# THE USE OF INFLUENZA VIRUS LABELLED WITH RADIOSULPHUR IN STUDIES OF THE EARLY STAGES OF THE INTERACTION OF VIRUS WITH THE HOST CELL

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(With 1 Figure in the Text)

Hoyle & Frisch-Niggemeyer (1955) using influenza virus labelled with radiophosphorus showed that virus particles disintegrated on entry into the host cell. Radiophosphorus labels the virus lipid and nucleic acid and it was found that on entry into the host cell the virus phospholipid was broken down with the release of water-soluble phosphorus compounds, while the virus ribonucleoprotein was split with the release of free nucleic acid which appeared to become associated with the nuclear material of the cell. No direct evidence of the fate of the virus protein could be obtained from this work.

In the present studies influenza virus has been labelled with radiosulphur and by the use of such virus direct evidence has been obtained regarding the behaviour of the virus protein on entry into the host cell. The D.S.P. strain of influenza virus A was used.

## PREPARATION OF VIRUS LABELLED WITH $^{35}S$

Influenza virus can be labelled with radiosulphur by cultivation in fertile eggs in which radioactive methionine has been introduced. The 12-day fertile egg will tolerate about 250 microcuries of radioactive methionine; larger doses kill the embryo.

Radioactive methionine was introduced into the allantoic sac of the 12-day fertile eggs in a dose of 200 microcuries. After 4 hr. incubation the eggs were inoculated with D.S.P. virus (0·1 ml. of a 1:100 dilution of 18 hr. infected allantoic fluid), and incubated for 40 hr. The allantoic fluid was then collected and the virus purified by two cycles of adsorption-elution from guinea-pig red cells, using the same technique as that previously used in the preparation of virus labelled with $^{32}P$ (Hoyle, Jolles & Mitchell, 1954).

The final concentrated product was tested for contamination with non-viral $^{35}S$ by determining the Geiger count before and after adsorption with red cells. It was found easy to produce preparations in which 99 % of the radioactivity was removed by red cell adsorption and in which the Geiger count per minute was two to three times the haemagglutinin titre. This degree of radioactivity corresponded to the presence of one atom of $^{35}S$ in about seven virus particles.

### *Technique of Geiger counting*

In previous work with $^{32}P$, Geiger counts were made on 1 ml. fluid samples placed in shallow dishes beneath an end-window type counter. Owing to the low energy of $\beta$-particles emitted by $^{35}S$ liquid counting is very inefficient, and a thirty-fold

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increase in count occurred if the samples were dried. The most satisfactory results were obtained by counting 0·5 ml. samples which were dried in slightly concave shallow dishes, the dried material covering an area of 2 cm. diameter. The dishes were placed beneath an end-window type counter of maximum sensitivity (window 1·9 mg./cm.$^2$). Corrections were necessary for the differences between the self-absorption of different types of dried material and these correction factors were determined by adding constant amounts of radioactive methionine to each type of fluid used and measuring the counts when the samples were dried.

## PROPERTIES OF VIRUS LABELLED WITH $^{35}S$

Almost the whole of the radioactivity of purified labelled-virus preparations is due to the virus particles, and can be removed by adsorption with red cells or by centrifugation at 26,000 *g*. A sample of labelled virus was diluted 1:5 with saline and one portion adsorbed twice with 10% guinea-pig red cells, while a second portion was centrifuged at an average centrifugal force of 26,000 *g* for 1 hr. against a sucrose density gradient. Geiger counts gave the following results:

| | |
|---|---|
| Original virus preparation (1:5) | 661 c.p.m. |
| Supernatant after red cell adsorption | 4 c.p.m. |
| 26,000 *g*. supernatant | 7 c.p.m. |

### *Chemical precipitation*

Sulphur-labelled virus is readily precipitated by protein precipitants. A sample of labelled virus was mixed with an equal volume of a saline extract of normal chorio-allantoic membrane. Samples of the mixture were then (*a*) adsorbed with 10% guinea-pig red cells, (*b*) precipitated with an equal volume of saturated ammonium sulphate, and (*c*) precipitated by addition of 5% trichloracetic acid. The fluids were centrifuged and the deposits dissolved in normal sodium hydroxide, neutralized, dried and counted with the following results:

| | |
|---|---|
| Original fluid | 1410 c.p.m. |
| Virus adsorbed on red cells | 1395 c.p.m. |
| Ammonium sulphate precipitate | 1365 c.p.m. |
| Trichloracetic acid precipitate | 1400 c.p.m. |

It was also found that labelled virus could be almost completely precipitated by denaturation with 2 vol. of alcohol at room temperature, or by heat denaturation.

### *Fractionation of labelled virus with ether*

Influenza virus particles can be disintegrated by shaking with ether with the release of separate haemagglutinating and complement-fixing soluble antigen particles (Hoyle, 1952). The disintegration appears to result from the denaturation of a lipoprotein virus envelope. A sample of virus labelled with $^{35}S$ was shaken with half its volume of pure ethyl ether, incubated at 37° C. for 2 hr. and then centrifuged. A layer of denatured protein accumulated at the ether-water interface. The ethereal and aqueous layers were removed and the denatured protein dissolved in

normal sodium hydroxide. The aqueous ether-treated suspension was then adsorbed with 10% guinea-pig red cells and centrifuged. The supernatant fluid constituted the soluble antigen fraction. The haemagglutinin was eluted from the deposited red cells by suspending in saline and incubating for 4 hr. at 37° C. Serological tests gave the results shown in Table 1.

Table 1

| | Haemagglutinin titre | Complement-fixing antigen titre |
|---|---|---|
| Original virus suspension | 12,800 | 48 |
| Ether-treated suspension | 25,600 | 64 |
| Haemagglutinin fraction | 25,600 | 3 |
| Soluble antigen fraction | 64 | 48 |

As a result of ether treatment the haemagglutinin titre was doubled and there was a slight increase in complement-fixing antigen titre. Adsorption of the ether-treated suspension with red cells resulted in almost complete separation of the haemagglutinating and complement-fixing properties. Geiger counts of the preparations were as follows:

| | | |
|---|---|---|
| Original virus | 3520 c.p.m. | |
| Ethereal extract | 0 c.p.m. | |
| Denatured envelope protein | 2020 c.p.m. | 3260 |
| Haemagglutinin fraction | 580 c.p.m. | |
| Soluble antigen fraction | 660 c.p.m. | |

The results show that all the virus protein becomes labelled with $^{35}S$. Most of the labelled material appears in the envelope protein. It is, however, probable that the envelope protein does not constitute so high a proportion of the whole as the present results suggest, since it is difficult to disintegrate virus with ether without some loss of haemagglutinin and soluble antigen by denaturation. Three experiments on ether fractionation of virus gave the distributions shown in Table 2 of $^{35}S$ between the haemagglutinin, soluble antigen and denatured envelope protein.

Table 2

| Experiment | Haemagglutinin $^{35}S$ (%) | Soluble antigen $^{35}S$ (%) | Envelope protein $^{35}S$ (%) |
|---|---|---|---|
| 1 | 17·7 | 20·2 | 62·6 |
| 2 | 13·3 | 14·6 | 72·1 |
| 3 | 16·0 | 10·3 | 73·7 |
| Average | 15·7 | 15·0 | 69·3 |

*The behaviour of influenza virus labelled with $^{35}S$ when introduced as a primary inoculum*

Twelve-day fertile eggs were de-embryonated and inoculated with 1000 haemagglutinin units of sulphur-labelled virus in 5 ml. of glucosol. A large dose of virus was used in order to ensure the maximum possible take up of virus by the cells of

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the chorio-allantoic membrane. The eggs were incubated for 1½ hr. on a roller machine. The fluid was then removed, the chorioallantoic membranes washed in the shell, removed, frozen and thawed three times, and a saline extract made using 1 ml. of saline per membrane. After centrifugation the residual membranes were dissolved in normal sodium hydroxide. Samples of the various fluids were dried and Geiger counts per egg are given below:

| | | |
|---|---|---|
| Original inoculum | 1270 c.p.m. | |
| 1½ hr. roller fluid | 654 c.p.m. | |
| Saline membrane extract | 128 c.p.m. | 1157 |
| Residual membranes | 375 c.p.m. | |

Of the original $^{35}S$ inoculated 9 % was lost. 40 % was taken up by the cells and of this 25·4 % appeared in the saline extract while 74·6 % remained in the membrane residue. Similar results were obtained when the experiment was carried out using intact eggs instead of de-embryonated eggs.

*Chemical and physical state of the* $^{35}S$ *in the saline membrane extract*

The particle size of the $^{35}S$ in the extract was studied by centrifuging samples at an average centrifugal force of 26,000 *g* for 1 hr., and 100,000 *g* for 4 hr. against a sucrose density gradient; as a control labelled virus, suspended in a saline extract of normal chorioallantoic membrane, was centrifuged in parallel. Geiger counts gave the following results:

| | | |
|---|---|---|
| Control (labelled virus in normal CAM extract) | Original fluid | 697 c.p.m. |
| | 26,000 *g* supernatant | 4·4 c.p.m. |
| 1½ hr. infected CAM extract | Original fluid | 115 c.p.m. |
| | 26,000 *g* supernatant | 71 c.p.m. |
| | 100,000 *g* supernatant | 70 c.p.m. |

Over 99 % of the $^{35}S$ in the control preparation was deposited at 26,000 *g*. With the infected membrane extract only 38 % was deposited at 26,000 *g*, and even at 100,000 *g* only 39 % was deposited.

The chemical state of the $^{35}S$ in a 1½ hr. saline membrane extract was determined by precipitating samples by half saturation with ammonium sulphate and by addition of 5 % trichloracetic acid. The precipitate was dissolved in normal sodium hydroxide. Geiger counts (corrected for volume and self-absorption) on these samples were:

| | | |
|---|---|---|
| Original membrane extract | 96 c.p.m. | |
| Ammonium sulphate precipitate | 45 c.p.m. | 90 |
| Supernatant | 45 c.p.m. | |
| Trichloracetic acid precipitate | 47 c.p.m. | 102 |
| Supernatant | 55 c.p.m. | |

The results show large experimental errors, due to the irregular drying of samples containing large amounts of salt, and difficulties in accurately determining the corrections for self-absorption in the different samples; nevertheless, it is clear that about half the $^{35}S$ in the extract is not precipitated by protein precipitants.

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*State of the $^{35}S$ present in the residual membranes*

In previous work with radiophosphorus (Hoyle & Frisch-Niggemeyer, 1955) it was found that when a physiological (0·85 %) saline extract of the chorioallantoic membrane was made 1½ hr. after inoculation of eggs with virus labelled with $^{32}P$, much of the radiophosphorus remained in the residual membranes. A large part of this appeared to be associated with the cell nuclear material and could be recovered by extraction of the residual membranes with molar sodium chloride solution. It was, therefore, of interest to determine if a similar result occurred with virus labelled with radiosulphur. De-embryonated eggs were inoculated with virus labelled with $^{35}S$ and after 1½ hr. incubation the chorioallantoic membranes were removed and divided into two equal batches. Both were frozen and thawed three

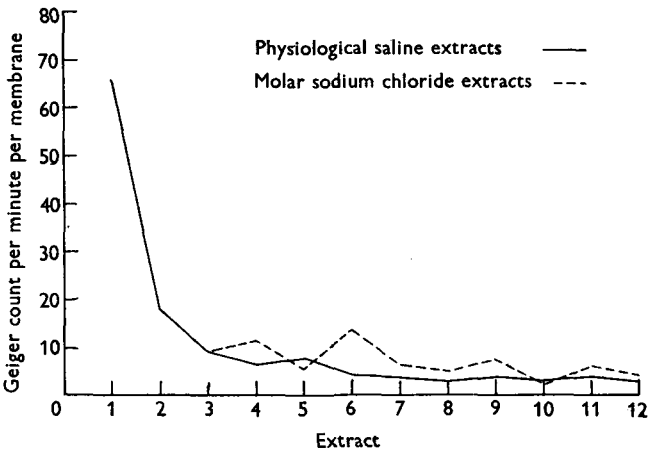


Fig. 1. Recovery of radiosulphur in serial extracts of the chorioallantoic membranes of eggs 1½ hr. after inoculation of influenza virus labelled with $^{35}S$.

times and then one batch was serially extracted twelve times using physiological saline (1 ml. per membrane), while with the second batch three extracts were made with physiological saline and these were followed by nine serial extracts with molar sodium chloride solution. The residual membranes were then dissolved in normal sodium hydroxide. Geiger counts of the extracts gave the result shown in Fig. 1. When the membranes were extracted with physiological saline the amount of $^{35}S$ recovered fell sharply in the early extracts to a low level which was then maintained over a long series of extracts. The results suggest an adsorption equilibrium, with $^{35}S$ being slowly released from some insoluble cell constituent. When physiological saline was replaced with molar sodium chloride there was no striking increase in the yield of $^{35}S$ as would be expected if radiosulphur was present in the cell nuclear material. Molar sodium chloride was, however, slightly more effective than physiological saline, so that while after twelve successive extracts using normal saline 33 % of the total membrane $^{35}S$ was still present in the residue, with molar sodium chloride only 14 % remained.

Samples of the extracts were precipitated with 5 % trichloracetic acid. In the

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first saline extract 50 % of the $^{35}S$ was precipitated, in the second 70 %, in the third 90 %, while in the later extracts all the $^{35}S$ was precipitable.

The experiments show that 1½ hr. after infection with sulphur-labelled virus the radiosulphur in the chorioallantoic membranes is present in two sharply distinct states. Part of the $^{35}S$ is present as material readily extracted by saline, not precipitable by protein precipitants, and not deposited by centrifugation at 100,000 *g*; this is almost certainly amino-acid. The larger part of the $^{35}S$ is present as material which appears to be adsorbed to some insoluble cell constituent, which is not the cell nucleus, and is extractable only with difficulty. This material is protein of large particle size, since it is deposited by centrifugation at 26,000 *g*. In three of our experiments it was possible to determine the relative amounts of $^{35}S$ appearing in the two forms. These were experiments in which a long series of saline extracts had been made and each extract precipitated by trichloracetic acid. The results are given in Table 3.

Table 3

| | Total CAM $^{35}S$ | Non-protein $^{35}S$ | Protein $^{35}S$ |
|---|---|---|---|
| (1) | 708 c.p.m. | 122 (17 %) | 586 (83 %) |
| (2) | 196 c.p.m. | 40 (20 %) | 156 (80 %) |
| (3) | 330 c.p.m. | 38 (12 %) | 292 (88 %) |

*Infectivity of 1½ hr. chorioallantoic membrane extracts*

Since most of the $^{35}S$ present in the chorioallantoic membranes 1½ hr. after infection with labelled virus appeared to be present as protein of particle size similar to that of the original virus, it was necessary to consider the possibility that it might be unaltered infective virus. Infectivity tests were, therefore, made on a representative sample of the extracts and on the original inoculum and the results compared with the Geiger counts (Table 4).

Table 4

| | Infectivity ($ID_{50}$/ml.) | Geiger count/ml. | Ratio $ID_{50}$ Geiger count | |
|---|---|---|---|---|
| Inoculum | 790,000,000 | 2012 | 392,000 | |
| 1st saline extract | 590,000 | 44 | 13,400 | Average of all extracts 8080 |
| 4th saline extract | 20,000 | 6·4 | 3,100 | |
| 7th saline extract | 15,500 | 2·6 | 6,000 | |
| 4th molar NaCl extract | 100,000 | 10·1 | 9,900 | |
| 7th molar NaCl extract | 49,000 | 6·15 | 8,000 | |

The results show that the original virus is fifty times more infective in relation to its $^{35}S$ content than the material present in the 1½ hr. membrane extracts. It is clear, therefore, that only about 2 % of the $^{35}S$ present in the membrane is unaltered virus; the remaining 98 % is non-infective. The material in the membrane extracts also differs from the original virus in that it does not agglutinate red blood cells.

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## DISCUSSION

Chemical analysis of purified D.S.P. virus (Frisch-Niggemeyer & Hoyle, 1956) showed it to contain 34% lipid, 3·5% carbohydrate, 0·74 ribonucleic acid (R.N.A.) and 62% protein. Ether fractionation of the virus indicates the presence of three protein containing components, namely, soluble antigen, haemagglutinin and envelope protein. The soluble antigen component carries all the nucleic acid and contains 5·3% of R.N.A. It therefore accounts for 14% of the entire virus and contains about 20% of the virus protein. The haemagglutinin was found to be a mucoprotein containing 4·2% polysaccharide; it also contains about 20% of the virus protein. The remaining 60% of the protein is combined with the lipid and most of the carbohydrate to form the envelope material. When virus labelled with $^{35}S$ is disintegrated by ether treatment the haemagglutinin and soluble antigen fractions each contain about 15% of the $^{35}S$, while 70% appears in the denatured envelope protein. As it is difficult to disintegrate virus by ether without some loss of haemagglutinin, and soluble antigen by denaturation, it is probable that the real $^{35}S$ content of haemagglutinin and soluble antigen approaches the value of 20% which would be expected from the results of chemical analysis.

Within $1\frac{1}{2}$ hr. of infection of cells with virus labelled with $^{35}S$, only a trace of the radiosulphur in the cells remains as infective virus. Between 10 and 20% is present in a non-protein non-sedimentable form, probably amino-acid, readily extracted from the cells by normal saline. The remainder is present as protein of a particle size similar to that of the original virus, but devoid of infectivity. This protein is extractable from the cells only with difficulty, the results suggesting that it is adsorbed to some insoluble cell constituent.

The simplest explanation of the observed results would be that on entry into the cell, one of the three protein components of the virus is hydrolysed to amino-acid while the others remain intact. Since most of the radiosulphur in the cells can be recovered as protein of particle size similar to the original virus it seems probable that the envelope protein remains intact. Hydrolysis of the envelope protein would not only completely disintegrate the particle, but would be expected to give much larger yields of amino-acid. Previous work with virus labelled with radiophosphorus (Hoyle & Frisch-Niggemeyer, 1955) showed that on entry into the cell the virus nucleoprotein disintegrated with the release of free nucleic acid, much of the nucleoprotein phosphorus becoming associated with the cell nuclear material. In the present studies there is no evidence of association of $^{35}S$ with the cell nucleus. Hydrolysis of the soluble antigen protein on entry into the cell would satisfactorily explain the observed results with either virus labelled with $^{32}P$ or with $^{35}S$.

The fate of the virus haemagglutinin protein is less certain. In the intact virus the haemagglutinin is probably closely associated with the surface of the particle and may in fact be part of the envelope. However, no haemagglutinin can be recovered from the cells $1\frac{1}{2}$ hr. after infection, even by treatment with receptor-destroying enzyme. Hoyle (1952) showed that purified influenza virus haemagglutinin is much more resistant to proteolytic enzymes than is soluble antigen, but the haemagglutinin was modified by trypsin so that although it still agglutinated

red cells it could not be eluted from them. It is possible, therefore, that the failure to recover haemagglutinin from the infected cell may be due to a modification by intracellular proteases without complete hydrolysis to amino-acid.

It seems most probable that the 10–20 % of $^{35}S$ appearing as amino-acid in the cells $1\frac{1}{2}$ hr. after infection with sulphur-labelled virus is derived from the virus nucleoprotein, and that the virus haemagglutinin and envelope protein are not hydrolysed, but remain intact on the cell surface.

## SUMMARY

1. Influenza virus can be labelled with $^{35}S$ by cultivation in fertile eggs into which radioactive methionine has been introduced.

2. When virus labelled with $^{35}S$ is fractionated by ether treatment the nucleoprotein soluble antigen fraction contains 15 % of the $^{35}S$, the haemagglutinin also contains 15 %, while the denatured envelope protein contains 70 % of the $^{35}S$.

3. When sulphur-labelled virus is introduced as a primary inoculum in fertile eggs and extracts of the chorioallantoic membrane are made $1\frac{1}{2}$ hr. later it is found that practically none of the $^{35}S$ is present as infective virus. Some 10–20 % of $^{35}S$ is recovered as amino-acid, while the remainder is present as non-infective material of particle size similar to that of the original virus.

4. It is suggested that on entry into the cell the virus nucleoprotein is hydrolysed with the release of amino-acid and free nucleic acid, while the virus envelope protein and haemagglutinin remain on the cell surface.

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