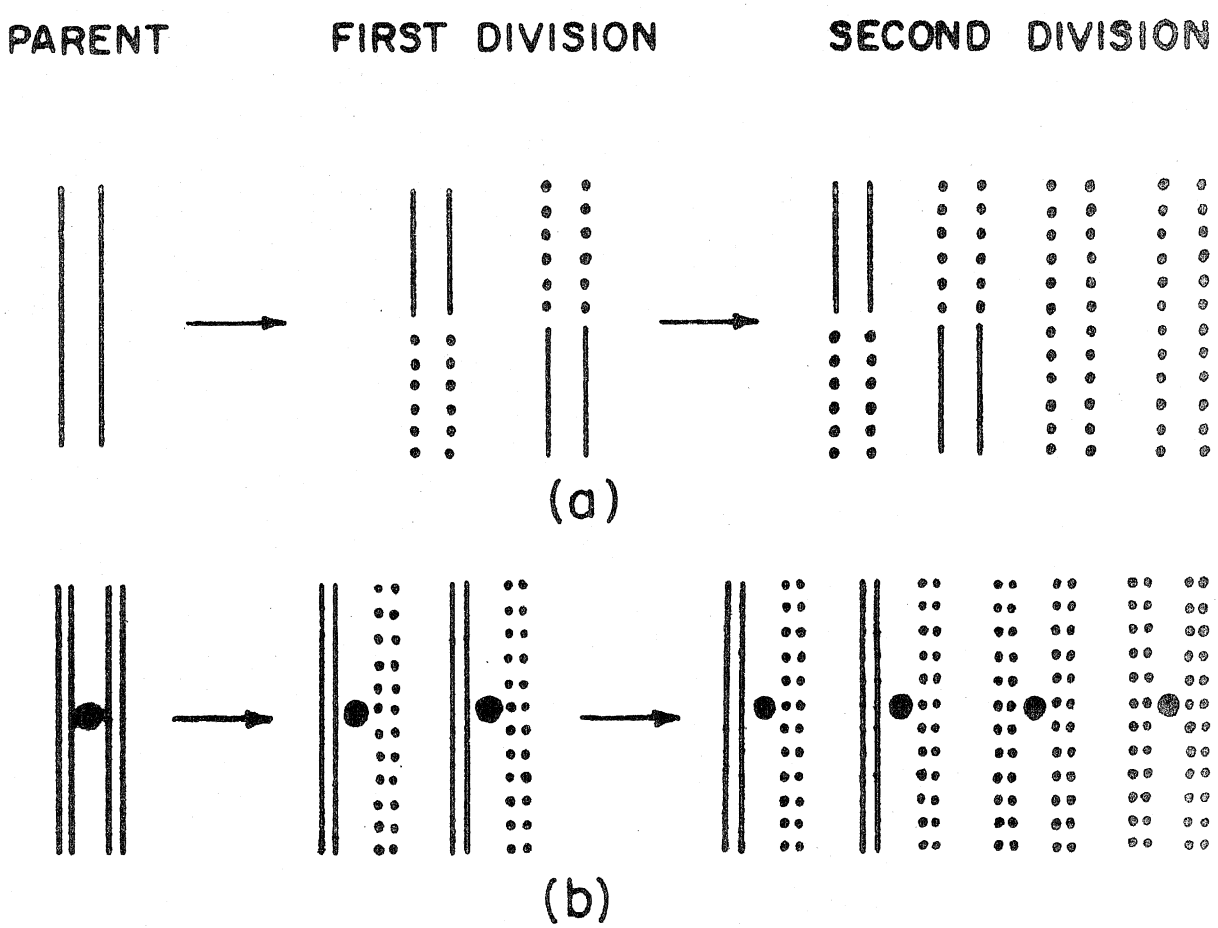


Figure 3

Densitometer trace of normal DNA from HeLa cells. The DNA was isolated by the dupanol method as described in the text, and banded in a CsCl density gradient at a speed of 44,770 rpm.

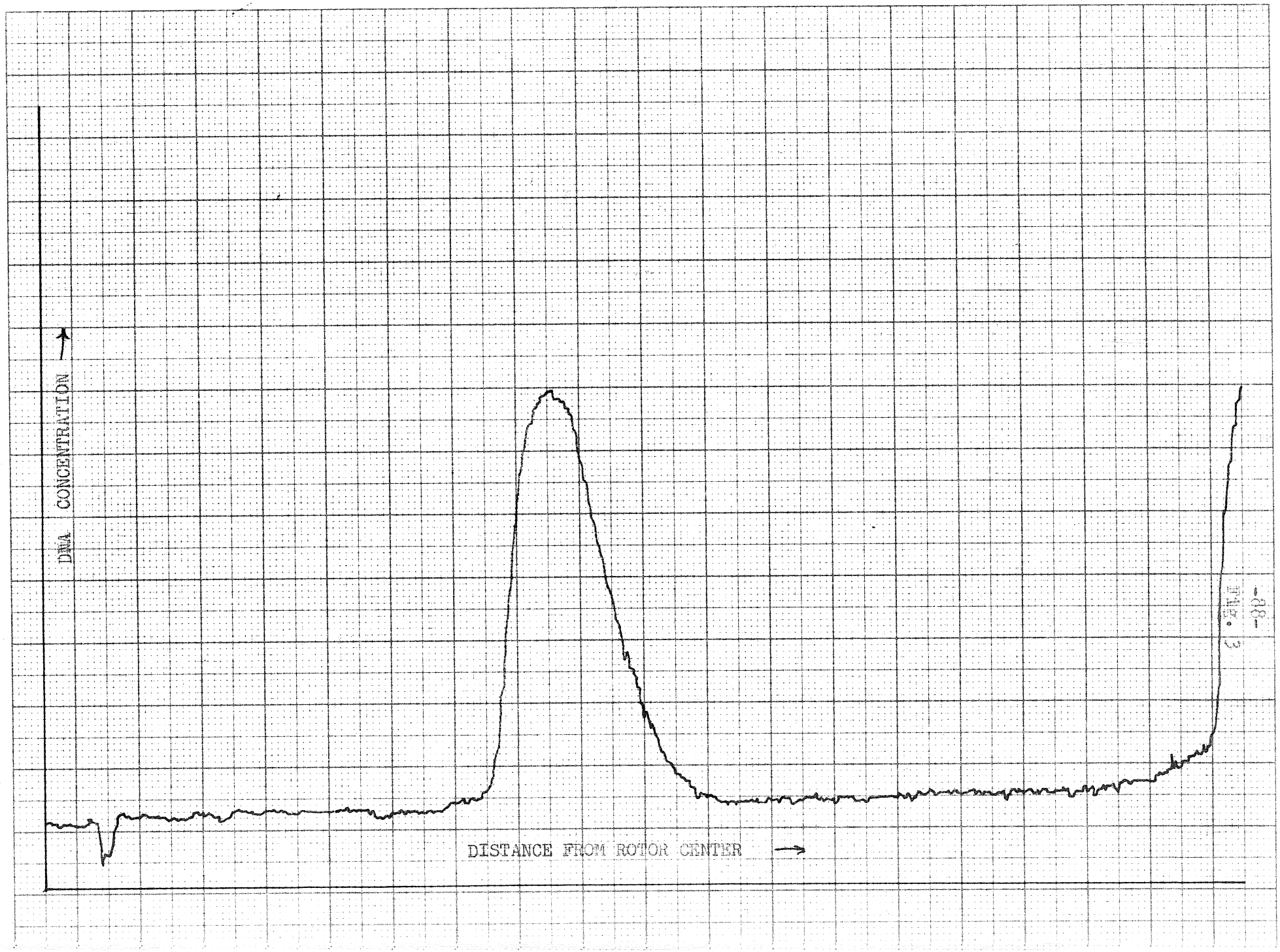

DNA CONCENTRATION
DISTANCE FROM ROTOR CENTER

Figure 4

Densitometer trace of normal DNA from HeLa cells. The DNA was isolated by the phenol method as described in the text, and banded in a CsCl density gradient at a speed of 44,770 rpm.

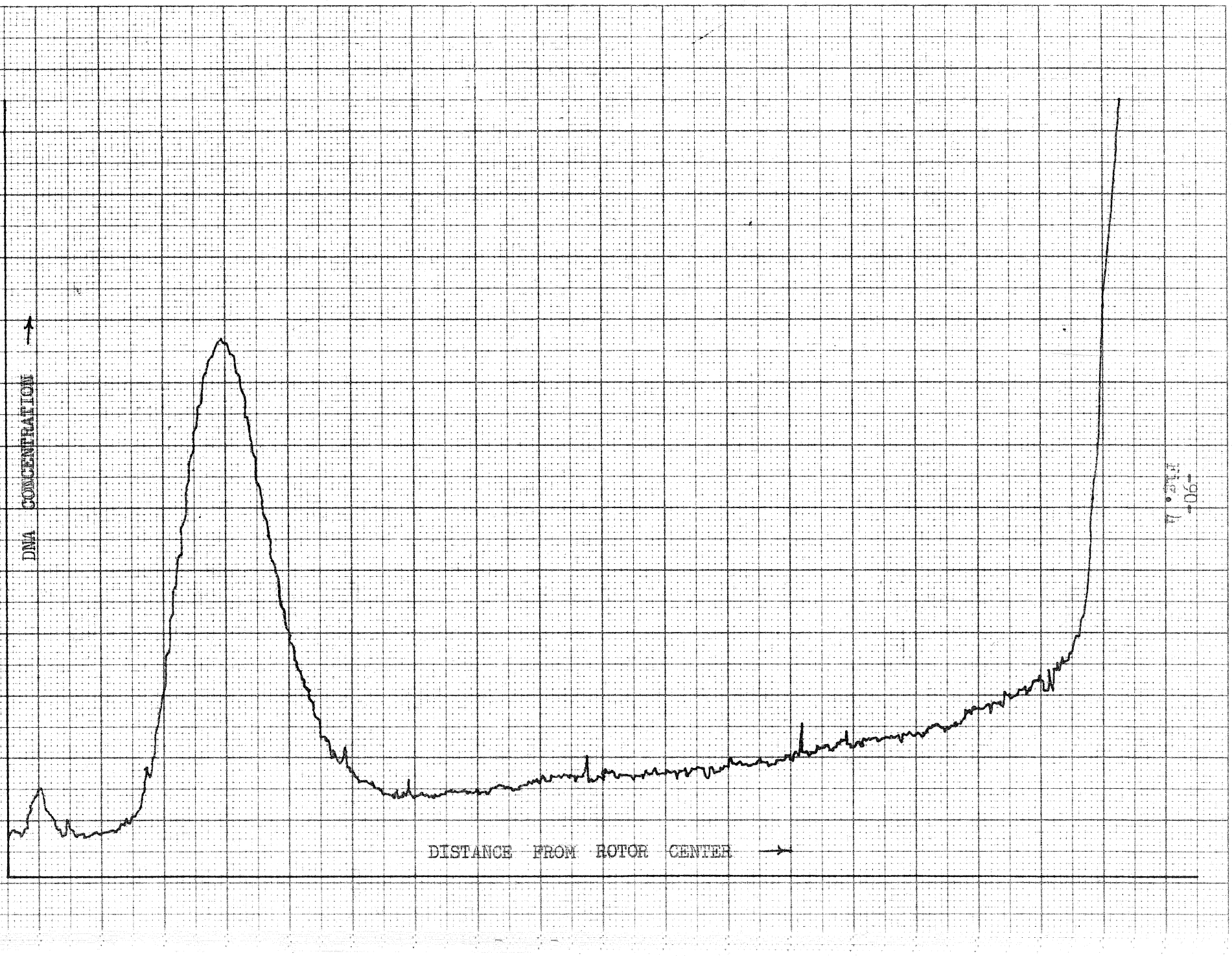
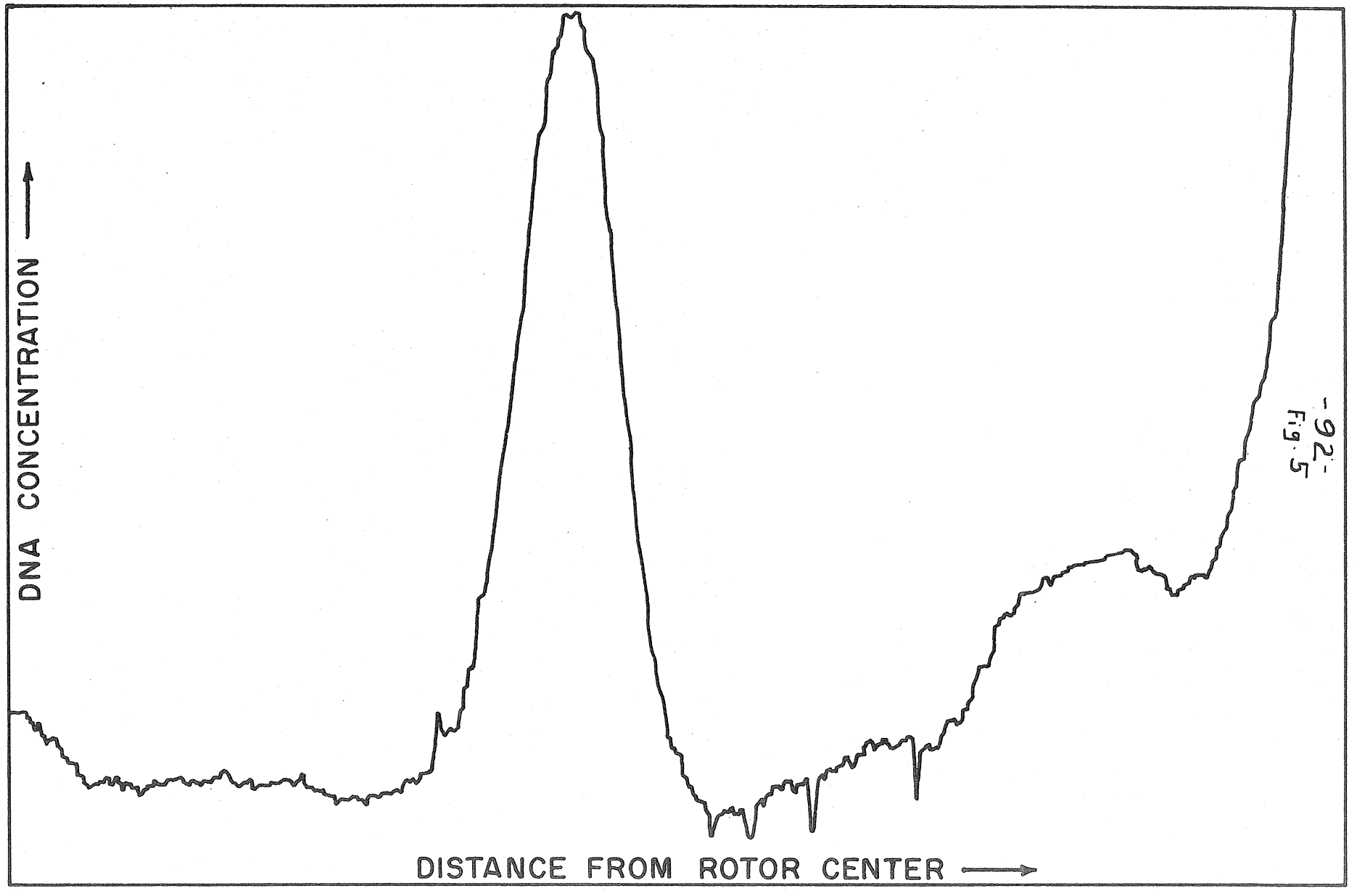

DNA CONCENTRATION
DISTANCE FROM ROTOR CENTER

Figure 5

Densitometer trace of DNA from HeLa cells after one division in BDU. Cells were harvested 36 hours (one division) after being plated into medium containing 20 γ/ml. Their DNA was isolated by the direct method as described in the text and banded in a CsCl density gradient at a speed of 44,770 rpm. The features of the band are independent of how the DNA was isolated. The hump to the right of the band is an artifact (See Fig. 6).

Fig 6



-92-
Fig. 5

Figure 6

Densitometer trace of normal and half substituted DNA from HeLa cells. Normal cells were added to the centrifuge cell containing the material of Fig. 5. Their DNA was isolated by the direct method, and banded at a speed of 44,770 rpm. The trace permits ready comparison of the differences between the two bands. Note that there is no trace of material to the right of the half substituted band.

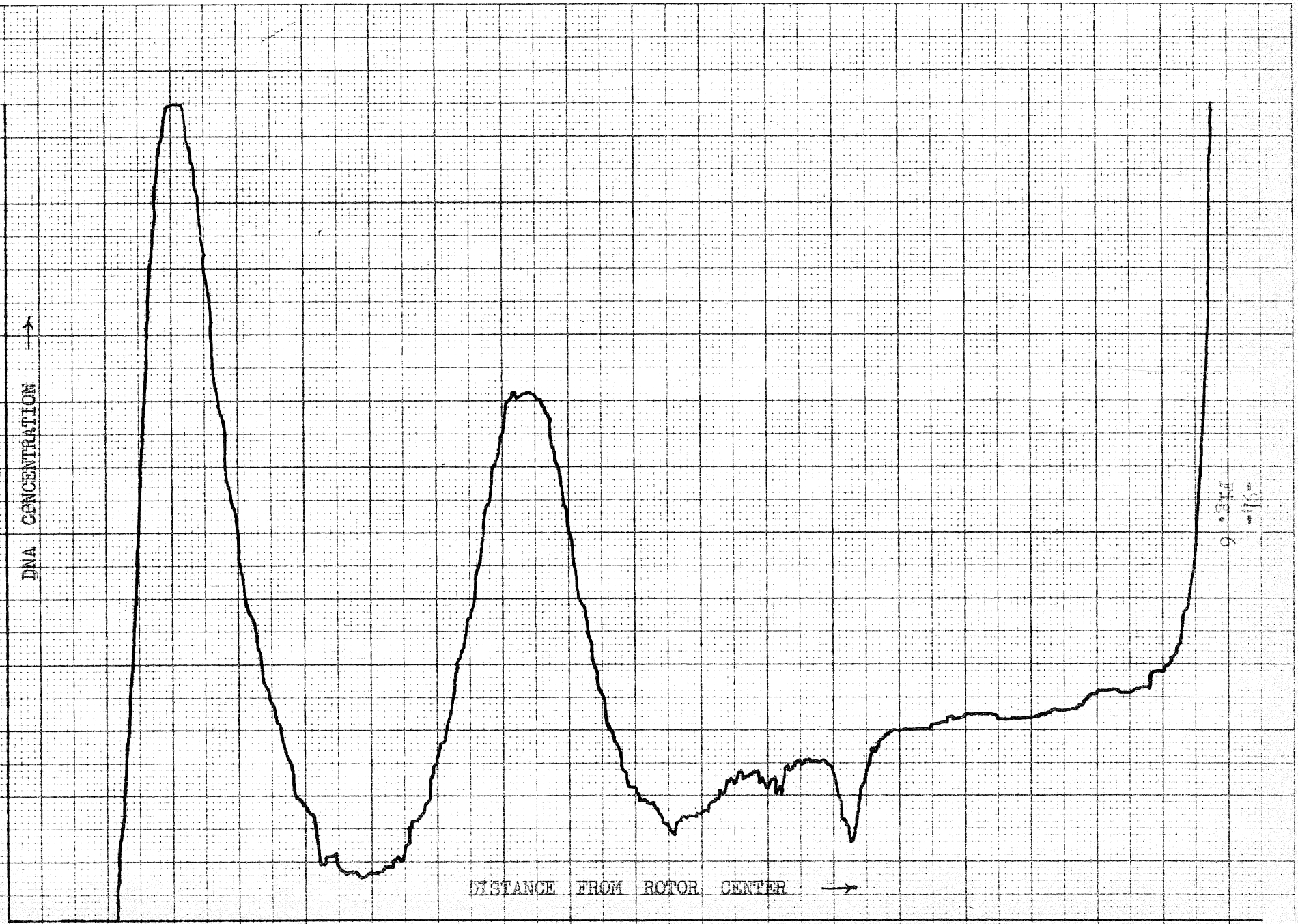

DNA CONCENTRATION →
DISTANCE FROM ROTOR CENTER →

Figure 7

Densitometer trace of CsCl blank. A centrifuge cell containing only CsCl of density 1.76 was run in the ultracentrifuge at 44,770 rpm for 14 hours. The trace shows the amount of differential UV light scattering caused by the CsCl gradient.

Fig. 7

Figure 8

Densitometer trace of DNA from HeLa cells after two divisions in BDU plus added normal DNA. Cells were harvested 55 hours after being plated into medium containing 20 γ/ml 5-bromodeoxyuridine. At this time all of the cells had divided once, and some twice. Their DNA was isolated by the phenol method as described in the text and banded in a CsCl density gradient at a speed of 44,770 (see Fig. 11). Normal DNA was then added to this preparation and the material again banded at a speed of 44,770 rpm.


DNA CONCENTRATION
DISTANCE FROM ROTOR CENTER

Figure 9

Densitometer trace of DNA from HeLa cells after two divisions in BDU. Cells were harvested 51 hours after being plated into medium containing 12 γ/ml 5-bromodeoxyuridine. At this time all of the cells had divided once, and some twice. Their DNA was isolated by the direct method as described in the text with normal HeLa DNA added as a marker; and banded in a CsCl density gradient at a speed of 44,770 rpm.

Fig. 9

Figure 10

Densitometer trace of DNA from HeLa cells after two divisions in BDU. Cells were harvested 51 hours after being plated into medium containing 12 γ/ml 5-bromodeoxyuridine. At this time all of the cells had divided once, and some twice. An aliquot of normal cells grown at the same time were mixed with the treated ones at a ratio of 1:4, and the DNA isolated by the phenol method as described in the text. The broken line shows the same run after absorption due to the CsCl base line (Fig. 7) is subtracted.

Fig 10

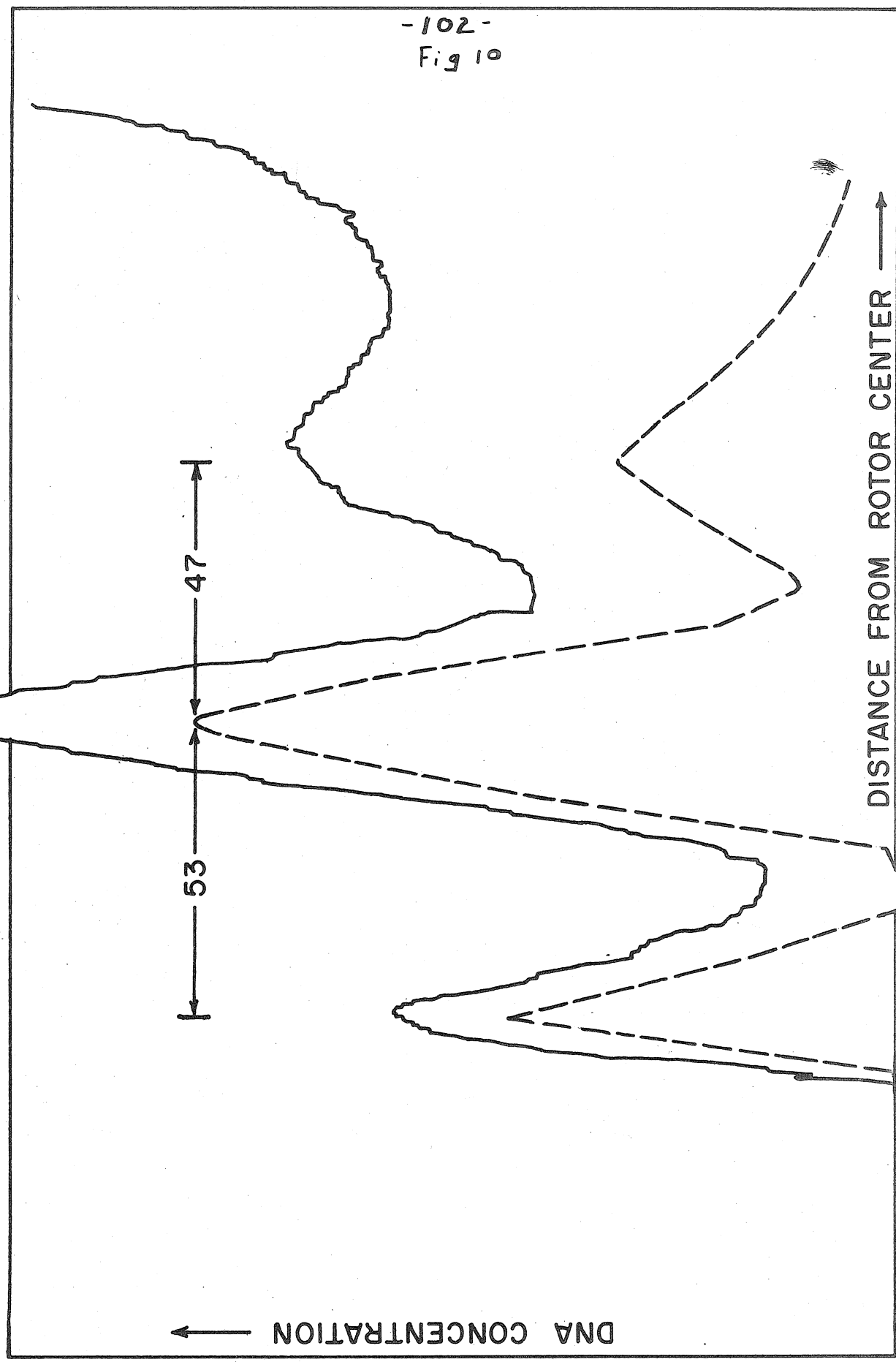


Fig 10,12

Figure 11

Densitometer trace of DNA from HeLa cells after two divisions in BDU. Cells were harvested 55 hours after being plated into medium containing 20 γ/ml 5-bromodeoxyuridine. At this time all of the cells had divided once, and some twice. Their DNA was isolated by the phenol method as described in the text and banded in a CsCl density gradient at a speed of 44,770 rpm.

DNA CONCENTRATION
DISTANCE FROM ROTOR CENTER

Figure 12

Alternative models for semi-conservative replication. Two models of DNA replication which are alternative to the one proposed by Watson and Crick are shown. Each predicts a semi-conservative mode of DNA replication.

(a) Medial model: the upper and lower halves of the DNA duplex are the conserved units.

(b) Two-strand model: Each DNA molecule is composed of two Watson-Crick double helices, each of which replicates by a conservative mechanism.

Fig. 12

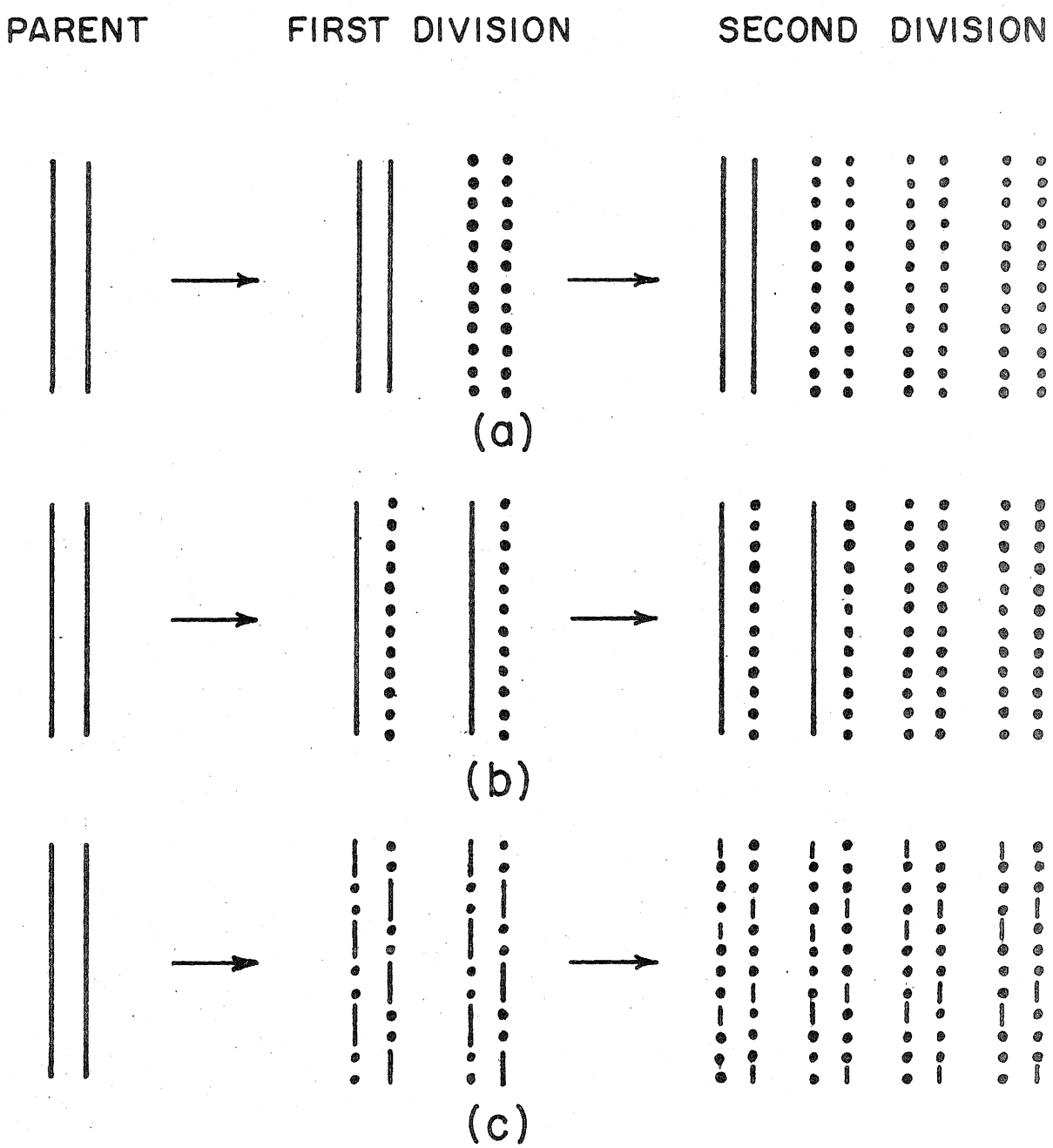


Figure 13

The effect of aminopterin on HeLa cells. Cells were transferred into 60 mm petri dishes at a concentration of 10^5 per plate. The curves represent growth of cells in:

Control = absence of analogs

A = 5 γ/ml aminopterin

A(C) = 10 γ/ml aminopterin for 15 hours, cells then washed 3 times with 2 ml TD and normal medium put on them

C(A) = control medium until second days count, then 5 γ/ml aminopterin added (arrow)

AT = 5 γ/ml aminopterin, 40 γ/ml thymidine

ATAdGl = 5 γ/ml aminopterin, 40 γ/ml thymidine, 40 γ/ml adenosine, 40 γ/ml glycine

Each point on the graph represents the ratio of the number of cells/2 circles/2 plates on day of count to the number of cells within the circles on the first day after plating.

Fig.13

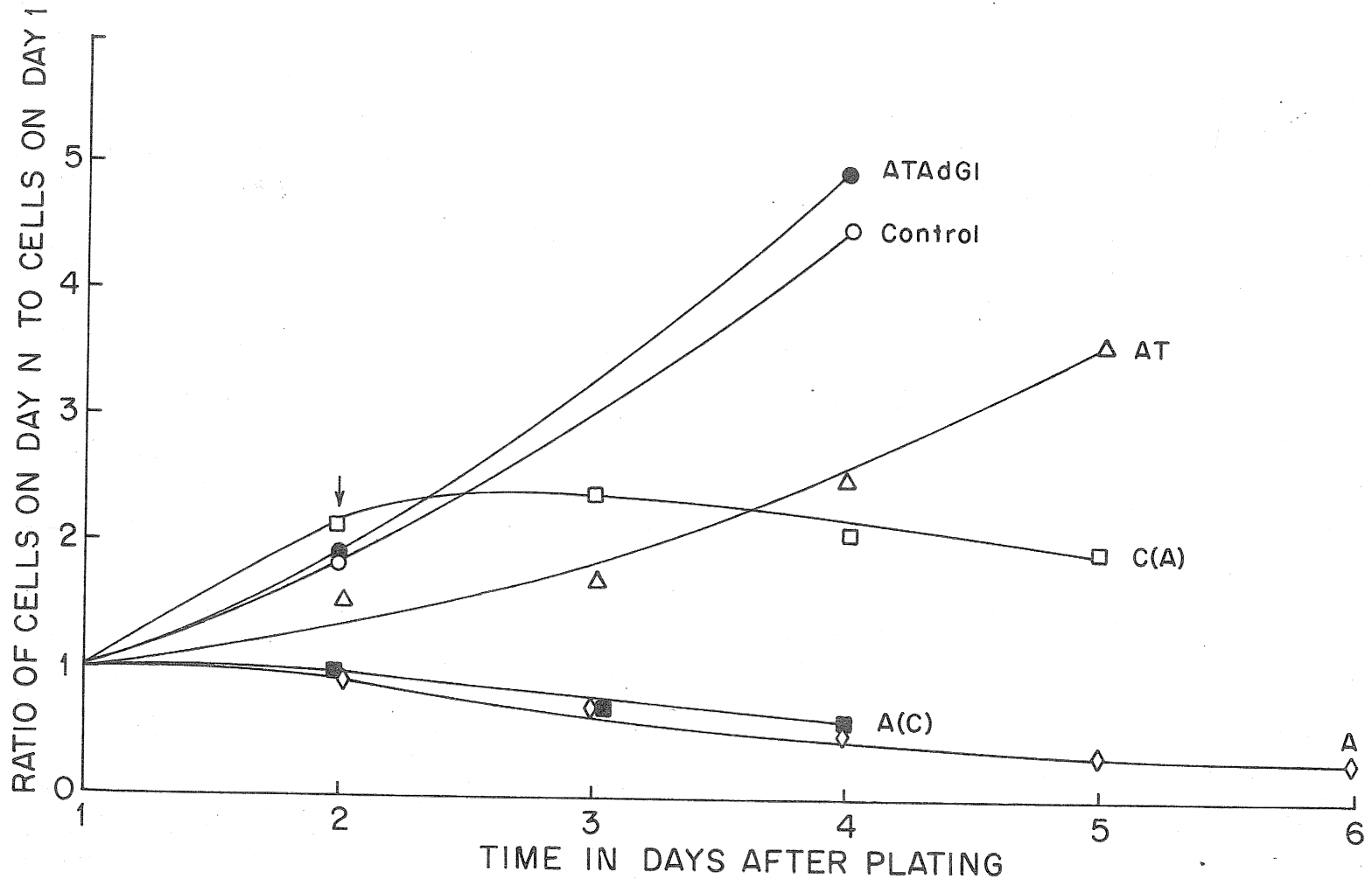

RATIO OF CELLS ON DAY N TO CELLS ON DAY 1
0
1
2
3
4
5
1
2
3
4
5
6
TIME IN DAYS AFTER PLATING
ATAdGI
Control
AT
C(A)
A(C)
A

Figure 14

The effect of 5-fluorouracil on HeLa cells. Cells were transferred into 60 mm petri dishes at a concentration of 10^5 per plate. The curves represent growth of cells in:

Control = absence of analogs

F = 1 γ/ml 5-fluorouracil (5FU)

FT = 1 γ/ml 5FU, 20 γ/ml thymidine

FUr = 1 γ/ml 5FU, 20 γ/ml uridine

F(C) = 1 γ/ml 5FU for 15 hours, cells then washed 3 times with 2 ml TD and normal medium put on them

C(F) = control medium until the second day's count then 1 γ/ml 5FU added (arrow)

Each point on the graph represents the ratio of the number of cells/2 circles/2 plates on day of count to the number of cells within the circles on the first day after plating.

Fig. 14

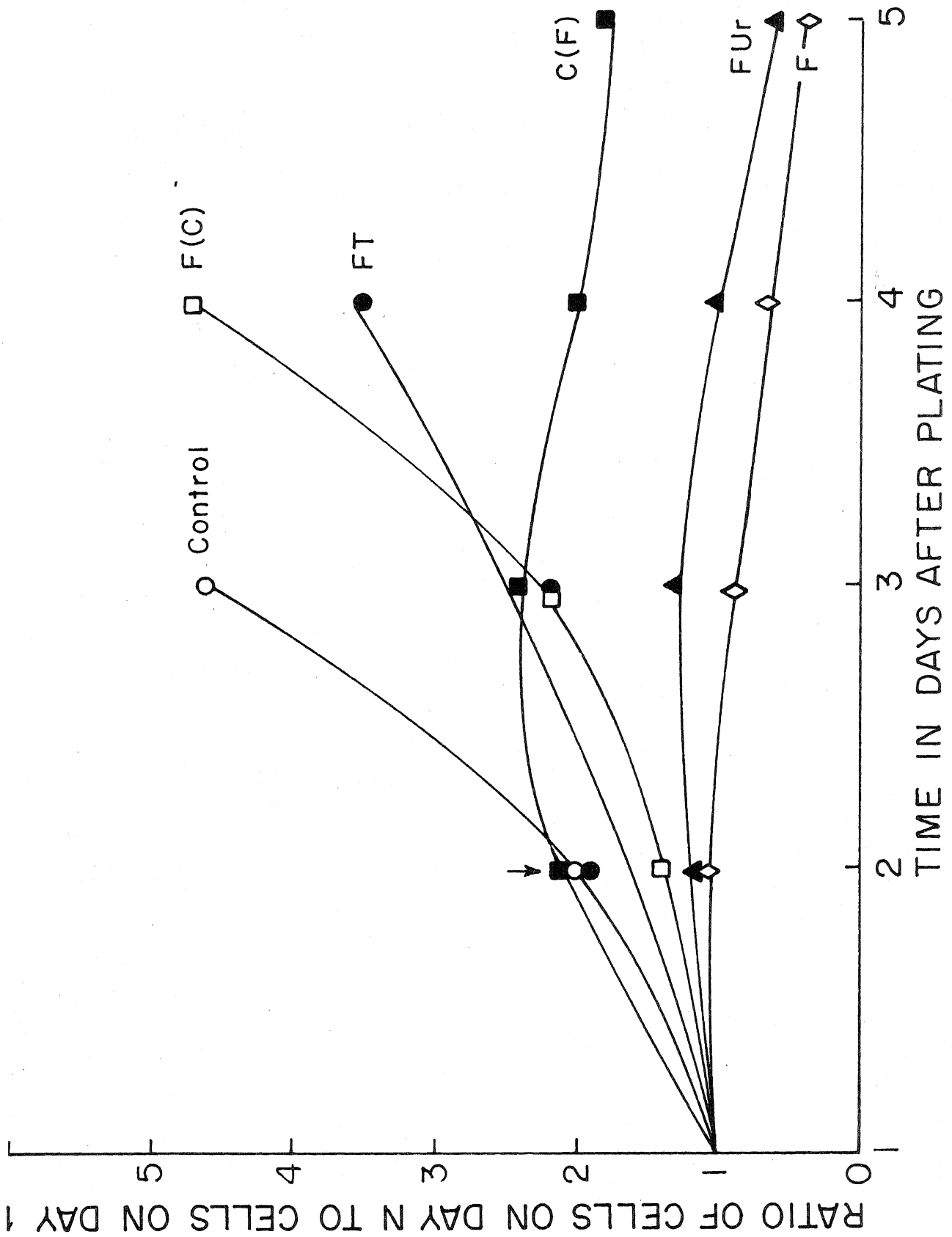

RATIO OF CELLS ON DAY N TO CELLS ON DAY 1
0
1
2
3
4
5
TIME IN DAYS AFTER PLATING
2
3
4
5
Control
F(C)
FT
C(F)
FUr
F

Figure 15

Effects of 5-bromodeoxyuridine on HeLa cells. Cells were plated directly into the indicated concentration (in γ/ml) of 5-bromodeoxyuridine (BDU) (first passage). 4 days after plating, the cells were trypsinized, and a suitable aliquot put into new plates (second passage) with the same concentration of BDU as the first passage cultures. Each point on the graph represents the ratio of the number of cells/2 circles/2 plates on day of count to the number of cells within the circle on the first day after plating.

Fig. 15

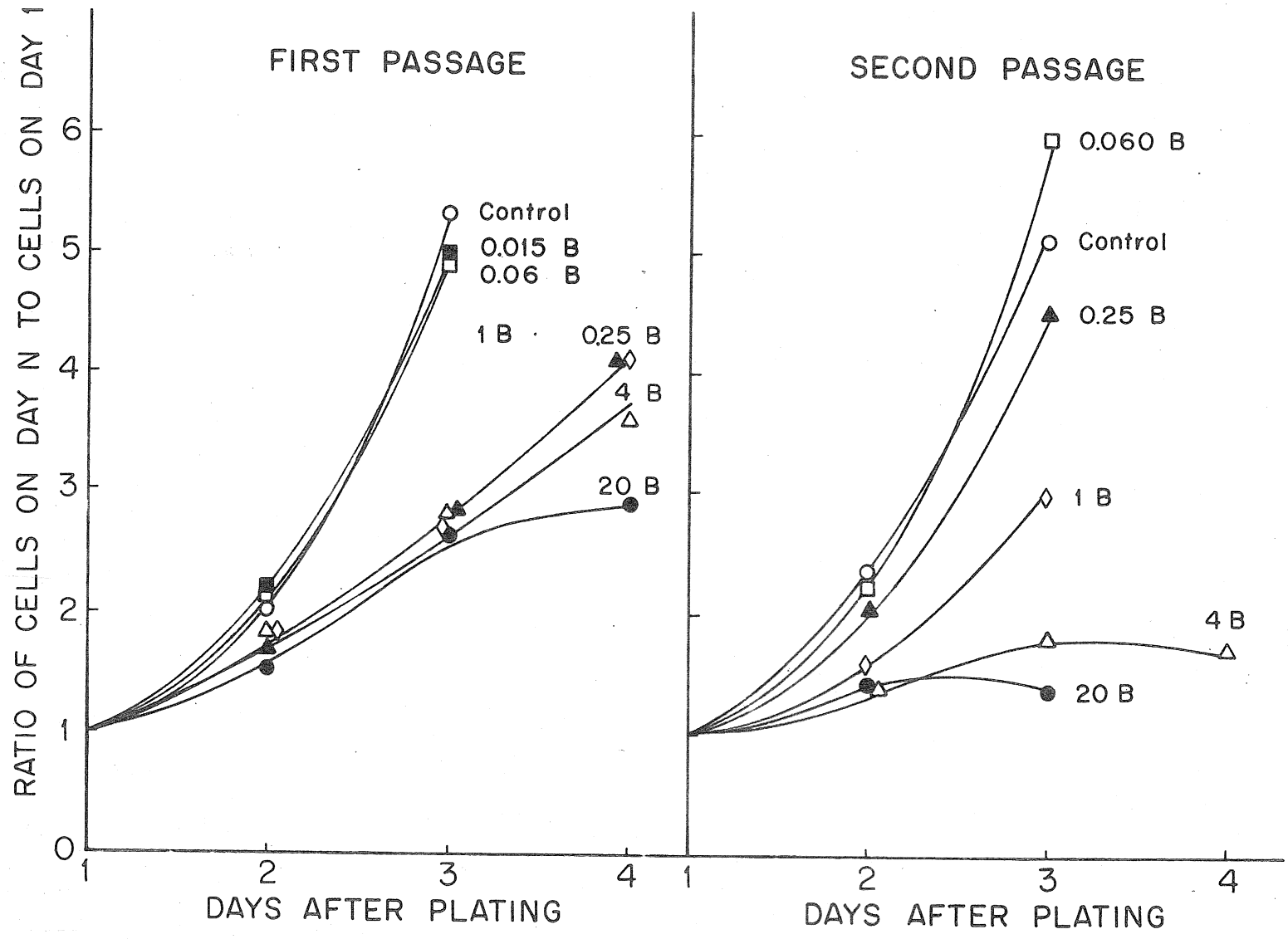

FIRST PASSAGE
SECOND PASSAGE
RATIO OF CELLS ON DAY N TO CELLS ON DAY 1
0
1
2
3
4
5
6
Control
0.015 B
0.06 B
1 B
0.25 B
4 B
20 B
0.060 B
Control
0.25 B
1 B
4 B
20 B
1
2
3
4
DAYS AFTER PLATING
DAYS AFTER PLATING

Figure 16

Densitometer trace of DNA from BDU resistant cells. The DNA of cells from a BDU resistant culture which had undergone approximately 38 divisions in the presence of 20 γ/ml BDU was isolated by the "direct" method (Thesis: part I, sec M) and banded in a CsCl density gradient at a speed of 44,770 rpm. The standard deviation of the band is 2 times larger than that of normal DNA isolated under the same conditions.

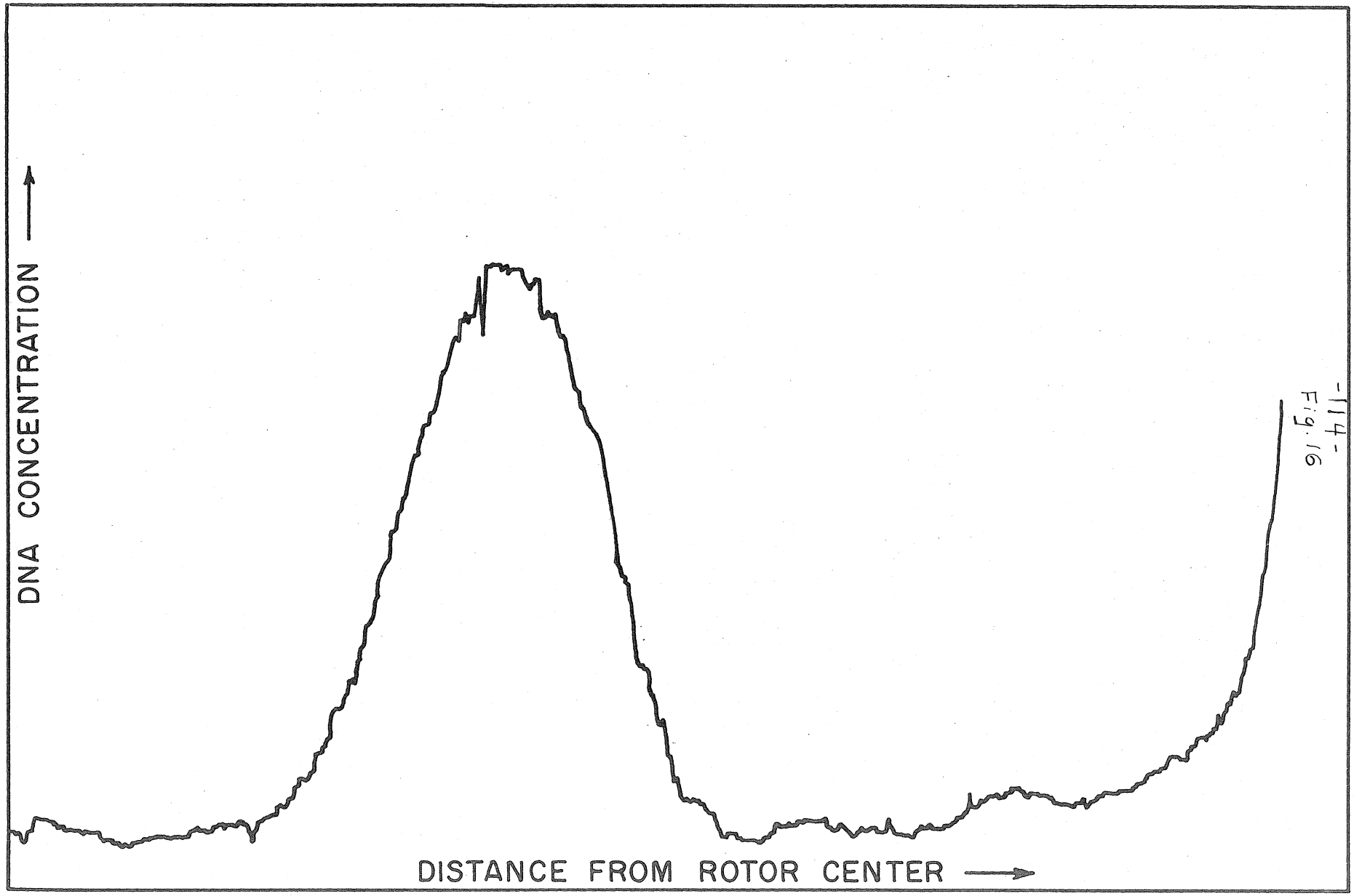


Fig. 16

Figure 17

Effects of aminopterin + BDU on HeLa cells. Cells were transferred into 60 mm petri dishes at a concentration of 10^5 per plate. The curves represent growth of cells in:

Control = absence of analogs

AB = 5 γ/ml aminopterin, 20 γ/ml 5-bromodeoxyuridine (BDU)

ABAdGl = 5 γ/ml aminopterin, 20 γ/ml BDU, 40 γ/ml adenosine, 40 γ/ml glycine

ATAdGl = 5 γ/ml aminopterin, 20 γ/ml thymidine, 40 γ/ml adenosine, 40 γ/ml glycine

Each point on the graph represents the ratio of the number of cells/2 circles/2 plates on day of count to the number of cells within the circles on the first day after plating. The first count of these experiments was made 11 hours after plating.

Fig. 17

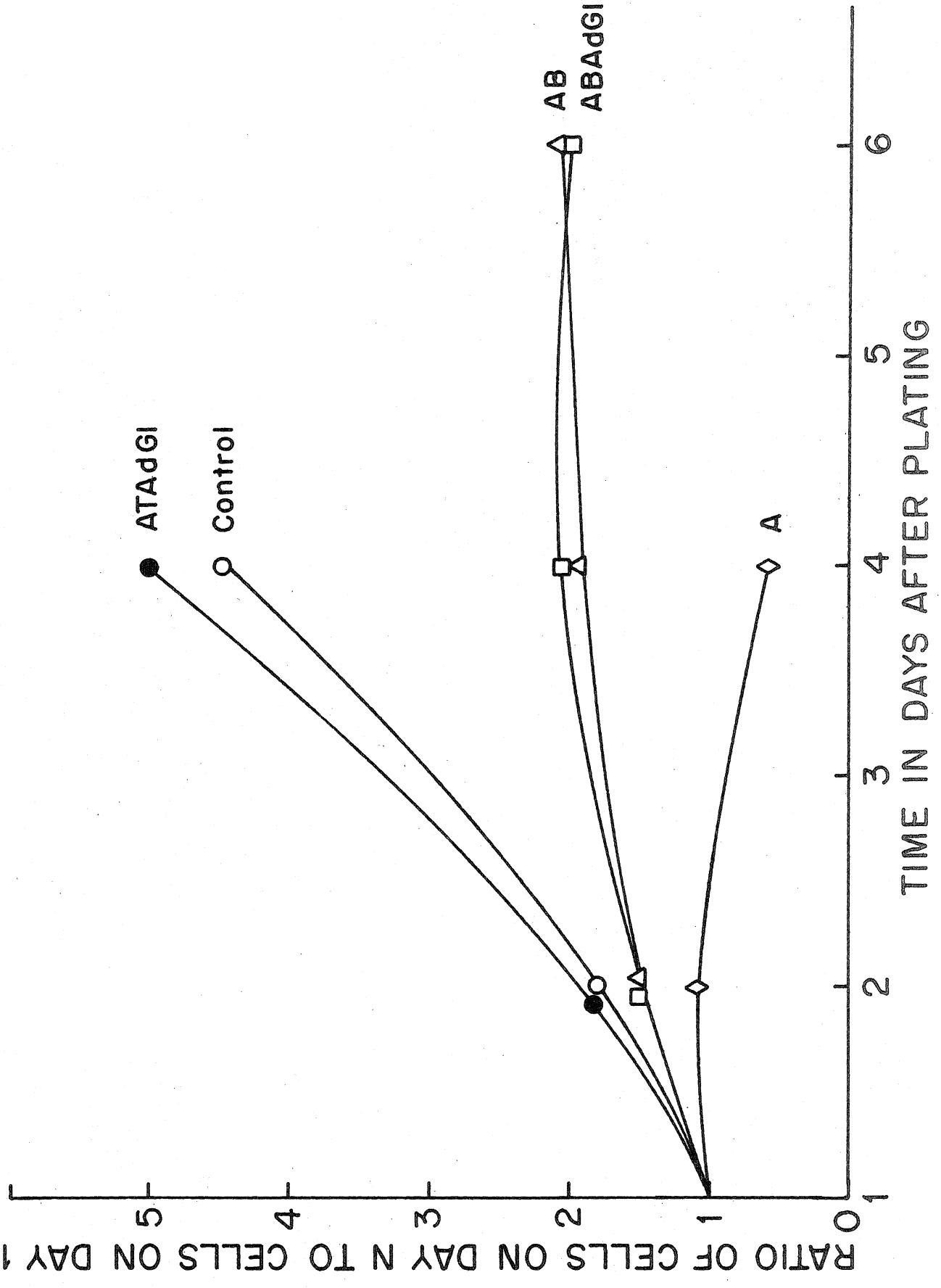

RATIO OF CELLS ON DAY N TO CELLS ON DAY 1
0
1
2
3
4
5
TIME IN DAYS AFTER PLATING
1
2
3
4
5
6
ATAdGI
Control
AB
ABAdGI
A

Figure 18

Effect of aminopterin on the growth curve of poliovirus. Plates containing 10^6 HeLa cells were infected with 5 pfu of poliovirus per cell. After an adsorption period of 30 minutes the cells were washed to remove unadsorbed virus. Cells plus supernates of the plates were sampled at the indicated times after infection and freeze-thawed before assaying. The virus was grown in normal cells (o) and cells which had been pre-treated for 24 hours with 10 γ/ml aminopterin and post treated during the infection period with the same amount (Δ). The final yield was approximately 100 pfu/cell from both treated and untreated cultures.

Fig. 18

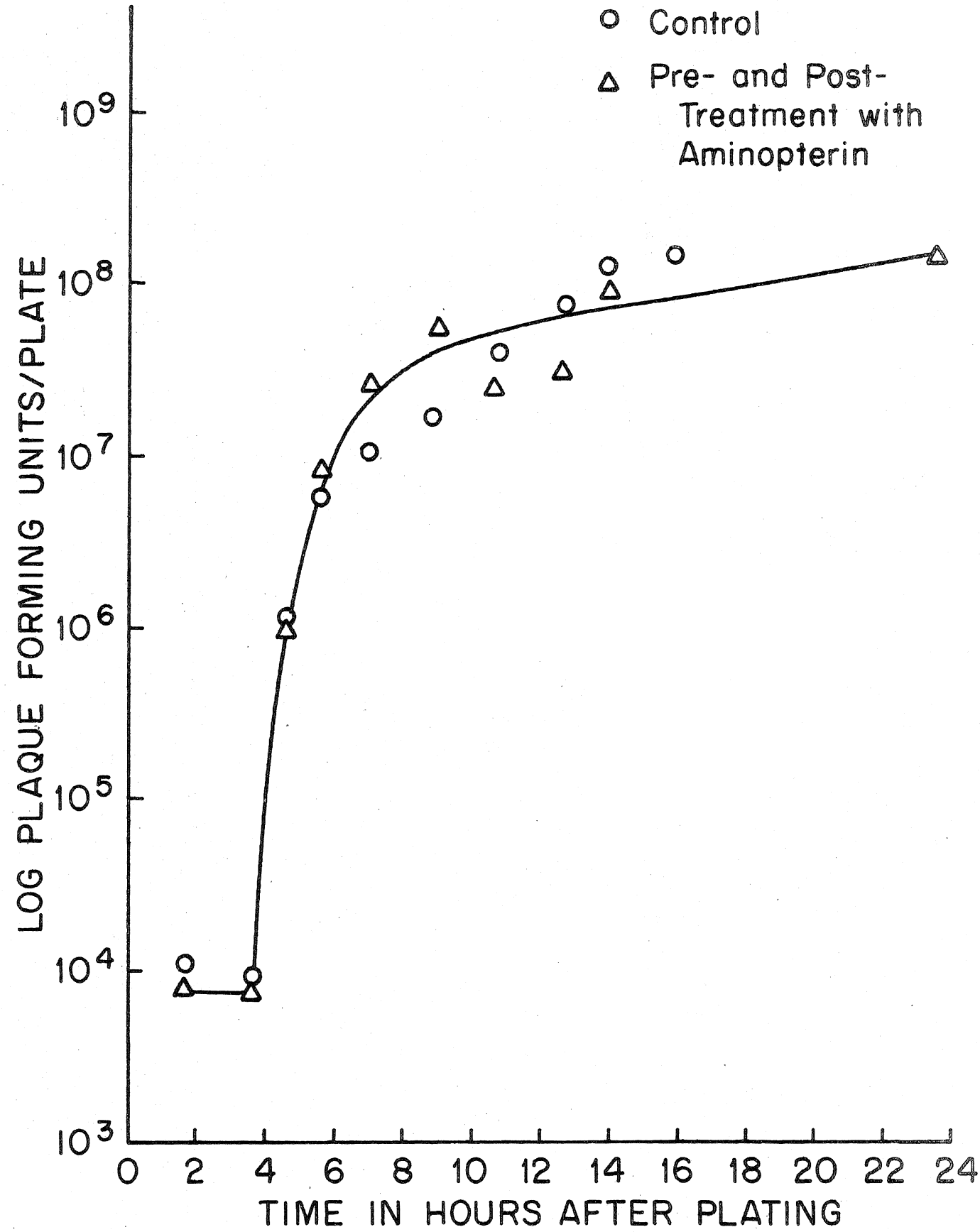
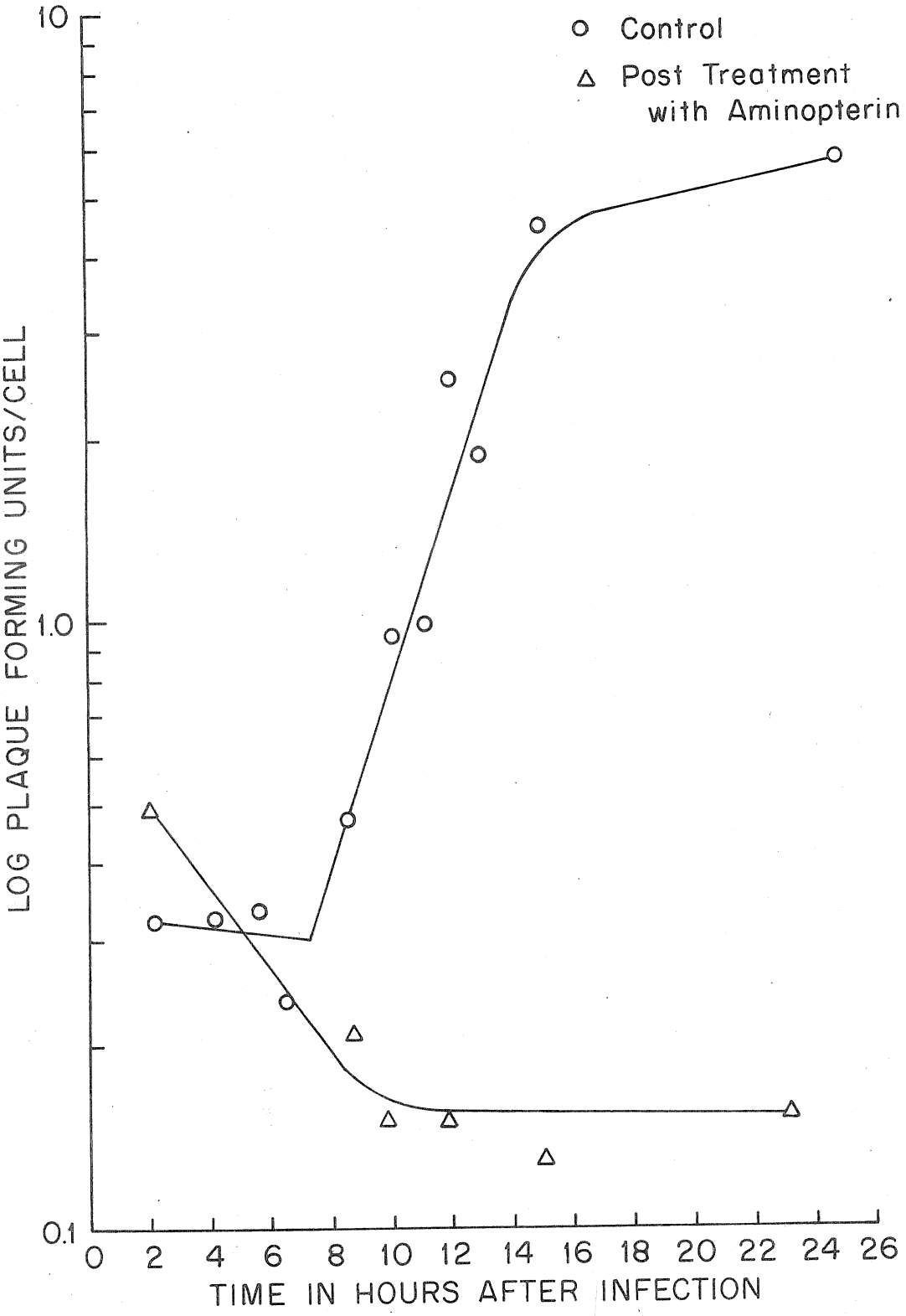

Control
Pre- and Post-Treatment with Aminopterin
LOG PLAQUE FORMING UNITS/PLATE
10^9
10^8
10^7
10^6
10^5
10^4
10^3
0 2 4 6 8 10 12 14 16 18 20 22 24
TIME IN HOURS AFTER PLATING

Figure 19

Effect of aminopterin on the growth curve of vaccinia virus. Plates containing 10^6 HeLa cells were infected with 5 pfu of vaccinia virus per cell. After an adsorption period of 60 minutes the cells were washed to remove unadsorbed virus. Cells plus supernates of the plates were sampled at the indicated times after infection and freeze thawed before assaying. The virus was grown in normal cells (O) and in cells which were post-treated during the infection period with 10 γ/ml aminopterin.

- 120 -
Fig 18


Control
Post Treatment with Aminopterin
LOG PLAQUE FORMING UNITS/CELL
10
1.0
0.1
0 2 4 6 8 10 12 14 16 18 20 22 24 26
TIME IN HOURS AFTER INFECTION

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