Measurement of Intracellular Albumin in Rat Liver*

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The albumin within the cells of the liver consists partly of albumin which is newly synthesized but not yet secreted, and partly of plasma albumin which has been taken up from the surrounding plasma or lymph and may be either stored or in the process of catabolism. Newly formed albumin occurs presumably in the parenchymal cells only, whereas ingested albumin is likely to be found in Kupffer cells and probably in parenchymal cells also (Freeman, Gordon & Humphrey, 1958). Since homologous proteins appear to be broken down very rapidly after uptake into cells (Humphrey & McFarlane, 1954), the actual quantity of ingested albumin intact within the cells is probably very small, but this is uncertain.

To calculate turnover rates of intracellular albumin, and for some other purposes, knowledge is required of the size of the pool of newly formed and yet unsecreted intracellular albumin. To measure this, it must be possible to extract albumin quantitatively from the liver, and to distinguish between that part which has not been secreted and that which consists of ingested or of extracellular albumin. Information about the distribution of albumin in the subcellular fractions of chicken liver (from which much but not necessarily all of the extracellular albumin had been removed by perfusion) has been reported by Peters (1957) in his study of serum-albumin synthesis. We have attempted to improve the method by injecting rat albumin labelled with ^{131}I into rats, and allowing the labelled albumin to come into equilibrium (after 48 hr. or longer) in the plasma and lymph. In this way, the presence of the radioactive label allows albumin in or derived from the extracellular fluid to be identified. Albumin was recovered from homogenized liver and from the plasma by specific precipitation with antiserum, and the proportion of unlabelled albumin in the liver albumin was calculated by comparison of the ^{131}I specific activities of the recovered liver albumin with that of the albumin recovered from the plasma. The extent of recovery of albumin from the samples could be calculated accurately from measurements of the protein-bound ^{131}I before and after precipitation, and hence the

size of the unlabelled intracellular-albumin pool could be estimated. The validity of the underlying assumptions is discussed below.

EXPERIMENTAL

Rats. Male hooded rats (about 300 g.) bred at the National Institute for Medical Research, Mill Hill, were used. The animal used for the perfusion *in vitro* was starved overnight before use, but the others had unrestricted access to water (containing 4 g. of NaCl and 100 mg. of KI/l.) and to the pelleted diet of Parkes (1946).

Rat [^{131}I]albumin. Rat albumin was prepared by chromatography on a CM-cellulose column (Cohen & Gordon, 1958), and was labelled with ^{131}I at an average of one atom of I/mol. by the ICI method of McFarlane (1958). About 4 mg. (100–400 μC according to the duration of the experiment) was injected intraperitoneally into the rats 2–5 days before the experiment. When the interval was less than 2 days equilibrium between the contents of the liver cells and the plasma and lymph did not appear to be fully established, and there was evidence that traces of highly labelled ‘denatured’ albumin from the original injection remained in the liver.

Anti-rat albumin. This was prepared in rabbits by intramuscular injection of rat albumin in Freund’s adjuvant (Freund & McDermott, 1942) followed by an intravenous course of rat albumin adsorbed on alum. By absorption with rat α -globulin it was made almost, but not completely, specific for rat albumin as judged by immunoelectrophoresis. In the experiment in which human blood was used for perfusion the antiserum was also absorbed with human plasma. It was shown that the reaction with rat albumin was not significantly affected by the presence of 1% of sodium deoxycholate.

Preparations from liver and serum

The liver of a rat which had received rat [^{131}I]albumin 2–5 days previously was removed. It was either not perfused, or perfused briefly with 0.9% NaCl through the portal vein, or perfused with normal rat blood *in vitro* for 4 hr. (Cohen & Gordon, 1958). The liver was then sliced coarsely, blotted to remove free fluid and weighed and homogenized at 0° with an equal weight of 0.15 M-phosphate buffer, pH 7.8. In most cases 1% of sodium deoxycholate was also added at this stage. The homogenate was frozen and thawed three times, well mixed and centrifuged at 2° for 2 hr. at 1500 g. The volumes of the supernatant fluid and deposit, and the ^{131}I radioactivity of each, were measured. To the supernatant fluid was next added N-HCl to bring the pH to 2.5–5.4, and the flocculated nucleoprotein was sedimented by centrifuging at 0°. The deposit was removed, redissolved and its radioactivity measured. The

* A preliminary account of some of these experiments was reported at the 8th Colloquium on The Protides of Biological Fluids, Bruges, 1960.

pH of the supernatant was readjusted by addition of N-NaOH to 7.5–8.0; 10 mg. of KI was added, with Merthiolate (Eli Lilly and Co.) and disodium ethylenediaminetetraacetate to concentrations of 0.01 and 1% respectively; the volume and radioactivity were measured. Although about half of the albumin remained in the pH 5.2 precipitate, treatment in this way yielded a clear supernatant fluid from which non-specific co-precipitation with the antigen-antibody precipitate was minimized. In two experiments, a portion of the liver extract was cleared by centrifuging at 5° for 2 hr. at 15 000 g, instead of by acid precipitation, and rat albumin was precipitated from the supernatant, in parallel with that from the acid-treated supernatant. The ^{131}I specific radioactivities of the albumins recovered were similar, and the estimates of intracellular albumin did not differ significantly (see Table 3).

Radioactivities were measured in a standard volume in a well-type scintillation counter. At each stage a portion of the material was also treated with an equal volume of 20% (w/v) trichloroacetic acid, in the presence of a few milligrams of KI, and the non-protein radioactivity was estimated separately.

Samples of the serum taken from the rat at death, or of the perfusion plasma at the end of the perfusion *in vitro*, were suitably diluted and their radioactivities measured. From these an approximate estimate could be obtained of the amount of albumin corresponding to a given amount of protein radioactivity which could be used as a guide in deciding the volume of the corresponding liver extract to be taken for precipitation of albumin. To serum and liver-extract samples, estimated to contain 0.2–1 mg. of rat albumin, was added sufficient antiserum to precipitate 75–95% of the radioactivity (by choosing conditions under which the albumin was not precipitated completely it was hoped that precipitation of contaminating proteins would be minimized). After being kept overnight at 2° the precipitated antigen-antibody complex was washed thrice with 0.9% NaCl at 0° , and the rat albumin was extracted from the precipitate by stirring at 2° with 2 ml. of 1% A.R. trichloroacetic acid in aqueous 95% (v/v) ethanol (Delaville, Delaville & Delaville, 1953). After standing for 4–24 hr. insoluble material was removed by centrifuging at 0° , and the albumin was precipitated from the extract by addition of 2 vol. of ether at 0° . The protein was collected by centrifuging and dissolved in 1 ml. of water containing a drop of m-NaHCO_3 . The radioactivities of the final solutions, of the materials remaining in the supernatant after ether precipitation and of the trichloroacetic acid-treated antigen-antibody precipitates were measured. The amount of rat albumin in the final solutions was measured by the method of Lowry, Rosebrough, Farr & Randall (1951), or by the specific absorption at $280\text{ m}\mu$, or both. From these measurements were calculated the ^{131}I specific activities of the recovered rat albumin and the overall recoveries of albumin from the original sera or liver extracts.

Preparation of mitochondrial and microsomal fractions

In the last experiment the whole liver was used for preparation of mitochondrial and microsomal fractions by the method of Schneider & Hogeboom (1951). The supernatant fluids at each stage were set aside for measurement of radioactivity and preparation of albumin. The mitochondrial and microsomal pellets were suspended in 0.9%

NaCl at pH 8.0 and treated with 1% of sodium deoxycholate. The disrupted mitochondrial and microsomal fractions were removed by centrifuging at 9 000 g and 105 000 g respectively, and the supernatant fluids were preserved. Neither the mitochondrial nor the microsomal fragments contained significant ^{131}I radioactivity. The mitochondrial residue was not further examined, but the microsomal fragments (ribosomes) were washed once with phosphate buffer, pH 7.2, and then treated successively for 2 hr. at room temperature with 1% of sodium ethylenediaminetetraacetate, NaHCO_3 at pH 9.3 and 0.5 mg. of pancreatic ribonuclease to release any remaining attached albumin (e.g. Askonas, 1958). The pH was adjusted to 7 and the volume was decreased by pressure dialysis to 1 ml.

Rat albumin was precipitated from the fractions with antibody in the presence of KI, sodium ethylenediaminetetraacetate and Merthiolate as used in other experiments. The final extract of the washed disrupted microsomes was layered in a capillary tube over anti-rat-albumin serum. Since no precipitin band occurred, whereas a control solution containing $10\mu\text{g.}$ of rat albumin/ml. gave a definite band, the extract was presumed to contain less than $10\mu\text{g.}$ of rat albumin.

Immuno-electrophoresis of recovered albumin

The final albumin solutions, after measurement of their radioactivity and protein content, were concentrated by drying from the frozen state and redissolving in a minimum volume of water. They were then examined by immuno-electrophoresis (Grabar & Williams, 1953) with an antiserum prepared against whole rat serum. Although with this antiserum at least seven α -globulin and two β -globulin components were revealed besides albumin and γ -globulin in normal rat serum, there was no evidence of the presence of any component other than albumin in our preparations (Plate 1). As a further check, immuno-electrophoresis was also performed with an antiserum prepared against whole rat-liver homogenate, and absorbed before use with rat plasma. This serum contained antibodies against nine distinct components of liver tissue, but gave no lines with the final extracted albumin preparations. For these reasons we consider that the preparations are unlikely to have contained substantial quantities of components other than albumin.

RESULTS

Seven experiments were made. In two the liver, after homogenization but before freezing and thawing, was divided into two approximately equal portions. To one portion 1% of sodium deoxycholate was added and to the other no further addition was made, and the two portions were thereafter treated similarly. In one such experiment most of the blood was perfused out of the liver beforehand, and in the other the blood was left in. This was done (a) to test whether more intracellular albumin would be extracted if deoxycholate were present to disrupt the microsomes, and (b) to test the validity of our method, which should be unaffected by the amount of free blood present. The results are given in Table 1. The amount of intracellular albumin (defined as above) was calculated as follows: [$\alpha = ^{131}\text{I}$

Immunoelectrophoresis of extracted albumin. *A-C*, Diffused against anti-rat serum; *D*, diffused against anti-rat liver. *A*, Albumin extracted from serum; *B*, albumin extracted from microsome fraction; *C* and *D*, albumin extracted from homogenized liver.

specific activity of plasma albumin (assumed equal to the specific activity of lymph albumin); $y = {}^{131}\text{I}$ specific activity of albumin recovered from the liver; $x = \text{total } [{}^{131}\text{I}] \text{ protein radioactivity in the liver plus contained blood and lymph (all due to } [{}^{131}\text{I}]\text{albumin)}$; $p = \text{mg. of intracellular albumin in liver}$; $q = \text{mg. of extracellular albumin in liver}$. Then, since the only $[{}^{131}\text{I}]$ protein is extracellular albumin, $q = x/a$ and $y = x/(p+q)$, hence

$$p = x(a-y)/ay.$$

This formula is independent of the degree of recovery. Applying the formula to liver 1 (wet wt. taken 11.5 g.; total protein radioactivity 20 900

counts/min.) and liver 2 (wet wt. taken 9.3 g.; total protein radioactivity 126 500 counts/min.) the amounts of intracellular albumin are calculated as follows: liver 1 (bile extract) 12.9 mg., (0.9% sodium chloride extract) 7.6 mg.; liver 2 (bile extract) 11.1 mg., (0.9% sodium chloride extract) 5.13 mg. These figures are in good agreement, despite the fact that one liver was perfused and the other was not, and they also indicate that treatment with deoxycholate revealed the presence of nearly twice as much intracellular albumin as did extraction with buffer alone. The results of the determinations of extracellular and intracellular albumin in the various subcellular fractions of liver are given

Table 1. *Recovery from rat livers 2 days after administration of 75 μC of rat $[{}^{131}\text{I}]$ albumin*

	Liver 1 perfused with 0.9% NaCl		Liver 2 not perfused with 0.9% NaCl	
	Half extracted with deoxycholate	Half extracted without deoxycholate	Half extracted with deoxycholate	Half extracted without deoxycholate
Total radioactivity (counts/min.)	11 930	12 080	65 800	77 024
Total protein radioactivity (counts/min.)	10 400	10 500	60 000	66 500
Total protein radioactivity in sediment (unwashed) after homogenization (counts/min.)	2 730	5 320	11 600	28 660
Total protein radioactivity in 'nucleo-protein' precipitate (counts/min.)	5 620	3 020	24 200	10 940
Total protein radioactivity in supernatant before precipitation of albumin with antibody (counts/min.)	2 150	2 120	24 200	27 000
Counts precipitated with antibody (%)	75	74	94	93
Amount of albumin recovered from precipitate (mg.)	0.086	0.043	0.39	0.51
Counts recovered from antigen-antibody precipitate (%)	45	67	78	92
Specific activity of albumin recovered from liver (counts/min./mg.)	1.340	4 000	4 220	5 300
Specific activity of albumin recovered from serum (counts/min./mg.)	7 700		6 750	
Total protein radioactivity/ml. of serum (counts/min.)	382 500		250 000	

Table 2. *Recovery of albumin from subcellular fractions of rat liver 3 days after administration of 200 μC of rat $[{}^{131}\text{I}]$ albumin*

	Total radioactivity (counts/100 sec.)	Radioactivity present as protein (%)	Specific activity of recovered albumin (counts/mg./100 sec.)	Amount of intracellular albumin in fraction (mg.)	Amount of extracellular albumin in fraction (mg.)
Mitochondrial washings	29 000	78	12 800	1.24	0.51
Deoxycholate extract of mitochondria	8 570	87	2 350	3.0	0.17
Supernatant from microsomes and mitochondria	236 000	86.4	37 000	0.92	4.6
Deoxycholate extract of microsomes	15 250	98.7	4 450	3.05	0.34
Extract of ribosomes	Nil	—	—	< 0.01	0
Serum (counts/ml.)	1 098 600	97.8	44 400	—	24.5 (mg./ml.)

in Table 2. Although the amounts of extracellular albumin are to some extent arbitrary, since they depend upon the efficiency with which blood was perfused out from the liver before homogenization, the figure shows that the isolated mitochondrial and microsomal fractions contained relatively little extracellular but approximately equal amounts of intracellular albumin. Most of the extracellular albumin was found, as expected, in the supernatant fluid after sedimentation of mitochondria and microsomes. The mitochondrial fraction, but not the microsomes, contained substantial amounts of ^{131}I not precipitable with trichloroacetic acid, which were derived presumably from breakdown products of ^{131}I albumin.

The results of the determinations in each experiment are summarized in Table 3, from which it may be seen that the values for liver intracellular albumin ranged from 0.3 to 1.19 mg./g. wet wt. The value for a liver which had been perfused with human blood *in vitro* for 4 hr. (during which it was shown to have secreted about 7 mg. of albumin/hr.) also fell within this range.

DISCUSSION

The validity of our calculations depends on the assumptions that the albumin isolated was pure, that all the intracellular albumin became freely mixed with the labelled extracellular albumin during homogenization and deoxycholate treatment, that the specific radioactivity of albumin isolated from plasma taken immediately before homogenization of the liver was the same as that in the extravascular spaces of the liver, and that the liver contained no selectively accumulated albumin

of high radioactivity. Evidence that the final albumin was not detectably contaminated with other proteins has been given above. In favour of the validity of the second assumption may be adduced our failure to extract significant amounts of albumin from the microsomes disrupted with deoxycholate by further drastic treatment (Table 2). The third assumption is difficult to prove, since the amount of extracellular extravascular protein in a rat liver is equivalent only to 0.06 ml. of plasma or less, and too small, compared with the total blood in this organ, to permit accurate estimation of the rate of equilibration between blood and lymph *in vivo*. However, the anatomical structure of the liver, and the rapid blood flow through it, both favour relatively rapid achievement of equilibrium of ^{131}I albumin between blood plasma and liver lymph. In the experiment in which the liver, taken from a rat 2 days after administration of ^{131}I albumin, was perfused with blood *in vitro* the ^{131}I albumin carried over in the liver from the donor animal had reached equilibrium with the perfusion plasma in about 3 hr. The last assumption is certainly not true if the liver is taken within a few hours of administering labelled albumin, since, however carefully the albumin has been prepared and iodinated, a small fraction is so altered as to be selectively taken up and later broken down by the liver (Cohen & Gordon, 1958). By 48 hr. this fraction appears to have been eliminated, but in an experiment similar to those reported above, except that the liver was removed only 24 hr. after administration of the ^{131}I albumin, the specific radioactivity of albumin recovered from the liver homogenate was actually higher than that from the blood plasma. This must have been due to the

Table 3. *Intracellular-albumin content of rat livers*

Expt. no.	Details	Time interval between injection of [¹³¹ I]albumin and test (days)	Albumin (mg./g. of wet liver)	Albumin in whole liver (mg.)	
1 (a)	Blood perfused out, deoxycholate added	2	1.12	12.9	
(b)	Blood perfused out, no deoxycholate		0.66	7.6	
2 (a)	Blood not perfused out, deoxycholate added	2	1.19	11.0	
(b)	Blood not perfused out, no deoxycholate		0.55	5.1	
3	Perfused <i>in vitro</i> for 4 hr. with human blood, deoxy- cholate added	2	0.74	5.9	
4	Blood perfused out and { extract clarified at pH 5.2 deoxycholate added { extract clarified by high-speed centrifuging	3	{ 0.284 } { 0.326 }	0.305	3.33
5	Blood perfused out and { extract clarified at pH 5.2 deoxycholate added { extract clarified by high-speed centrifuging	3	{ 0.388 } { 0.278 }	0.333	3.93
6	See Table 2	3	0.585	8.17	
7	Blood perfused out, deoxycholate added	5	0.30	3.6	

persistence in the liver of traces of albumin much more highly labelled than that currently in the circulation.

The range of values (0.3–1.19 mg./g.) obtained for the size of the intracellular-albumin pool is rather wide, and it is not clear how far this is due to real variations in the functional activity and rates of secretion of the liver-parenchymal cells. In the four experiments made 3–5 days after injection of [^{131}I]albumin the estimates were quite close (mean 0.38 mg./g.; range 0.3–0.585), and differed from those of the three experiments made 2 days after injection (mean 1.01 mg./g.; range 0.74–1.19). The experiments were made at different times of year and they involved different amounts of radioactivity: 80–130 μC and 25–40 μC /100 g. body wt. for the longer and shorter experiments respectively. The sensitivity of liver to irradiation is known to vary very widely. Thus when cell division is occurring, as in regenerating liver, doses of radiation approximately three times the maximum employed in the present experiments have been found to be just sufficient to decrease the ability of tissue slices to oxidize glucose to carbon dioxide. On the other hand, with normal rat liver, irradiation with the same dose (600 rad.) did not affect glucose oxidation (Holmes, 1956).

The finding that intracellular albumin was about equally distributed between the mitochondrial and microsomal fractions is in agreement with the observation of Peters (1957), who, in chicken liver from which blood had been removed by perfusion, found 1.02 mg. of albumin/g. of which 0.211 mg. and 0.147 mg. were associated with the microsomal and mitochondrial fractions respectively. Dr P. N. Campbell (personal communication) has also found albumin in the mitochondrial fraction of rat liver. In our own experiments the mitochondrial fraction was subjected to only one washing with 0.3M-sucrose. An estimate of the degree of contamination with microsomes of the mitochondrial fraction prepared in this way has been provided by Dr B. D. Roodyn (personal communication) on the basis of the distribution of glucose 6-phosphatase. On the accepted assumption that this enzyme is confined to the microsomes it appears that the mitochondrial fraction would contain a maximum of 23% of microsomal material. Even if 25% of the albumin found in the deoxycholate extract of the mitochondrial fraction is attributable to contamination, some 2.3 mg. remains to be accounted for. Whether or not such albumin was synthesized in the mitochondria is an open question. In experiments with chicken-liver slices, incubated *in vitro* for 20 min. with radioactive glycine, Peters (1957) found relatively little radioactivity in albumin isolated from the particulate fraction composed mainly of mitochondria, compared with that from the micro-

somal fractions. Keller, Zamecnik & Loftfield (1954) found that, 10 min. after administration of [^{14}C]leucine *in vivo*, the protein of the mitochondrial fraction of rat liver had only one-fifth of the radioactivity of that of the microsomal fraction. It is clear that synthesis of albumin in the mitochondria, if it occurs at all, must be slow compared with that in microsomes. In our experiments, if the albumin in the mitochondrial fraction was neither synthesized there nor due to microsomal contamination, it might presumably have been derived from extracellular albumin; if so it would have been expected to contain ^{131}I . The fact that it did not do so (implicit in our definition of 'intracellular' albumin) implies either that it was derived from within the cell or that iodine had been split from extracellular albumin in the mitochondria without degradation of the protein.

The finding of [^{131}I]albumin-breakdown products in the mitochondrial but not in the microsomal fractions indicates that microsomes are probably not sites of albumin breakdown, or that non-protein iodine is very rapidly lost from them. The actual amounts of non-protein iodine (Table 2) correspond to 0.625 mg. of [^{131}I]albumin in the supernatant fluid from which mitochondria and microsomes had been largely removed, to 0.112 mg. in the washings from the mitochondrial fraction and to 0.022 mg. in the mitochondrial fraction itself. These figures are compatible with, but do not prove, the idea that ^{131}I was split off from albumin in the mitochondria. The probability that this occurred is strengthened by the observations of Penn (1960) that isolated liver mitochondria (but not microsomes) in the presence of suitable cofactors can rapidly degrade rat albumin labelled with [^{14}C]phenylalanine, largely to free amino acids.

Taking the mean value obtained in the third- and fifth-day experiments, 0.4 mg. of albumin/g. wet wt., for the amount of intracellular albumin, it is possible to calculate approximately the turnover time of the liver albumin. According to Gordon & Humphrey (1960) the liver from a rat weighing 300 g. secretes about 12 mg. of albumin/hr. The total intracellular albumin in the liver of such a rat is about 4.5 mg. and the time taken for renewal of the intracellular-albumin pool is thus about 22 min.

It has been observed by several workers that, after addition of labelled amino acids, labelling of secreted proteins such as albumin or γ -globulin is not detectable either *in vivo* or *in vitro* until after 15 or more min. For chicken liver, Peters (1957) found that about 20 min. was required before added [^{14}C]glycine appeared in the extracellular albumin, although he could recover labelled albumin from liver microsomes within 10 min., and extrapolation of his data indicated that some labelled albumin

might already have been present after 3–4 min. The period of time required for the first appearance of secreted labelled albumin is only a little shorter than that which we calculate to be required for complete replacement of the intracellular albumin. In our experiments three-quarters of the newly synthesized intracellular albumin was bound to subcellular particles, but the true proportion was probably greater, inasmuch as any disruption or failure to sediment the particles would increase the amount of albumin apparently unbound.

These facts taken together suggest that the process of secretion involves rapid synthesis of albumin molecules, which remain attached to subcellular structures for a relatively much longer period, before being rapidly released from the cell. The process must resemble travel along a pipe line, since the time taken for the first labelled albumin molecules to be secreted in the presence of labelled amino acids is little less than the time taken for secretion of an amount of albumin equal to the whole of the intracellular pool. Furthermore, in an active liver, the time required for synthesis of albumin molecules and their passage to the surface of the cell cannot vary very widely from cell to cell or even within each cell.

SUMMARY

1. An estimate of the amount of intracellular albumin in rat livers has been obtained by an isotope-dilution method, in rats which had received rat [^{131}I]albumin and been kept until equilibrium had been reached between the blood and the liver.

2. Albumin was recovered, by precipitation with antibody and dissociation in a trichloroacetic acid-ethanol mixture, from the livers after homogenization and treatment with deoxycholate, and the specific radioactivity was measured and compared with that of plasma albumin.

3. The estimates of the intracellular albumin

pool were 0.3–1.19 mg./g. wet wt. In four of seven experiments the value was close to 0.4 mg./g.

4. The distribution was examined of intracellular albumin between mitochondrial and microsomal fractions and the supernatant. At least three-quarters of the intracellular albumin was associated with the particulate fractions, and was evenly divided between the two.

5. The bearing of these findings on the process of albumin synthesis and secretion are discussed.

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The Purification of Interferon

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Virus interference may be defined as an effect of a live or inactivated virus on host cells which results in that cell being unable subsequently fully to support the growth of a second virus. In 1957, during a study of interference induced by heat-

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inactivated influenza virus, Isaacs & Lindenmann (1957) observed the formation of a virus-interfering substance, to which they gave the name 'interferon'. Interferon could not be detected until several hours after treatment with inactivated virus, when it appeared first in the cells and was subsequently released into the surrounding buffer