THE DISINTEGRATION OF INFLUENZA VIRUS PARTICLES ON ENTRY INTO THE HOST CELL

STUDIES WITH VIRUS LABELLED WITH RADIOPHOSPHORUS

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Studies of the growth of influenza virus in the chorio-allantoic membrane of the fertile egg in recent years have shown that it is probable that on entry into the host cell the virus particle becomes disintegrated into smaller units which are replicated and later aggregated into complete virus.

The evidence for the disintegration of the infecting particle is indirect.

(1) Saline extracts of the infected chorio-allantoic membrane made 1 hr. after inoculation contain no virus demonstrable by serological methods, and the infectivity of such extracts is less than 1 % of that which would be expected from the amount of virus shown to have entered the cells (Hoyle, 1948, 1950; Henle & Henle, 1949).

(2) The first evidence of multiplication of virus in the cells is the appearance in cell extracts of complement-fixing particles of smaller size than the infecting virus (soluble antigen) and later the appearance of haemagglutinating particles which are lacking in infectivity. (Hoyle, 1948, 1950; Henle & Henle, 1949).

(3) If cells are infected simultaneously with two different strains of virus, infective particles may later be recovered which have properties derived partly from one strain of the virus and partly from the other, suggesting preliminary disintegration followed by cross recombination (Burnet & Lind, 1951).

(4) No particles of the size and morphology of infective virus can be demonstrated in the infected cell by electron microscopy in the early stages of multiplication (Wyckoff, 1953).

(5) Infective virus particles can be disintegrated *in vitro* by treatment with ether with the release of complement-fixing 'soluble antigen' particles and also of haemagglutinating particles of small size (Hoyle, 1950, 1952).

The demonstration by Graham & McLelland (1949, 1950), Hoyle, Jolles & Mitchell (1954) and Liu, Blank, Spizizen & Henle (1954) that influenza virus could be labelled with radiophosphorus afforded a possible method of detecting the fate of the infecting particle on entry into the host cell. This paper describes the behaviour of ^{32}P labelled virus introduced as a primary inoculum in fertile eggs and affords direct evidence that the infecting particle becomes disintegrated on entry into the host cell.

PREPARATION OF VIRUS LABELLED WITH ^{32}P

Graham & McLelland (1949) showed that when influenza virus was grown in fertile eggs in which radioactive phosphorus had been introduced into the allantoic sac the virus incorporated ^{32}P into its structure, and Graham (1950) showed that both

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the nucleic acid and the phospholipid were labelled. By increasing the dose of ^{32}P Hoyle *et al.* (1954) and Liu *et al.* (1954) produced virus preparations much more heavily labelled than those of Graham. For the purpose of the present work it was necessary to obtain very heavily labelled virus, and it was found that the dose of ^{32}P could be increased to 1 millicurie per egg, and when this was done preparations of virus could be obtained in which the Geiger count per minute per ml. was of the same order as the haemagglutinin titre. With such preparations it became possible to follow the fate of radioactive virus introduced as a primary inoculum, though it was necessary to use relatively large inocula in order to obtain the maximum take up of virus by the chorio-allantoic membrane.

Most of the work in this paper has been done with virus labelled by the technique used by Hoyle *et al.* (1954) with the modification that the dose of ^{32}P per egg was increased from 100 microcurie to 1 millicurie. The technique consists of growing the virus in eggs inoculated into the allantoic sac with 1 millicurie of ^{32}P in the form of disodium phosphate, collecting the infected allantoic fluid and concentrating and purifying the virus by two cycles of adsorption-elution from guinea-pig red cells, the cells carrying the adsorbed virus being washed several times with ice-cold saline before elution to remove contaminating inorganic ^{32}P. The D.S.P. strain of influenza virus A was used throughout the work.

Geiger counts were made by the technique used by Hoyle *et al.* (1954), 1 ml. fluid samples being placed in shallow metal dishes beneath a Geiger counter of the end-window type.

PROPERTIES OF LABELLED VIRUS PREPARATIONS

(1) *Adsorption with red cells*

The labelled virus preparations usually contained some residual inorganic ^{32}P. The amount of this could be assessed by adsorbing the preparation with red cells and determining the residual radioactivity of the supernatant. Table 1 shows the results obtained with five preparations. The preparations had an average haem-

Table 1. *Radioactivity of concentrated virus preparations made from eggs inoculated with* 1 *millicurie of* ^{32}P

Preparation	Haemagglutinin titre	Geiger count per min/ml.	Geiger count after R.B.C. adsorption	Virus radioactivity (c.p.m./ml.)
1	12,800	3,655	315	3,340
2	10,240	10,740	385	10,355
3	15,360	6,260	590	5,670
4	7,680	4,210	220	3,990
5	16,385	10,472	664	9,808
Average	12,500	7,068	435	6,633

agglutinin titre of 12,500 and an average Geiger count of 7068 c.p.m./ml. Of this radioactivity 94 % was carried by the virus particles and was adsorbable on the red cells, while 6 % was due to contaminating non-viral ^{32}P.

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(2) *Centrifugation*

The above result was confirmed by a centrifugation experiment. A 1/5 saline dilution of a labelled virus preparation was divided into two parts. One part was adsorbed twice with 10 % guinea-pig red cells and the radioactivity of the original and adsorbed fluids tested. The second part was centrifuged for 1 hr. in a Spinco centrifuge against a sucrose density gradient at an average centrifugal force of 26,000 *g*. The radioactivity of the original fluid and the 26,000 *g* supernatant was measured:

Preparation	Geiger count (c.p.m./ml.)	Non-virus ^{32}P (%)
Original labelled virus	731	8·6
Adsorbed with red cells	63	
Original labelled virus	728	8·6
26,000 *g* supernatant	63	

91·4 % of the ^{32}P of the labelled virus preparation was carried by the virus and could be adsorbed on red cells or sedimented by centrifugation for 1 hr. at 26,000*g*.

(3) *Chemical precipitation*

If labelled virus is suspended in a saline extract of normal chorio-allantoic membrane the virus can be precipitated by half saturation with ammonium sulphate.

0·1 ml. of labelled virus preparation was added to 1·0 ml. of saline extract of normal chorio-allantoic membrane. The 0·1 ml. of labelled virus had a Geiger count of 272 c.p.m., of which 257 represented virus ^{32}P adsorbable on red cells. An equal volume of saturated ammonium sulphate was added and the fluid centrifuged at 6000 *g* for 10 min. The deposit redissolved in water gave a Geiger count of 250 c.p.m., representing 97 % of the virus ^{32}P.

(4) *Determination of lipid and nucleic acid radioactivity*

The radioactivity of ^{32}P labelled virus is partly due to phospholipid and partly to nucleic acid. The relative amounts of lipid and nucleic acid ^{32}P can be determined by the following method.

1 ml. of a 1/5 dilution of labelled virus is adsorbed with 10 % guinea-pig red cells. The cells are centrifuged out and suspended in a minimum quantity of saline. Ten volumes of ethanol are then added and the mixture allowed to stand overnight. After removal of the supernatant the residual denatured red cells are again extracted with ethanol. The Geiger count of the pooled ethanol extracts gives a measure of the phospholipid ^{32}P. Nucleic acid ^{32}P is determined by dissolving the denatured red cells in N/1-sodium hydroxide and counting after dilution with saline. The following example illustrates the method:

Preparation	Geiger count (c.p.m./ml.)	
Original labelled virus 1/5	563	
Adsorbed with red cells	14	
Virus ^{32}P, by subtraction	549	
Ethanol extract of red cells	190	553
Residual red cells in NaOH	363	

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It was concluded that 34 % of the virus ^{32}P was present as phospholipid and 66 % as nucleic acid.

The proportion of virus radioactivity due to phospholipid varies in different preparations from 20 to 50 %. It was found that when ^{32}P was introduced into fertile eggs the nucleoproteins of the cell became labelled more rapidly than the phospholipid. As a result of this it is possible to prepare labelled virus in which the ratio of lipid to nucleic acid ^{32}P varies considerably. If the virus is inoculated into eggs immediately after inoculation of ^{32}P and in a relatively large dose so that maximum yield of virus is rapidly attained the resulting labelled virus will show a high content of nucleic acid ^{32}P with a relatively low lipid ^{32}P. If, however, virus is inoculated 24 hr. after introduction of ^{32}P and in small dosage, so that a greater length of time is taken to attain maximum titre, the resulting labelled virus will have a relatively high lipid ^{32}P.

RECOVERY OF ^{32}P IN SALINE EXTRACTS OF THE CHORIO-ALLANTOIC MEMBRANE OF EGGS INOCULATED WITH LABELLED VIRUS

One of the outstanding problems in the growth cycle of influenza virus is that of the so-called 'dark period' immediately following entry of virus into the host cell. Saline extracts of the chorio-allantoic membrane made 1–2 hr. after inoculation show no demonstrable virus by serological methods. A series of experiments was therefore carried out to determine if ^{32}P could be recovered in such extracts when eggs were inoculated with labelled virus.

Four 13-day-old fertile eggs were inoculated into the allantoic sac with 960 haemagglutinin units of labelled virus. After incubating $1\frac{1}{2}$ hr. the allantoic fluids were collected and the chorio-allantoic membranes removed, washed with saline, suspended in saline (1 ml. per membrane) and frozen and thawed three times. In order to destroy cell receptors which might prevent recovery of haemagglutinins 200 Australian units of crystalline receptor destroying enzyme (R.D.E.) were added to the membrane suspension which was then incubated for 4 hr. at 37° C. The fluid was then centrifuged, the supernatant extract removed and the residual membranes dissolved in N/1 sodium hydroxide. The radioactivity of the various samples was then measured:

Preparation	Geiger count (c.p.m./ml.)	
Original inoculum	270	of which 261 was adsorbable on red cells, i.e. virus ^{32}P
$1\frac{1}{2}$ hr. allantoic fluids	137	of which 128 was virus ^{32}P
Membrane extract	50	
Residual membranes	54	

The result indicates that 51 % of the inoculated virus was taken up by the cells. 19 % of the original virus ^{32}P was recovered in the membrane extract and 20 % in the residual membranes. 12 % of the ^{32}P was lost, probably owing to the impossibility of collecting the whole of the chorio-allantoic membranes.

Since the original inoculum had a haemagglutinin titre of 960 the membrane extract, containing 19 % of the ^{32}P, should have contained 182 haemagglutinin

units per egg if the virus had remained intact. As the volume of extract per egg was 1·3 ml. the haemagglutinin titre would have been 140. On test no haemagglutinin could be demonstrated. It was therefore clear that the ^{32}P recovered in the membrane extract was not present as unaltered virus.

When experiments of this type are carried out with intact eggs it is inevitable that the whole of the inoculated ^{32}P will not be recovered owing to the difficulty of collecting the whole of the chorio-allantoic membrane. For this reason de-embryonated eggs have been used in much of the work. With such eggs quantitative recovery of the inoculated ^{32}P is possible.

Twelve de-embryonated eggs were inoculated with 1024 haemagglutinin units of labelled virus contained in 5 ml. of 0·5 % glucose saline. The eggs were incubated for 1½ hr. on a roller machine. Fluids and membranes were then collected. Saline extracts were prepared from each membrane individually. With six of the membranes the extracts were prepared by freezing and thawing only, without any period of incubation, while with the other six the frozen and thawed membrane suspensions were incubated for 5 hr. at 37° C. to allow possible elution of haemagglutinin from cell receptors. Geiger counts were then made with the following results:

	Total Geiger c.p.m.	
Original inoculum	1094	Virus ^{32}P 1035
Inoculum adsorbed with red cells	59	
(*a*) Membrane extracts prepared without incubation		
Roller fluid	710	1111
Membrane extract	82	
Residual membranes	319	
(*b*) Membrane extracts prepared with incubation		
Roller fluid	710	1090
Membrane extract	128	
Residual membranes	252	

The roller fluid adsorbed with red cells gave a Geiger count of 65 c.p.m. Therefore 645 c.p.m. represented virus ^{32}P not taken up by the cells. 38 % of the inoculated virus was taken up by the cells, and the virus ^{32}P was completely recovered. When membrane extracts were prepared by freezing and thawing only, 20 % of the virus ^{32}P was recovered in the extract and 80 % in the residual membranes. Incubation of the membrane suspensions after freezing and thawing increased the recovery of ^{32}P in the extract to 33 %.

Neither membrane extract contained any demonstrable haemagglutinin.

PARTICLE SIZE OF THE MEMBRANE EXTRACT ^{32}P

The above experiments show that on entry into the host cell the virus is modified. Radioactive phosphorus derived from the inoculated virus can be found in membrane extracts made at 1½ hr. but no haemagglutinin is detectable. Two possible explanations can be offered. The virus may be disintegrated on entry into the cell with a separation of the phosphorus-containing lipid and nucleoprotein from the

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non-phosphorus-containing haemagglutinin. Or the virus may remain intact but become combined irreversibly with some inhibitor which prevents its demonstration by haemagglutination and renders it non-infective. A decision between these alternatives can be made by determining the particle size of the ^{32}P which appears in the membrane extracts. If the virus remains intact but linked to an inhibitor, the ^{32}P would be present as material of a particle size at least equal to that of the original virus, while if the virus is disintegrated the ^{32}P would appear as material of smaller particle size.

De-embryonated eggs were inoculated with labelled virus and saline extracts of the chorio-allantoic membranes made after $1\frac{1}{2}$ hr. incubation by freezing and thawing but without incubation. A control preparation was made by adding labelled virus to a saline extract of normal chorio-allantoic membrane. The infected membrane extract and the control were then centrifuged for 1 hr. in the Spinco centrifuge at an average centrifugal force of 20,000 *g* against a sucrose density gradient. Samples of the supernatant fluids were removed and again centrifuged at 100,000 *g* for 3 hr. Geiger counts gave the following results:

	Geiger count (c.p.m./ml.)
Control (normal membrane extract + labelled virus)	
Original fluid	793
20,000 *g* supernatant	16
100,000 *g* supernatant	6
$1\frac{1}{2}$ hr. infected membrane extract	
Original fluid	212
20,000 *g* supernatant	184
100,000 *g* supernatant	166

98 % of the ^{32}P of the control preparation was sedimented at 20,000 *g*. By contrast only 14 % of the ^{32}P in the infected membrane extract was sedimented at 20,000 *g* and 78 % was not sedimentable even at 100,000 *g*.

The result indicates that the failure to demonstrate intact virus in saline extracts of infected chorio-allantoic membranes made during the 'dark period' is not due to combination of the virus with an inhibitor of haemagglutination but is due to disintegration of the virus on entry into the cell. The greater part of the ^{32}P found in $1\frac{1}{2}$ hr. infected membrane extracts is present as material of much smaller particle size than intact virus.

CHEMICAL STATE OF THE ^{32}P IN INFECTED MEMBRANE EXTRACTS

The ^{32}P in labelled virus preparations is present partly as phospholipid and partly as nucleic acid. The chemical state of the ^{32}P in infected membrane extracts made $1\frac{1}{2}$ hr. after inoculation of labelled virus was determined in the following way:

(1) A sample of the extract was dried and extracted with ethanol–ether (2:1). The extract radioactivity gave a measure of the lipid ^{32}P.

(2) A sample was precipitated by half saturation with ammonium sulphate. The precipitate radioactivity was taken to be due to nucleoprotein + lipoprotein.

(3) A sample was precipitated with two volumes of ethanol. The precipitate radioactivity was taken to be due to nucleoprotein + nucleic acid. The supernatant radioactivity represented lipid + phosphorus compounds of small molecular weight. The following result was obtained with a saline extract of the chorio-allantoic membranes of ten eggs made 1½ hr. after inoculation of labelled virus. Intact eggs were used and the extract prepared by freezing and thawing without incubation.

		Total Geiger c.p.m.	
Membrane extract		545	
Ethanol-ether extract of dried membrane extract		17 (*a*)	
Ammonium sulphate precipitation	Precipitate	64 (*b*)	544
	Supernatant	480 (*c*)	
Ethanol precipitation	Precipitate	306 (*d*)	546
	Supernatant	240 (*e*)	

From these results it was calculated that the total radioactivity of 545 c.p.m. was made up in the following way:

	c.p.m.	%
Phospholipid (*a*)	17	3·1
Nucleoprotein (*b* − *a*)	47	8·6
Nucleic acid (*d* − (*b* − *a*))	259	47·5
Phosphorus compounds of small molecular (*e* − *a*) weight	223	41·0
Total	546	100·2

A second similar experiment using intact eggs gave the following distributions of ^{32}P in the 1½ hr. membrane extract: lipid 2 %, nucleoprotein 17 %, nucleic acid 38 %, phosphorus compounds of low molecular weight 43 %.

A similar experiment was carried out with de-embryonated eggs, saline membrane extracts being made at 1½ hr., one extract by freezing and thawing only and one by freezing and thawing followed by incubation for 5 hr. at 37° C. The following results were obtained:

Extract prepared by freezing and thawing only (%)		Extract prepared by freezing and thawing and incubation at 37° C. (%)	
Lipid ^{32}P	1	Lipid ^{32}P	1
Nucleoprotein ^{32}P	15	Nucleoprotein ^{32}P	7
Nucleic acid ^{32}P	8	Nucleic acid ^{32}P	24
Small molecular weight ^{32}P	76	Small molecular weight ^{32}P	68

The results show that the ^{32}P found in infected membrane extracts during the dark period is not present as intact virus. Some 20–40 % of the ^{32}P of intact labelled virus is present as phospholipid, but only 1–3 % of the membrane extract ^{32}P is lipid. Labelled virus is almost completely precipitated by half-saturated ammonium sulphate, but only 8–17 % of the ^{32}P in the membrane extracts was precipitated. A greater amount of the ^{32}P was precipitated by ethanol than by ammonium sulphate, indicating the probable presence of free nucleic acid. The amount of this free nucleic acid was greater in membrane extracts from intact eggs than in extracts from de-embryonated eggs. In the de-embryonated eggs the amount of ^{32}P recoverable as free nucleic acid in the extracts was increased when the frozen and thawed membranes were incubated.

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A large amount of the ^{32}P in all the extracts was present as phosphorus compounds not precipitable by ethanol. The amount of this small molecular weight ^{32}P was greater in the extracts from de-embryonated eggs than in those from intact eggs. The significance of these findings is discussed below.

RECOVERY OF PHOSPHOLIPID ^{32}P

A large part of the ^{32}P in eggs inoculated with labelled virus is not found in the membrane extracts made at $1\frac{1}{2}$ hr. but in the residual membranes. As the amount of lipid ^{32}P present in the membrane extracts was very much less than in intact virus it was of interest to determine how much of the ^{32}P present in the residual membranes was present as phospholipid.

A preparation of labelled virus with a high content of phospholipid ^{32}P was made by inoculating virus 24 hr. after introduction of ^{32}P. The lipid ^{32}P of the labelled virus was determined by adsorbing the virus on red cells and extracting the red cells twice with ten volumes of ethanol:

	Total Geiger c.p.m.	
Original labelled virus	2618	
Supernatant after R.B.C. adsorption	166	
Ethanol extract of red cells	1058	2644
Residual red cells in N/1-NaOH	1520	

The result indicates that 40 % of the ^{32}P of the labelled virus was present as ethanol-soluble phospholipid.

Ten de-embryonated eggs were inoculated with the labelled virus and incubated $1\frac{1}{2}$ hr. Saline membrane extracts were then made by freezing and thawing. 36·4 % of the inoculated virus was taken up by the cells, and this amount was estimated to have a total content of phospholipid ^{32}P of 2118 c.p.m.

The membrane extract was dried *in vacuo* and extracted overnight with anhydrous ethanol. The extract gave a Geiger count of 38 c.p.m. The residual membranes were similarly dried and extracted with ethanol. The extract gave a Geiger count of 446 c.p.m. Thus only 1·8 % of the original virus phospholipid ^{32}P was recovered in the membrane extract and 21 % in the residual membranes. 77 % of the virus phospholipid entering the cells was not recoverable as ethanol-soluble phospholipid at $1\frac{1}{2}$ hr.

A second experiment was carried out in which de-embryonated eggs were inoculated with labelled virus containing 40 % of lipid ^{32}P. The chorio-allantoic membranes were collected at $1\frac{1}{2}$ hr. and the water content reduced by extracting with two volumes of ethanol. This first ethanol extract contained, in addition to water-soluble ^{32}P, a small amount of lipid which was recovered by extracting with toluene. The membranes were then boiled with anhydrous ethanol under reflux for 3 hr. After removal of the ethanol the membrane residue was dissolved in N/1-NaOH. Geiger counts gave the following result:

	Total Geiger c.p.m.	
First ethanol extract	281	
Toluene extract of first ethanol extract	9	
Second ethanol extract	232	Total lipid 332
Membrane residue in N/1-NaOH	1680	

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The total ^{32}P in the membranes was 2284 c.p.m. Of this 914 c.p.m. should represent phospholipid. The actual amount of lipid ^{32}P recovered was 332 c.p.m. or 36 %. 64 % of the original phospholipid ^{32}P was not recoverable as ethanol-soluble phospholipid.

It is clear that on entry into the host cell the virus phospholipid is disintegrated with release of its ^{32}P. It would be expected that ^{32}P derived from the lipid would appear as phosphorus compounds of small molecular weight. Such compounds are found in saline extracts of $1\frac{1}{2}$ hr. infected membranes, and it seems most probable that these are derived from the virus phospholipid. In all our experiments the amount of ^{32}P liberated by disintegration of the phospholipid was more than enough to account for the whole of the small molecular-weight phosphorus found in the membrane extracts.

FATE OF THE VIRUS NUCLEOPROTEIN PHOSPHORUS

The major part of the ^{32}P of labelled virus is present as ribonucleoprotein. Disintegration of virus particles by ether treatment results in the release of this ribonucleoprotein as soluble antigen particles which have no haemagglutinating power and no affinity for cell receptors (Hoyle *et al.* 1954). If the virus phospholipid is disintegrated on entry into the host cell it might be expected that nucleoprotein soluble antigen would be released. Such particles should be found in saline extracts of the infected membrane. A small part of the ^{32}P present in $1\frac{1}{2}$ hr. infected membrane extracts is present as nucleoprotein precipitable with half-saturated ammonium sulphate, but a larger part appears as free nucleic acid. The results suggest that nucleic acid may be split off the virus nucleoprotein on entry into the cell. However, it was also possible that the free nucleic acid found might be produced by synthesis from the ^{32}P released by disintegration of the phospholipid. Quantitative considerations, however, indicate that the whole of the free nucleic acid found could not be derived in this way. Thus in one experiment the amount of ^{32}P derived by disintegration of phospholipid and available for synthesis was measured as 330 c.p.m. The small molecular weight ^{32}P found in the $1\frac{1}{2}$ hr. membrane extract was 227 c.p.m. and the free nucleic acid 280 c.p.m. While the whole of the small molecular-weight phosphorus could be accounted for by the phospholipid disintegration the nucleic acid could not also be produced from phospholipid ^{32}P. Some disintegration of the virus nucleoprotein must have occurred. There is some evidence that the free nucleic acid found in $1\frac{1}{2}$ hr. membrane extracts is partly derived by disintegration of the virus nucleoprotein and partly by synthesis from ^{32}P derived from the phospholipid. The amount of free nucleic acid found in $1\frac{1}{2}$ hr. membrane extracts is increased if the extracts are incubated for some hours after freezing and thawing, while the nucleoprotein content is decreased. This would suggest that proteolytic enzymes are present in the cell extract and that they attack the virus nucleoprotein and release the nucleic acid. It is also found that the free nucleic acid content of $1\frac{1}{2}$ hr. membrane extracts from intact eggs is greater than in extracts from de-embryonated eggs, while the content of small molecular weight ^{32}P is less. This might suggest that nucleic acid

can be synthesized from ^{32}P derived from the virus phospholipid and that such synthesis is more rapid in the intact egg than in the de-embryonated egg.

The whole of the nucleoprotein and nucleic acid ^{32}P found in $1\frac{1}{2}$ hr. membrane extracts represents only a small part of the original virus nucleoprotein ^{32}P. The major part remains in the residual membranes after making the saline extracts. Both ribonucleoprotein and ribonucleic acid are readily soluble in physiological saline, so that failure to extract the nucleoprotein ^{32}P would seem to indicate some modification of the virus nucleoprotein on entry into the cell. The possibility was considered that the virus nucleoprotein was firmly adsorbed to the cell nucleus or even entered into it. The nuclear material of the cell is insoluble in physiological saline but soluble in molar sodium chloride. Experiments were therefore done to determine if ^{32}P could be extracted from the residual membranes with molar sodium chloride. Two experiments gave similar results. Washed chorio-allantoic membranes from intact eggs inoculated $1\frac{1}{2}$ hr. previously with labelled virus were frozen and thawed and three successive extracts made with physiological saline (1 ml. per membrane). Four successive extracts were then made with molar sodium chloride. These extracts were very viscous. Three successive extracts were then made with absolute ethanol and the final membrane residue dissolved in normal sodium hydroxide. Geiger counts gave the following results:

	Total Geiger c.p.m.	
1st physiological saline extract	106	
2nd physiological saline extract	12	118
3rd physiological saline extract	0	
1st molar NaCl extract	69	
2nd molar NaCl extract	48	138·5
3rd molar NaCl extract	21·5	
4th molar NaCl extract	0	
1st ethanol extract	13	
2nd ethanol extract	62	75
3rd ethanol extract	0	
Residue in NaOH	213	

A considerable amount of ^{32}P can be extracted from the membranes with molar sodium chloride even after complete extraction with physiological saline. Of the total membrane ^{32}P, 22 % was extractable with physiological saline, 25·2 % with molar sodium chloride, 13·7 % was present as ethanol-soluble material, presumably lipid, while the residue after all the extractions still contained 39 % of the ^{32}P.

The second experiment gave similar results, 26 % of the ^{32}P being extractable with physiological saline, 22 % with molar sodium chloride, 25 % with ethanol while 27 % remained in the residue.

Attempts were made to determine the chemical state of the ^{32}P in the molar sodium chloride extracts. Dilution of a sample with six volumes of water produced a stringy precipitate of deoxyribonucleoprotein which carried 30 % of the radioactivity. Half-saturation with ammonium sulphate produced a precipitate carrying 40 % of the radioactivity. Addition of two volumes of ethanol produced a stringy precipitate of nucleoprotein. The supernatant fluid was opalescent, and even after

standing overnight it proved difficult to centrifuge out all the precipitate, but nevertheless the ethanol precipitate carried 60 % of the radioactivity.

It seemed probable that the ^{32}P in the molar sodium chloride extracts was partly present as nucleoprotein and partly as free nucleic acid, but the possibility that phosphorus compounds of simpler structure were also present could not be excluded.

DISCUSSION

Work on the multiplication of influenza virus in recent years has produced a steadily increasing body of evidence indicating that the disintegration of the infecting particle on entry into the host cell is an essential prerequisite for virus reproduction. The work described here affords the first direct evidence of this disintegration.

Within $1\frac{1}{2}$ hr. of infection with virus labelled with ^{32}P, radiophosphorus can be recovered from cell fractions which could not conceivably contain intact virus. Thus saline extracts of the frozen and thawed cells contain radiophosphorus which cannot be precipitated with protein precipitants, and which cannot be sedimented at a centrifugal force of 100,000 *g*. This small molecular-weight phosphorus is probably mainly derived by disintegration of the virus phospholipid. The amount of ethanol-soluble ^{32}P recoverable from the infected cells is considerably less than the amount present in the infecting virus. It seems probable that on entry into the cell the virus phospholipid is destroyed with the release of phosphorus compounds of small molecular weight. Radiophosphorus released in this way may be used by the cell for synthesis and might ultimately appear in any phosphorus-containing cell fraction, and in fact $1\frac{1}{2}$ hr. after infection any phosphorus containing cell fraction which can be made by any form of chemical or physical treatment appears to be radioactive. However, the amount of ^{32}P released by disintegration of the virus phospholipid cannot account for all the non-protein radiophosphorus found in the cell at $1\frac{1}{2}$ hr.; in particular, the free nucleic acid found must be largely derived from the virus nucleoprotein.

Destruction of the virus phospholipid would, by analogy with the results of ether treatment of virus, be expected to release the virus nucleoprotein in the form of particles of soluble antigen. Saline extracts of the cells made $1\frac{1}{2}$ hr. after infection contain radiophosphorus in the form of nucleoprotein precipitable by half-saturation with ammonium sulphate, but they also contain ^{32}P which is precipitable with two volumes of ethanol but not by ammonium sulphate. This material is probably nucleic acid. Incubation of the frozen and thawed cell suspension during the saline extraction decreases the amount of ^{32}P recoverable as nucleoprotein but increases the nucleic acid. It may be that on entry into the cell the virus nucleoprotein is attacked by intracellular protease with release of the nucleic acid.

The total amount of nucleoprotein and nucleic acid ^{32}P which can be recovered in saline extracts of the infected cells is much less than the amount present in the infecting virus; in fact, most of the nucleoprotein ^{32}P is not recoverable by extraction with physiological saline. This unextractable ^{32}P appears to be largely associated with the cell nuclear material and much of it can be extracted when the

insoluble nuclear material is dissolved in molar sodium chloride. In such molar sodium chloride extracts the ^{32}P appears partly as nucleoprotein and partly as free nucleic acid. When the deoxyribonucleoprotein in the extract is precipitated by dilution with water the precipitate is found to be radioactive. Although it is impossible to decide whether this means that ^{32}P from the virus ribonucleoprotein has become transferred to the nuclear deoxyribonucleoprotein or whether the precipitated deoxyribonucleoprotein carries down adsorbed ribonucleic acid, nevertheless, the finding would indicate a very intimate association between the virus nucleic acid and the cell nucleus. It is of interest that Watson & Coons (1954), studying the location of influenza virus in tissues by means of fluorescent antibody, found that specific staining was first demonstrable in the cell nuclei, and Liu (1955) showed that the nuclear staining was due to soluble antigen.

The main impression we have gained during the course of this work has been an appreciation of the extremely dynamic state of the constituents of the living cell, a condition in which the larger molecules present are being continuously broken down and resynthesized. It is probable that many of these changes continue even in cell extracts prepared by freezing and thawing. It is obviously very difficult in these conditions to elucidate exactly the fate of any particular molecule introduced into the cell. It seems clear that on entry into the cell the virus particle is disintegrated and its component units enter into the dynamic cell system. We had not, however, expected that the disintegration would be so complete and the integration with the cell system quite so intimate. Previous work (Hoyle 1952) had suggested that the virus lipid was similar to the lipid of the host cell. If this is so then the finding that within $1\frac{1}{2}$ hr. of entry into the cell some 60–80 % of the virus lipid is destroyed would indicate a very rapid phospholipid turnover in the cell.

The recovery from the infected cell of free labelled nucleic acid indicates that the virus nucleoprotein is largely disintegrated. The recovery of a large amount of labelled nucleoprotein and nucleic acid by extraction with molar sodium chloride after complete extraction with physiological saline suggests that the virus nucleic acid becomes intimately associated with the cell nucleus. It is possible that entry of virus nucleic acid into the cell nucleus is responsible for the turnover from synthesis of cell ribonucleoproteins to virus nucleoprotein which occurs in the infected cell.

SUMMARY

Influenza *A* virus labelled with radiophosphorus was introduced as a primary inoculum into the allantoic sac of fertile eggs. Virus so introduced enters the cells of the chorio-allantoic membrane, and a study has been made of the chemical state of ^{32}P present in the membranes $1\frac{1}{2}$ hr. after inoculation.

It was found that on entry into the host cell the virus particle disintegrates probably as a result of destruction of its phospholipid. Radiophosphorus derived from the virus phospholipid can be recovered from the infected membranes by extraction with physiological saline in the form of compounds of small molecular weight which are not precipitated by protein precipitants and are not sedimentable at a centrifugal force of 100,000 *g*.

Saline extracts of the infected membranes also contain labelled nucleic acid which appears to be derived from the virus nucleoprotein.

A large part of the nucleoprotein phosphorus of the inoculated labelled virus cannot be recovered from the infected membranes by extraction with physiological saline but can be recovered if the cell nuclear material is dissolved in molar sodium chloride. The ^{32}P in such molar chloride extracts is partly precipitated with the deoxyribonucleoprotein on dilution with water, and is partly present as free nucleic acid.

It is suggested that on entry into the host cell the virus particle is broken down, the phospholipid being destroyed and the nucleoprotein disintegrated with the release of free nucleic acid which enters into a close relation with the cell nuclear material.

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