Isolation of Glucosamine from the Capsids of a Picornavirus

By S. HALPEREN* H. O. STONE† and B. D. KORANT

Central Research Department, Experimental Station, E. I. du Pont de Nemours and Company, Wilmington, Delaware 19898, U.S.A.

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SUMMARY

The purified capsids of echovirus 12 were found to contain radioactivity when radioactive glucosamine was provided for virus-infected cultures. Radioactivity was identified with glucosamine, after recovery from the virus particles by acid hydrolysis and paper chromatography. Although the label was not removable from the isolated virus particles without destruction of virus, polyacrylamide gel electrophoresis showed that it was not covalently bound to any of the four virus polypeptides since it migrated more rapidly and was identical electrophoretically to the glucosamine-labelled fraction isolated from uninfected cells. Glucosamine labelling of virus capsids was only achieved during virus multiplication, since labelled fractions from uninfected cells did not bind to virus capsids. Its apparently low mol. wt., lack of associated virus or cellular proteins, lack of labelling of virus with other radioactive sugars, and the similar electrophoretic migration of free N-acetyl glucosamine suggested that the component is an acetylated product that is trapped during the assembly of the virus.

INTRODUCTION

Evidence in several reports has suggested that carbohydrate or carbohydrate derivatives may be present in the capsids of picornaviruses (Lerner, Bailey & Tillotson, 1965; Rueckert, 1965; Lerner & Miranda, 1968) and there is direct chemical evidence for a carbohydrate constituent in echovirus 12 (Stevens et al. 1968). We have reported that echovirus 12 became radioactively labelled when radioactive glucosamine was provided for infected cell cultures (Halperen, Stone & Korant, 1970). We now report evidence suggesting that the radioactivity associated with virus is in the form of N-acetyl-d-glucosamine after its acetylation from the original glucosamine by the cell.

METHODS

Cell cultures. The LLCMK2 continuous line of monkey kidney cells (Hull, Cherry & Tritch, 1962) were propagated as monolayers in plastic bottles or in 100 × 20 mm plastic Petri dishes (Falcon Plastics) in a growth medium consisting of Eagle's Minimal Essential Medium (MEM) containing 6 % calf serum and 100 μg/ml streptomycin and 100 units/ml penicillin. Trypsin (0.25 %) was used to disperse the cells for passage.

Virus. Echovirus 12 (Travis strain) was propagated in monolayers of LLCMK2 cells maintained in serum-free MEM. In all experiments virus infection was affected by adsorption to monolayers at multiplicity of 30 to 40 p.f.u./cell. Virus titrations were performed by a

* Present address: Department of Oral Pathology, Baylor College of Dentistry, 800 Hall Street, Dallas, Texas 75226, U.S.A.
† Department of Microbiology, University of Kansas, Lawrence, Kansas 66044, U.S.A.
haemagglutination procedure in serial twofold dilutions with a half-tube precision in successive titrations (Halperen, Eggers & Tamm, 1964). For storage, cells were scraped up into 0.5 ml/plate of phosphate buffered saline (PBS, pH 7.2 to 7.4) and kept at -20 °C. For virus purification, infected cells suspended in PBS were extracted by three cycles of freezing and thawing to release virus; extracts were clarified by centrifuging at 6000 g for 10 min; supernatant fluids were homogenized twice with ethyl ether and twice with Freon®, (E. I. du Pont de Nemours & Co.), mixed with PBS-saturated CsCl solution at 4 °C to an average density of 1.33 g/ml and centrifuged in a Spinco SW39 or SW50.1 rotor at 110000 g for 18 to 24 h. Fractions were collected after puncturing the bottom of the centrifuge tube. With this procedure, when radioactive amino acids were provided in the maintenance medium during the entire period of infection, the only peaks of TCA-precipitable radioactivity found in fractions of CsCl gradients were identical with peaks containing the virus particles. Thus virtually all contaminating cellular proteins were eliminated.

Gel electrophoresis. Samples for electrophoresis were dialysed against 0.01 M-phosphate buffer solution, pH 7.2, reconstituted to contain 0.1 % sodium dodecyl sulphate (SDS) and 0.1 % 2-mercaptoethanol, and then immersed in a boiling water bath for 1 min. The preparations were then electrophoresed through 10 % polyacrylamide gels containing 0.1 % SDS with 5 mA/gel for 18 to 20 h (Summers, Maizell & Darnell, 1965). Gels were fractionated by a crushing device (Maizel, 1966) and the radioactivity of the fractions suspended in Bray's solution was determined with a liquid scintillation counter.

Chromatography. The identification of glucosamine associated with virus was by chromatography on paper (Spiro, 1966). Radioactive glucosamine-labelled virus particles, obtained from peak fractions in CsCl density gradients, were dialysed against distilled water, hydrolysed (4 N-HCl, 120 °C, 6 h), and amino sugars separated by descending paper chromatography on Whatman no. 40 paper. The paper was cut into 7/8 x 1 7/8 in. strips which were then counted in a dioxane base scintillation solution.

Chemicals. 2-(α-hydroxybenzyl)benzimidazole (HBB) was obtained through the kindness of Dr H. J. Eggers, Giesen, West Germany. The compound was used as a 0.22 mM solution prepared by shaking overnight at 37 °C in protein-free medium. Radioactive chemicals were purchased from New England Nuclear Corporation, Boston, Massachusetts. In all experiments [3H]- or [14C]-glucosamine were used at 3 μCi/ml (sp. act. [3H] > 200 mCi/m-mol; [14C] sp. act. 40 to 50 mCi/m-mol). Mixtures of L-amino acids were used at the concentrations indicated in the figure legends.

RESULTS

Association of glucosamine with echovirus 12

Although the capsids of picornaviruses are assumed to contain no carbohydrate (Schwerdt & Schaffer, 1965) the evidence that the capsids of picornavirus may contain carbohydrates or derivatives led to the attempt to label echovirus 12 with amino sugars. [14C]-glucosamine was added to the medium of several cell cultures immediately after adsorption of echovirus 12. The cultures were then incubated for 8 h until marked c.p.e. developed. The media were then drained and the cells harvested in small vol. of PBS.

The samples of harvested virus were then purified as described in Methods. Before centrifuging the samples were dialysed against 500 vol. of PBS to remove ‘soluble’ radioactive material. Fractions were collected from CsCl density gradients and diluted with PBS. A sample of each fraction was removed for titration of haemagglutinating activity and the remainder precipitated with 5 % TCA and counted. A preparation of virus labelled with
Echovirus 12-bound cellular glucosamine

Fig. 1. Labelling of echovirus 12 capsids with radioactive glucosamine and amino acids. Haemagglutinating activity (×—×) and TCA-precipitable radioactivity from [14C]-glucosamine (●—●) (sp. act. 52 mCi/mmol) were determined in fractions from a single CsCl density gradient. Only the TCA-precipitable radioactivity from [14C]-amino acids (○—○) was determined in fractions from a separate gradient.

[14C]-amino acids was fractionated simultaneously in another similar gradient. The results are summarized in Fig. 1. The two types of haemagglutinating virus particles distinguished in fractions from CsCl gradients were previously shown to represent virus particles (density 1.33 g/ml in CsCl) and RNA deficient or empty capsids (density 1.29 g/ml). It was also shown that no virus-specific particles smaller than the virus capsid participated in the haemagglutination reaction (Halperen et al. 1964). These procedures show identical patterns of recovery for the haemagglutinating activity of the virus particles and the radioactivity due to either glucosamine or amino acids.

Dialysis was essential for removing peaks of glucosamine-labelled material that was not bound to virus, since multiple radioactive peaks were found in fractions from non-dialysed preparations. However, dialysis was not necessary for the preparation of amino acid labelled virus particles, since only homogenization with ether and Freon® was necessary to remove all non-virus radioactivity. These findings suggest that the glucosamine-labelled peaks not associated with virus were not glycoproteins. The slight disparity between the peak of haemagglutinating activity of the empty capsids and the corresponding radioactivity is probably due to uncertainties in the assay of haemagglutination. There was closer agreement between virus haemagglutinating activity and TCA-precipitable, glucosamine radioactivity when CsCl density gradient fractions containing peaks of haemagglutinating activity of [14C]-glucosamine-labelled virus were dialysed to remove the CsCl, and fractionated again by CsCl density gradient centrifuging (Fig. 2).
Fig. 2. Fractions from CsCl density gradient of Fig. 1, containing the peaks of haemagglutinating activity and TCA-precipitable radioactivity, were dialysed separately against 500 vol. of PBS, mixed with PBS-saturated CsCl to an average density of 1.33 g/ml, and centrifuged at 110000 g for 19 h. Fractions were assayed for haemagglutinating activity (×—×, △—△) and TCA-precipitable radioactivity (●—●, ○—○). (A) 'Full' capsids; (B) RNA-deficient capsids.

Attempts were also made to label virus with radioactive choline, fructose, fucose, galactose, galactosamine, or N-acetyl glucosamine, but in no case was virus-associated radioactivity found in CsCl density gradient fractions.

Further evidence for the close and specific association of the glucosamine label with virus was obtained when virus was reacted with SDS. In these experiments it was found that the glucosamine label remained non-dialysable as long as the integrity of the virus or empty capsids was retained. However, the glucosamine label became dialysable when virus haemagglutinating activity was lost, as when virus was heated with SDS in boiling water, or when empty capsids were dialysed against SDS. Similar results, not presented here, were obtained when glucosamine labelled virus was homogenized with butanol–water mixtures, or subjected to extreme changes in pH, or degradation with borate buffer at pH 10.

That the radioactivity associated with the virus particles remained as glucosamine was shown by chromatography of acid hydrolysed, glucosamine-labelled virus. Fractions from CsCl gradients containing peaks of [3H]-glucosamine labelled virus particles were dialysed against distilled water, hydrolysed and chromatographed as described in Methods. The results (Fig. 3) show the approximate correspondence of the profile of radioactivity from glucosamine-labelled virus with the peak of radioactivity from a parallel chromatogram of a mixture of radioactive and non-radioactive glucosamine. Although glucosamine was found in the hydrolysates of the virus, it was not known at that time whether glucosamine was present in the virus particles or as N-acetyl glucosamine, since the hydrolysis conditions would result in the loss of acetyl groups.

Although not shown in Fig. 3, non-radioactive glucosamine, in mixture with portions of radioactive control glucosamine and virus hydrolysate samples, was located by staining with 1% ninhydrin at the same position in the chromatogram as the radioactive control. When non-radioactive galactosamine and N-acetyl glucosamine were chromatographed together they were separated from glucosamine by these procedures, as were the carbohydrates glucose and galactose which were identified by staining with AgNO₃-NaOH (Block, Durrum & Zweig, 1958).

To determine whether the glucosamine was associated with a specific structural polypeptide of the virus, fractions from CsCl gradients containing virus doubly-labelled by [3H]-amino acids mixture and [14C]-glucosamine were dialysed. The virus was then disrupted.
Echovirus 12-bound cellular glucosamine

Fig. 3. Identification of glucosamine in echovirus 12. The virus was purified, hydrolysed, amino sugars separated by descending paper chromatography, and radioactivity counted as described in Methods. —, Virus; --, control glucosamine sample.

Fig. 4. Lack of covalent binding of virus-associated glucosamine to virus polypeptides. Virus was doubly labelled for 8 h during a single cycle of multiplication by inclusion in the maintenance medium of [3H]-l-amino acid mixture at 5 μCi/ml and [14C]-glucosamine at 3 μCi/ml. After purification as in Methods, a CsCl fraction containing radioactive virus was analysed by electrophoresis through a 10% polyacrylamide gel. The fractions obtained were examined for radioactivity. —, [3H]-amino acids; --, [14C]-glucosamine. A control sample of [14C]-glucosamine (control) was analysed on a parallel gel (-----).
Fig. 5. CsCl density-gradient fractionation of cellular glucosamine components. Ten monolayer cultures (~10⁸ cell culture) were maintained for 24 h at 37 °C in MEM containing 3 μCi/ml [3H]-glucosamine. The cultures were drained, the cells harvested in 5 ml PBS, disrupted by three cycles of freezing and thawing, and virus added to 2 × 10⁶ p.f.u./ml. The mixture was then incubated at 37 °C for 24 h and purified as in Methods, but without dialysis. CsCl density gradient fractions were assayed for TCA-precipitable radioactivity (×××) and haemagglutinating activity (○○○○).

Isolation of cellular glucosamine-containing material electrophoretically identical to that from virus particles

A series of experiments was set up to determine whether cellular components containing glucosamine could bind to virus particles. Cell cultures were maintained in MEM containing radioactive glucosamine for 24 h at 37 °C. The medium was then drained and the cells
Fig. 6. Comparison by polyacrylamide gel electrophoresis of glucosamine-containing components from uninfected cells and from labelled virus particles. An undialysed sample of fraction 18 from the gradient in Fig. 5 was prepared for polyacrylamide gel electrophoresis by the addition of SDS to 0.1% and 2-mercaptoethanol to 0.1% in 0.01 M-PO₄ buffer solution, pH 7.2. The sample was heated in a boiling water bath for 1 min before electrophoresis (---). A CsCl density-gradient fraction of [¹H]-glucosamine-labelled virus dialysed against 500 vol. of 0.1% SDS and 0.1% 2-mercaptoethanol before degradation by heating was analysed in a parallel gel (---); as were [¹H]-amino acid-labelled virus (---); and free N-acetyl glucosamine (-----). Right side, ordinates for glucosamine; left side, for amino acids.

Echovirus 12-bound cellular glucosamine

resuspended in PBS and disrupted by three cycles of freezing and thawing as for virus extraction. Extracts were prepared from uninfected cells and from virus-infected cells in which virus multiplication was prevented by inclusion in the medium of 0.22 mM-HBB (Eggers & Tamm, 1961). To these extracts non-radioactive virus was added to give 2 × 10⁹ p.f.u./ml and 2 × 10⁵ cells/ml, and the mixtures were incubated for 24 h. The mixtures were then dialysed against 300 vol. of PBS and the virus purified as described in Methods. Fractions from CsCl density gradients were examined for TCA-precipitable radioactivity and for virus haemagglutinating activity. No radioactivity was found in any of the fractions from these gradients although the usual two peaks of virus haemagglutinating particles were detected. Extracts of cells which had not been dialysed showed a number of peaks of glucosamine label in fractions from CsCl gradients, some of which coincided with peaks for virus particles (Fig. 5).

The material containing glucosamine from peak fraction 18 of the CsCl gradients of non-dialysed uninfected cells (Fig. 5) was compared with virus-associated glucosamine by polyacrylamide gel electrophoresis (Fig. 6). As a marker, amino-acid-labelled virus was electrophoresed at the same time in a parallel gel. The glucosamine-labelled materials from these sources were electrophoretically identical and identical electrophoretic patterns were
Fig. 7. Comparison of the rates of incorporation of radioactive amino acids and glucosamine into virus-infected cultures. Replicate monolayer cultures were infected with virus diluted in MEM or 'mock' infected with MEM, and incubated at 37 °C. At the indicated times [14C]-amino acids or [3H]-glucosamine was added to duplicate infected and uninfected cultures and incubation continued for 15 min. Plates were drained and immediately placed on crushed ice, washed twice with ice cold PBS, extracted twice with 10% TCA, twice with 5% TCA, harvested in 1 ml absolute ethanol and radioactivity counted in a dioxane based scintillation solution ([3H]-glucosamine: ●—●, infected; ○—○, uninfected; [14C]-amino acid: ▲—▲, infected; △—△, uninfected).

obtained with fractions 12 or 14 from the gradient shown in Fig. 5. These results suggest that the virus glucosamine label originated from a cellular component containing glucosamine. Since a primary cellular product of glucosamine is the acetylated form, and the conditions for hydrolysis and chromatographic identification could not distinguish between the two molecules, free N-acetyl glucosamine was also electrophoresed under identical conditions described in Methods. The results in Fig. 6 show that free radioactive N-acetyl glucosamine is electrophoretically identical with the glucosamine label extracted from virus particles and from the gradient fractions obtained from uninfected cultures. Thus it is concluded that the virus-associated component contains, in fact, N-acetyl glucosamine. The lack of association with virus of extracted radioactive glucosamine-containing components from uninfected cells or from cells infected in the presence of HBB may be due to the impermeability of the capsids after formation. Alternatively, the receptor sites on the virus for this component may become saturated during virus multiplication, or factors present only during virus multiplication may be essential.

It is possible that the labelling of virus during multiplication may be due to the stimulation by virus infection of the synthesis of glucosamine-containing components. The incorporation of glucosamine was compared with that of amino acids, and the results are shown in Fig. 7. It is evident that while virus infection sharply inhibits cellular protein synthesis there is little significant change in the synthesis of TCA-precipitable products containing glucosamine during a single cycle of virus multiplication.
DISCUSSION

A component of small mol. wt. containing glucosamine was found closely associated with the virus particles and empty capsids of echovirus 12. Our evidence suggests that this component originates in the cells, is acetylated and becomes associated with virus particles only during the synthesis and/or assembly of virus protein into capsids. It is notable that virus was not labelled when radioactive N-acetyl glucosamine was provided for the infected cultures. Since N-acetyl glucosamine did not label any component in uninfected or infected cells that was similar to that found in CsCl gradients of extracts from glucosamine-labelled cultures, it is probable that N-acetyl glucosamine is not significantly incorporated into these cells.

Although the exact nature of the glucosamine label obtained from the virus particles and from the isolated cellular components is not known, it is not believed to be a glyco- or muco-peptide because the label was not covalently linked to any of the virus polypeptides, and virtually all cellular proteins were removed by the purification methods. However, we cannot rule out the possibility that a cellular protein may have been glucosylated during infection.

The exact physical relationship between the virus and the cellular component containing glucosamine remains to be determined, as does its precise source. However, since the virus-associated labelled glucosamine could not be released from the virus without destruction of virus activity, it is reasonable to assume that the component is not merely loosely bound to the surface of the virus particles. It may be that this material is enclosed in virus capsids during maturation in some way analogous to the inclusion of cellular constituents in the capsids of small DNA viruses (Michel, Hirt & Weil, 1967; Winocour, 1968; Trilling & Axelrod, 1970; Estes, Huang & Pagano, 1972). Whether this material originates as a possible free membrane intermediate or cytoplasmic membrane constituent cannot as yet be determined. However, since cytoplasmic membranes have been shown to proliferate in cells as a result of infection with a number of picornaviruses (Dales et al. 1965; Amako & Dales, 1967a, b; Skinner, Halperen & Harkin, 1968), and the role of special membranes in the synthesis of specific virus products has been established (Caligiuri & Tamm, 1970a, b; Roumiantzef, Summers & Maizel, 1971), it may be suggested that membrane involvement in virus protein synthesis could lead to the binding of membrane constituents to the proteins in whose synthesis the membranes are functioning. In this respect it is known that membranes contain proteins bound in them with a variety of affinities, and some of these proteins function as enzymes for the synthesis of the membrane of which they are a constituent (e.g. Roelefsen, Zwaal & Van Deenen, 1971).

We have also found identical glucosamine labelling with echovirus 7 but not with poliovirus type 2 in the same cells. Whether or not this phenomenon is related to the property of virus haemagglutination remains to be determined.

Although the function of these components in picornavirus multiplication is still purely speculative, experiments to be reported separately will show that the sedimentation properties of cellular constituents labelled with radioactive glucosamine are changed sharply after infection with echovirus 12, a finding that may be related to the mobilization of membrane constituents recently reported to result from infection with poliovirus (Mosser, Caligiuri & Tamm, 1972).

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REFERENCES


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