*RECOMBINATION DURING TRANSFORMATION IN HEMOPHILUS INFLUENZAE**

BY MARY JANE VOLL† AND SOL H. GOODGAL‡

DEPARTMENT OF BIOCHEMISTRY, JOHNS HOPKINS UNIVERSITY SCHOOL OF HYGIENE AND PUBLIC HEALTH

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In bacterial transformation, the irreversible uptake by a cell population of DNA extracted from a variant strain is followed by integration and expression of the variant "information" carried by the transforming DNA. Little is known of the details or mechanism of transformation after uptake of the DNA by a cell, i.e., of the process of integration which results in a permanent hereditary change. One must conclude from studies on linked transforming factors, however, that some process of recombination is involved.[1–3] The studies reported in this paper were designed to determine the time and some of the conditions for recombination during transformation.

Goodgal and Herriott have shown that a population of cells which has irreversibly taken up transforming DNA releases a large part of this activity when lysed shortly after uptake.[4] In the experiments to be described, the intracellular fate of transforming DNA has been followed quantitatively by this technique in the case of two linked markers located on the same DNA molecule.

Goodgal (3) has shown that in *Hemophilus influenzae* the two factors responsible for resistance or sensitivity to the antibiotics streptomycin and cathomycin (Novobiocin) are linked. When DNA is extracted from donor cells resistant to both cathomycin and streptomycin and is used to transform cells sensitive to both antibiotics, some 20 per cent of the resistant clones are endowed genetically with resistance to both antibiotics. The remainder of the transformations are to streptomycin resistance alone and to cathomycin resistance alone. The degree of linkage obtained depends to some extent on the method of preparation of the DNA and the conditions of transformation, but for material treated in the same way, the fraction of linked transformations is the same. The failure to retain complete linkage may be attributed to loss or inactivation of one or the other marker in the process of extracting the DNA from the donor cells, in the process of transformation itself, or in both processes.

One can transform to streptomycin or cathomycin sensitivity as well as to resistance. The frequency of transformation to sensitivity is the same as to resistance, and the same linkage relationships obtain.[3] Resistance and sensitivity thus may be considered as alleles of the same locus, the frequency of genetic incorporation of either or both factors being a function of the spatial relationships between the loci and not of the particular forms utilized in the test.[3] When, in the course of transformation, both streptomycin and cathomycin loci are genetically incorporated, linked transformations are obtained. When only one locus is genetically incorporated, transformations result which are recombinant with respect to the two markers in question, one marker supplied by the donor population and the other by the recipient population. This is the restricted manner in which the term recombination will be used in this paper, i.e., recombination between two linked loci on a single DNA molecule.

In the experiments to be described, cells marked by resistance to one antibiotic are transformed with DNA from cells resistant to the other antibiotic. At various times after uptake, samples of the recipient culture are lysed. Since lysis releases the total DNA content of the cells, both donor and recipient cell DNAs are released. Their transforming activities are then assayed on a double-sensitive strain. This technique enables one to follow the initial appearance and increase of linked transforming activities and affords insight into the sequences of intracellular events following DNA uptake. The results demonstrate that half the maximum amount of recombinant transforming activity, as measured by linkage of donor and recipient transforming factors, can be recovered from the recipient cell population within 15 minutes after a short uptake period. Furthermore, this genetic recombination occurs in the absence of either growth or appreciable increase in recipient transforming activity. Generally similar results were recently reported for pneumococcus by Fox and Hotchkiss.[9]

Materials and Methods.—Culture strains: Rd: A stock strain of *Hemophilus influenzae*, sensitive to less than 3 μg/ml streptomycin and to less than 1 μg/ml cathomycin. This strain was obtained originally from Alexander and Leidy.[8] S: A strain of Rd resistant to greater than 500 μg/ml streptomycin and sensitive to less than 1 μg/ml cathomycin, obtained by transforming into Rd a factor for one-step high-level streptomycin resistance. This marker was originally isolated by Alexander and Leidy.[8] C: A one-step mutant of Rd resistant to at least 2.5 μg/ml of cathomycin and sensitive to less than 3 μg/ml streptomycin.

Media: The growth medium used was a 50:50 mixture of Levinthal Stock and three per cent Eugonbroth (BBL), to which was added 2 μg/ml DPN.[5] For plating, a final concentration of 1.25 per cent Bacto-agar (Difco) was added. These media are referred to as Elev and Elev agar respectively.

For dilution of cells for plating, 3 per cent Eugonbroth (BBL) was used.

Transforming preparations: C DNA: A purified preparation of DNA[5] extracted from C (concentration of 0.5 mg DNA/ml). S DNA: Deoxycholate lysates of concentrated suspensions of S (approximate concentration of 0.02 mg/ml DNA).

Preparation of competent cultures: Highly competent cultures, i.e., cultures in which the majority of cells are capable of absorbing DNA, were grown by the aerobic-nonaerobic technique.[5]

Transformation frequencies of markers used: The frequency of transformation per unit of DNA to cathomycin resistance is twice that to streptomycin resistance.

Terms: Viable cells and transformed cells: the figures given for the number of viable cells or transformed cells refer to the number of colonies obtained upon plating, i.e., to viable centers of growth.

Experimental procedure: A competent culture of strain S containing approximately 1.5×10^9 viable cells per ml was diluted tenfold into Elev medium. An excess of C DNA was added and the mixture incubated at 36 $\pm$ 1°C with gentle mixing for five minutes, after which time DNAase and Mg^{++} were added to give a final concentration of 1 μg/ml DNAase and 0.003 M Mg^{++}. Incubation of the culture was continued with shaking. After allowing two minutes for the DNAase to destroy the unabsorbed DNA and at intervals thereafter, samples were removed and used for the determination of the total number of viable cells, the number of transformed cells, and the preparation of cell lysates.

In the determination of the total number of viable cells per ml, samples were diluted appropriately, mixed with nonselective Elev agar, and incubated. The number of transformed cells per ml was detected by an agar layer technique: appropriate dilutions were mixed with nonselective Elev agar, and after two hours incubation to allow time for expression of the transformed characteristic, one set of plates was overlaid with agar containing cathomycin and another set with agar containing both cathomycin and streptomycin. After the overlayer had hardened, the plates were reincubated. The dilutions were selected to give about 200 colonies per plate.

Lysates were prepared from samples containing about 6×10^8 viable cells. The samples were immediately heated for 10 to 20 minutes at 63 $\pm$ 2°C in order to stop the process of recombina-

tion. Such heating reduced the viability of the samples to 0.2 per cent of the original level or less. The heated samples subsequently were centrifuged, and the cells were washed with physiological saline containing 0.014 *M* sodium citrate and then resuspended in 2.5 or 3.0 ml of the citrate-saline fluid. Lysis was achieved by the addition of 0.16 ml of 2.0 per cent sodium deoxycholate and 0.2 ml of 1 *N* ammonium hydroxide. Following 15 minutes incubation, 0.2 ml of 1 *M* KH_2PO_4 was added to reduce the pH to approximately 8.[5]

Reciprocal experiments, in which competent C cells were treated with S DNA, were also performed following the same procedure outlined above.

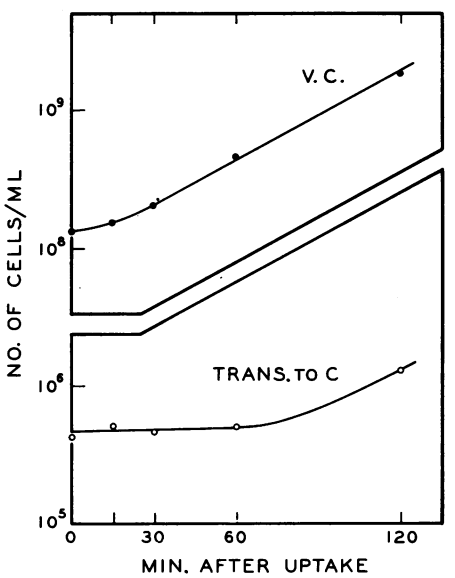

FIG. 1*a*.—Transformation of Recipient S Cells by C DNA. Closed circles are total number of viable centers (V.C.) and open circles are number of cathomycin-resistant viable centers (transformants).

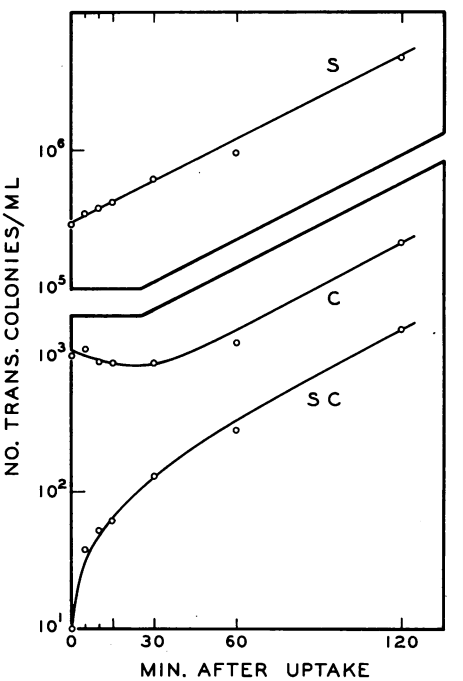

FIG. 1*b*.—Assay of Transforming Activity in Recipient Cell Lysates. *S*, streptomycin-resistant; *C*, cathomycin-resistant; *SC*, both cathomycin- and streptomycin-resistant. Assay mixture: 0.5 ml Rd + 4.45 ml 0.125 *M* saline + 0.05 ml lysate. To prepare the recipient cell lysates, 2.5 ml samples of the recipient culture were lysed at 0, 5, 10, 15, and 30 min. At 60 min, a $^1/_2$ dilution and at 120 min, a $^1/_{10}$ dilution of the recipient culture were lysed. In the graph, the 60-min and 120-min values have been multiplied by two and ten respectively. The *S* and *C* classes include the *SC* class.

The assay for transforming activity in the recipient cell lysates was performed by a slight modification of the routine transformation assay[5]—namely, by increasing the concentration of sensitive receptor cells in order to remove a larger part of the DNA from solution and thereby reduce the background of random doubles.[3] To 0.5 ml of competent Red cells diluted tenfold in 0.125 *M* saline, 0.05 or 0.1 ml of lysate was added, and the mixture was incubated for thirty minutes, after which time the numbers of transformations to streptomycin resistance, cathomycin resistance, and both streptomycin and cathomycin resistance were determined by the agar layer technique. The recovery of recipient transforming activity, i.e., the percentage of single cell equivalents in the lysates which gave recipient transformations in the assay, was 15 to 40 per cent, the

higher values being obtained when the recipient culture was C. The ratio of recovery of donor transforming activity to recovery of recipient transforming activity was high, closely approximating that expected on the basis of the number of cells transformed.

Experimental Results.—Recovery of recombinant transforming factor under conditions of normal growth of the recipient cell population: When a population of competent S cells was exposed to C DNA in Elev medium, the transforming activities present in the recipient cell population could be followed through several generations of growth. The results of such an experiment are presented in Table 1, in which the data from the assay are presented in the form of ratios, and in Figure 1, in which the numbers of transformed colonies obtained in the assay (1*B*) and the number of viable recipient cells and the number of transformed recipient cells (1*A*), are plotted as a function of time. Zero time in all experiments reported is two minutes after the addition of DNAase to the recipient cell population.

The data show that the recipient cell population, except for a possible slight lag, increased exponentially with the usual generation time of 30 minutes. The increase in recipient S DNA as measured by transforming activity, was proportional to the increase in recipient cells (Fig. 1). The recovery of donor C DNA remained constant or dropped slightly during the first 30 minutes after uptake (Fig. 1*B*), a fact indicating that there was either a lag in the replication of the introduced variant genetic information or that there was some loss of this information compensated for by a replication of the activity which remained. This lag showed up as a twofold drop in the donor/recipient ratio (C/S column, Table 1) during the first 30 minutes. By 30 minutes after uptake, the donor DNA was increasing at the same rate as the recipient DNA (Fig. 1*B* and Table 1, C/S).

A striking result was the early recovery of linked donor-recipient transforming activity (SC) from the recipient population (Fig. 1*B* and Table 1). Such linkage

TABLE 1

LINKAGE OF INTRODUCED C MARKER TO RECIPIENT S MARKER (ELEV):* RATIO OF TRANSFORMANTS IN THE ASSAY OF RECIPIENT CELL LYSATES

Time (min)	SC/C	C/S	SC/S
0	0.82×10^{-2}	3.4×10^{-3}	0.28×10^{-4}
5	3.5×10^{-2}	3.1×10^{-3}	1.1×10^{-4}
10	5.9×10^{-2}	2.3×10^{-3}	1.4×10^{-4}
15	7.2×10^{-2}	2.0×10^{-3}	1.5×10^{-4}
30	15×10^{-2}	1.4×10^{-3}	2.1×10^{-4}
60	23×10^{-2}	1.2×10^{-3}	2.9×10^{-4}
120	23×10^{-2}	1.4×10^{-3}	3.2×10^{-4}

Note: The S and C classes include the SC class.
* Elev is the growth medium used (see *Materials and Methods*).

could be detected within a few minutes after uptake. Study of the recombinant to recipient transformation ratio (SC/S column, Table 1) shows that about half the maximum amount of recombination, as measured by transforming activity, occurred within 15 minutes after uptake. Linkage of donor and recipient transforming factors appeared to be nearly complete at 30 minutes after uptake and to be complete by 60 minutes after uptake, since by this time the recombinant to recipient transformation ratio had reached a nearly constant value.

Similar results were obtained when the reciprocal experiment was performed, i.e., when the recipient cells were C and the donor DNA was S (Table 2).

TABLE 2

LINKAGE OF INTRODUCED S MARKER TO RECIPIENT C MARKER (ELEV)*

Time (min)	Recipient Population: Viable centers	Recipient Population: S transformants	Ratio of Transformants, Assay: SC/S	Ratio of Transformants, Assay: S/C	Ratio of Transformants, Assay: SC/C
0	1.8×10^8	4.4×10^5	0.62×10^{-2}	2.6×10^{-3}	0.16×10^{-4}
5	...	...	2.8×10^{-2}	1.7×10^{-3}	0.48×10^{-4}
10	...	...	5.3×10^{-2}	1.4×10^{-3}	0.73×10^{-4}
15	...	...	8.3×10^{-2}	0.71×10^{-3}	0.61×10^{-4}
30	3.4×10^8	9.1×10^5	15×10^{-2}	0.69×10^{-3}	1.1×10^{-4}
60	6.5×10^8	1.1×10^6	21×10^{-2}	0.53×10^{-3}	1.1×10^{-4}
120	2.6×10^9	3.4×10^6	20×10^{-2}	0.66×10^{-3}	1.3×10^{-4}

Note: The S and C classes include the SC class.
* Elev is the growth medium used (see *Materials and Methods*).

Recombination in the absence of growth of the recipient population: To see whether linkage of donor and recipient transforming factor was dependent on growth of the recipient population, experiments were performed in saline where growth was sharply reduced. Growth of the recipient population was inhibited by centrifuging and resuspending a competent recipient culture in an equal volume of saline. Donor DNA was added to the resuspended cells, uptake was terminated after five minutes, and the culture was further incubated for 60 minutes. In the experiment for which data are presented in Figure 2 and Table 3, there was essentially no growth of the recipient cell population after uptake of DNA, as measured both by colony count and turbidity, and there was little if any increase in recipient cell transforming activity (top curve, Fig. 2). Nevertheless by 30 minutes after uptake, a considerable amount of linkage of donor and recipient transforming factors

TABLE 3

LINKAGE OF INTRODUCED S TO RECIPIENT C (SALINE)

Four ml of a competent culture of C cells were centrifuged and resuspended in 3.5 ml of 0.125 *M* saline and placed in a 36°C water bath. Then 0.3 ml of S DNA was added and five minutes later 2.5 μg/ml DNAase and 0.003 *M* Mg^{++} were added and the culture was incubated further, without shaking. At the times listed 0.3 ml samples were removed, diluted into 1.2 ml of citrate saline, and immediately heated for twenty minutes at 64°C. The heated samples were centrifuged, resuspended in 3.0 ml of citrate saline, and lysed as described in the text. The recipient culture was also sampled at the stated times for a determination of viable and transformed cells. Turbidity measurements were made on a Coleman Junior Spectrophotometer.

The recipient cell lysates were assayed for transforming activity as described in the experimental procedure. Two assays were performed using 0.1 and 0.05 ml of lysate respectively. The results of the assay are plotted in Figure 2. In the table and in the graph, the S and C classes include the SC class.

RECIPIENT CELL CULTURE

Time after uptake (min)	Viable centers	S transformants	SC transformants	Turbidity
0	1.4×10^9	8.4×10^6	3.6×10^6	0.18
30	1.5×10^9	1.4×10^7	4.0×10^6	0.19
60	1.5×10^9	1.1×10^7	3.2×10^6	0.185
120	1.2×10^9	7.6×10^6	1.9×10^6	...

ASSAY: RATIO OF TRANSFORMANTS

Time after uptake (min)	SC/S $\times 10^{-2}$: 0.10 ml lysate	SC/S $\times 10^{-2}$: 0.05 ml lysate	S/C $\times 10^{-3}$: 0.10 ml lysate	S/C $\times 10^{-3}$: 0.05 ml lysate	SC/C $\times 10^{-4}$: 0.10 ml lysate	SC/C $\times 10^{-4}$: 0.05 ml lysate
0	0.59	0.48	2.8	3.3	0.17	0.16
30	9.2	8.6	1.6	1.8	1.5	1.5
60	11	9.4	1.9	2.2	2.0	2.0
120	13	14	1.6	1.7	2.1	2.3

had occurred (bottom curve, Fig. 2, and SC/S and SC/C columns, Table 3). The results of this experiment have been confirmed by a reciprocal experiment in which the recipient S population after resuspension in saline was preincubated for 15 minutes before being exposed to C DNA. Once again there was no detectable

increase in viable count, turbidity, or recipient transforming activity of the recipient cells after uptake. By 60 minutes after uptake, the recombinant/donor DNA assay ratio was 0.16, a value which compares favorably with the ratio obtained for a similar time under conditions of growth (see Table 1).

Nature of the recombinant linkage: Evidence that recombinants do not represent a spurious linkage was obtained by the following procedure. The DNA from the recipient population was isolated and purified following uptake of donor DNA. The ratio of recombinant to donor transforming activity in the purified DNA was not appreciably altered from that obtained with crude material. In addition, this ratio was independent of the concentration of DNA.

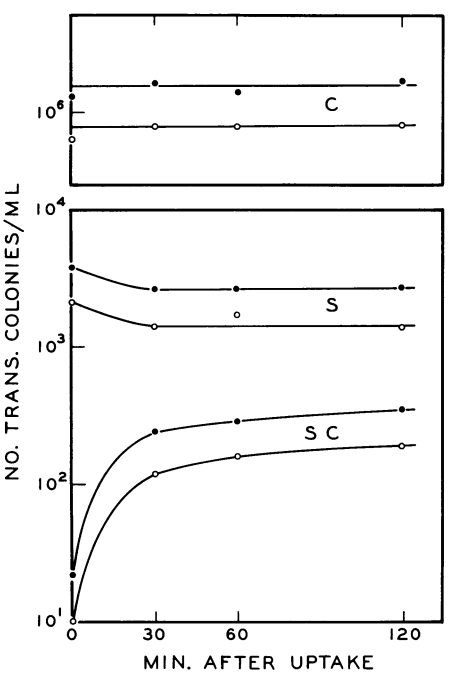

FIG. 2.—Figure 2 is explained in the Legend for Table 3. Open circles, 0.5 ml of lysate; closed circles, 0.10 ml of lysate. *S*, streptomycin-resistant; *C*, cathomycin-resistant; *SC*, treptomycin- and cathomycin-resistant.

A Sevag purification[7] of the recipient culture was performed following a 5-minute uptake period and 30 minutes' further incubation. Because of the small quantity of starting material, the recovery yield was low, constituting about 10 per cent of the total DNA in the cells. The ratio of U.V. absorption at 260 mμ to that at 230 mμ was 2.4, indicating that the DNA was not appreciably contaminated with protein, which absorbs highly at 230 mμ. The E260 and E230 values for purified DNA preparations given by Chargaff[10] are calculated to give ratios around 2.4, which value is obtained for purified DNAs in this laboratory. As shown in Table 4, a recombinant to donor ratio of 0.12 was obtained for the purified DNA. This value is close to those obtained for crude material under similar experimental conditions (Table 1). Furthermore, this ratio remained constant over a hundred-fold dilution range of the purified DNA preparation.

TABLE 4

CONSTANCY OF LINKAGE OF S AND C AT VARYING DILUTIONS OF NEWLY FORMED RECOMBINANT DNA

100 ml of a competent S culture was exposed to 0.1 ml of C DNA for five minutes at 36°C. DNAase was added and the culture was continued in incubation. 32 minutes after the addition of DNAase, the culture was heat-inactivated, and DNA was extracted from the cells by the Sevag procedure. 0.1 ml of the given dilutions of the extracted DNA was assayed for transforming activity by the method outlined under *Experimental procedure*.

DNA dilution	No. transformed colonies			Recombinant donor ratio
	S	C	SC	SC/C
2×10^{-1}	3.1×10^6	2.5×10^4	2.9×10^3	0.12
2×10^{-2}	7.6×10^5	7.1×10^3	8.2×10^2	0.12
2×10^{-3}	8.3×10^4	8.5×10^2	9.8×10^1	0.12

Note: The S and C classes include the SC class.

Discussion.—The process of recombination between linked loci in *Hemophilus*

influenzae takes place within a relatively short time after uptake of DNA by the recipient cells. The half-maximum amount of recombination is attained within 15 minutes after a short exposure of recipient cells to DNA. That the recombinant transforming activity found in the recipient cell lysates after uptake results from linkage of donor and recipient transforming factors inside the recipient cell and is not a result of events following lysis is shown by the following observations:

1. The recombinant transforming activity increases as a function of time after uptake even under conditions where there is little or no increase in donor or recipient transforming activities.

2. The recombinant activity, present 30 minutes after uptake in the crude DNA extracts (lysates), is retained during purification.

3. Upon dilution of crude or purified recipient cell lysates, the ratio of recombinant to donor, or to recipient, transformations remains constant.

If the recombinant transforming activity found in the recipient cell lysates resulted from agglomeration of DNA molecules in solution, such activity should have been lost in purification and in dilution of the lysate. If the recombinant transformations obtained in the assay were the result of random double transformations, their number should vary as the product of the frequency of the single transformations to streptomycin and cathomycin resistance, i.e., as a square function.[3] As noted above, both kinetic and dilution considerations eliminate this possibility. A small number of random double transformations is expected under the conditions of the assay. However, they represent a significant portion of the SC transformations only when the number of recombinants is still low (0 minute lysates).

Under conditions of growth it was found that by 30 minutes after uptake, donor transforming factor was increasing at the same rate as recipient transforming factor. This was surprising, since in the *Hemophilus influenzae* transformation system there is a characteristic lag of two to three generations before the number of transformants in the recipient population begins to increase (Fig. 1*A*). Only the initial delay in increase of transformants following uptake must be due to lag in the formation or increase of donor DNA replicas. Some of the additional lag after this time may be attributed to the fact that the viable center of growing cultures consists largely of two joined cells.[5] This would mask an initial increase in the number of transformed cells, since the daughter progeny would tend to remain together and give rise to only one resistant clone. If in addition it is assumed that there are two nuclei or half units per cell, only one of which is altered by the incoming DNA, the entire delay in increase of transformed recipient viable centers would be adequately accounted for.

We conclude from the experiments performed in saline that recombination can occur in the absence of growth and with less than 15 per cent synthesis of genetically functional DNA. This DNA was determined by measuring the transforming activity of the recipient cells. No more than 15 per cent of such synthesis should have escaped our attention by this procedure. Since increase in total DNA and increase in transforming activity are closely correlated,[6] it is clear that a large fraction of the recombination which occurs under conditions of growth also occurs when not more than 15 per cent of normal DNA synthesis is occurring.

Whatever mechanism of recombination might be proposed, it is clear that a

normal level of DNA synthesis is not necessary for that process. Two possibilities suggest themselves: (1) that the mechanism of recombination is a conservative one, with little or no concomitant DNA synthesis, or (2) there occurs a special kind of synthesis, restricted primarily to DNA molecules which have penetrated the cells and a portion of the DNA inside the cells.

The results indicate that transforming factors provide a most fruitful approach to the study of genetic recombination on the molecular level.

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*PURIFICATION AND IMMUNOLOGICAL CHARACTERIZATION OF TYPES 4 AND 5 ADENOVIRUS-SOLUBLE ANTIGENS**

BY WESLEY C. WILCOX AND HAROLD S. GINSBERG

DEPARTMENT OF MICROBIOLOGY, SCHOOL OF MEDICINE, UNIVERSITY OF PENNSYLVANIA

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It has become increasingly apparent in many systems that the interaction of animal virus and host cell results in the production of antigenic materials readily separable from virus.[1–5] Although in most instances little is known about the nature or origin of these antigens, evidence has been presented in at least two instances which may be interpreted to indicate that certain of these materials are related to infectious virus, either as virus precursor materials,[6] or alternatively, as materials released by the degradation of newly synthesized virus.[7]

A primary obstacle to a more thorough study of such antigens as regards their relationship to the virus synthetic process has been the problem of separating these materials, first from normal cell components, and secondly, one from the other, so that each could be characterized independently of the others.