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GLUCOSAMINE BINDING TO SERUM PROTEINS: ITS POSSIBLE RELEVANCE TO CELL SURFACE AND CONDITIONED MEDIUM STUDIES

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SUMMARY

Radioactive species in commercial preparations of [^{14}C]glucosamine bind to macromolecular components in horse, fetal calf and human sera, as well as to bovine serum albumin. The binding appears to be non-enzymatic and is stable through extensive dialysis and acid precipitation. On the basis of competition and chromatographic experiments glucosamine appears to be the major reactive species.

When chick muscle fibroblasts are grown in serum-containing medium with [^{14}C]glucosamine, at least some of their radioactive cell surface components as well as radioactive components in the overlying conditioned medium, display chromatographic properties which are similar to those detected in complete medium which has been exposed to [^{14}C]glucosamine in the absence of cells. Preliminary results with [^{3}H]fucose indicate that it also binds to acid-precipitable medium proteins in the absence of cells. The possible relevance of such "anomalously labeled" serum components to the study of cell surface and conditioned medium glycoproteins is discussed.

INTRODUCTION

The use of radioactive sugar precursors to label the glycoproteins and mucopolysaccharides of tissue culture cells has greatly facilitated the study of cell surface components [1–5]. In experiments of this type, and in those involving cell surface "turnover" [5, 6], radioactive macromolecular material has usually been interpreted to result from cellular biosynthesis. Although it seems likely that most of the macromolecular radioactivity decribed in such experiments does indeed result from biosynthesis, to our knowledge the formal possibility that anomalously labeled serum proteins might also contribute to the results has not been considered.

In a series of experiments with chick muscle cells in which we were attempting to measure the extent to which putative cell surface glycoproteins were released into the culture medium, we ran the seemingly trivial control of incubating fresh culture medium with [^{14}C]glucosamine in the absence of cells. Unexpectedly, a significant amount of acid-precipitable radioactivity was found, and the total precipitable radioactivity increased with incubation time over a 24 h period. This observation suggested the possibility of a direct association between serum proteins and radio-

active sugar precursors. In addition, since there had been reports that serum proteins bind to cell surfaces [7, 8], it raised the possibility that a percentage of the cell surface radioactivity measured in cultures exposed to sugar precursors could be due to the adhesion of serum protein–sugar complexes to the cell surface and/or to the direct association of radioactive sugar precursors with serum proteins which had adsorbed previously to the cell surface.

In this paper we describe a series of experiments which indicate a non-enzymatic association between serum proteins and radioactive species present in commercial preparations of [^{14}C]glucosamine and [^{3}H]fucose. In addition to glucosamine itself, radioactive impurities in commercial preparations of [^{14}C]glucosamine are described which can bind to macromolecular serum components. At least some of the anomalously labeled serum peptides are chromatographically similar to radioactive components isolated from the surfaces of cells grown in medium containing commercial [^{14}C]glucosamine. Previous chemical studies have described the occurrence of direct sugar–protein interactions [9]; however, we are not aware that such interactions have been considered in recent studies of cell surface biochemistry.

MATERIALS AND METHODS

Cell culture medium

The cell culture medium routinely employed in these studies contains, by volume: 44 % Hank's balanced salt solution, 40 % NC nutrient solution, 10 % horse serum, 5 % chick embryo extract [10], and 1 % penicillin–streptomycin (10 000 units penicillin G-salt, potassium-salt and 0.5 mg/ml streptomycin sulfate).

Chemicals

Horse serum, fetal calf serum and newborn calf serum were purchased from Grand Island Biological Company (Grand Island, N.Y.); human serum was obtained from pooled hospital samples.

Radioactive hexosamines were purchased from New England Nuclear (Boston, Mass.) and from Amersham/Searle Corp. (Arlington Heights, Ill.). Preparations from both sources gave qualitatively the same results. Specific activities of the various batches were: D-[1-^{14}C]glucosamine · HCl, 40–50 Ci/mole; D-[6-^{3}H]glucosamine (3.6 Ci/mmole); D-[1-^{14}C]glucosamine · HCl, 55 Ci/mole and D-[1-^{3}H]glucosamine · HCl, 2.3 Ci/mmole. Only one batch of L-fucose (L-[1,5,6-$^{3}H_3$]fucose; 4.8 Ci/mmole) was tested and it was purchased from N.E.N.

D(+)Galactosamine · HCl was purchased from J. T. Baker Co. (Phillipsburg, N.J.); D-(+)-glucosamine · HCl from Nutritional Biochemicals Corp. (Cleveland, Ohio); trypsin (twice crystallized) from Worthington Biochemical Corp. (Freehold, N.J.); bovine serum albumin (Pentex crystallized) from Miles Laboratories, Inc. (Kankakee, Ill.).

Labeling techniques

Whole culture medium was labeled in the absence of cells in either plastic culture dishes (Falcon Plastics; Oxnard, Calif.) or in glass prescription bottles with equivalent results. Serum was labeled equally well in either balanced salt solution (Hank's

and NC stock solution) or in phosphate buffer (0.1 M containing 0.1 M NaCl (pH 7.4).

When Dowex column fractions were to be analyzed for serum binding ability, 1.0-ml fractions were neutralized with 3 M NaOH; phosphate buffer (pH 7.4) was added to give a final concentration of 0.1 M, and finally, serum was added to 10 % (v/v).

Cells were labeled at confluency in 3 ml of medium on 100-mm plastic culture dishes. The dishes were rocked at medium speed on a rocker platform (catalogue No. 6600, Bellco Glass, Vineland, N.J.).

All incubations were at 37 °C in a water-saturated, air–CO_2 (95 : 5; %) incubator.

Competition experiments

Competition experiments were performed in 0.1 M NaH_2PO_4 buffer (pH 7.4) containing 0.1 M NaCl. A 10 % (v/v) solution of horse serum was incubated at 37 °C for 28 h with 1 μCi/ml of commercial [^{14}C]glucosamine and increasing concentrations of cold glucosamine. The unlabeled glucosamine stock solution itself was also made up in the phosphate buffer and adjusted to pH 7.4. Following acid precipitation, the pellets were washed, solubilized and counted as described below.

Acid precipitation and dialysis

All acid precipitations were accomplished by adding an equal volume of cold 20 % trichloroacetic acid to the sample. Pellets were washed four to seven times with 5 vol. of cold 5 % trichloroacetic acid by resuspension and centrifugation. The washed pellets were solublized in 1 ml of 5 % sodium dodecylsulfate and subsequently counted or dialyzed further.

Some incubation mixtures were sequentially dialyzed prior to acid precipitation versus: (a) 10^{-2} M glucosamine–10^{-3} M Tris (pH 7.4); (b) 1 M NaCl; (c) 6 M guanidine · HCl; and finally (d) against glass-distilled water.

Before ion-exchange chromatography, trypsin digests were first exhaustively dialyzed (without acid precipitation) against 10^{-3} M Tris containing $5 \cdot 10^{-3}$ M NaCl (pH 7.4), and then against DEAE starting buffer.

Column chromatography

(*A*) *Dowex column chromatography.* 0.9 cm × 40 cm Dowex 50 W-X12 (H^+ form) columns were used for separation of amino sugars, according to Gardell [11]. Glucosamine and galactosamine markers were detected by the Elson-Morgan reaction [12].

(*B*) *DEAE column chromatography.* DEAE Sephadex A-25 (1.5 cm × 30 cm column) was used for separation of labeled trypsin digests. Starting buffer was 0.02 M Tris (pH 7.5) containing 0.09 M NaCl; limit buffer was 0.02 M Tris (pH 7.5) containing 1.0 M NaCl. A 600 ml linear gradient was employed and 1-ml fractions were collected at a flow rate of 15 ml/h and counted directly.

NH_3 detection

Glucosamine (0.5 M) in phosphate buffer (0.1 M phosphate buffer–0.1 M NaCl; pH 7.2) was incubated at 37 °C. Incubated solutions were adjusted to pH 11

with 10 M NaOH. NH_3 was detected by means of an ammonia electrode (Model 95-10; Orion Research, Inc., Cambridge, Mass.), using the expanded scale of a Beckman Zeromatic pH meter (Beckman Instruments, Inc.; Fullerton, Calif.). Standards consisted of increasing concentrations of NH_4Cl in phosphate buffer.

Trypsin digestion

(*A*) *Media.* Before trypsin digestion, labeled medium was dialyzed exhaustively against 10^{-3} M Tris (pH 7.4) containing $5 \cdot 10^{-3}$ M NaCl, and then against 0.02 M Tris (pH 7.4)–0.05 M $CaCl_2$–0.03 M NaCl. Dialysis was continued until a background level of dialyzable radioactivity was obtained. In a typical experiment this required four 2-l dialysis changes over a 2 day period. Medium was digested with 2 mg/ml trypsin for 2 h at 37 °C and the pH was readjusted periodically to 7.8–8.0 with NaOH. Fresh trypsin (1 mg/ml) was added after 1 h of incubation.

(*B*) *Cells.* After five washes with 5 ml of Puck's saline G, each monolayer culture was trypsin digested for 15 min with 3 ml of trypsin (1 mg/ml) in 0.02 M Tris (pH 7.4) –0.05 M $CaCl_2$–0.03 M NaCl. The loosened cells and trypsin digest were transferred to a test tube and cells were separated from the trypsinate by centrifugation at 800 rev./min in a Model PR-2 international refrigerated centrifuge (rotor No. 269). The digests were centrifuged at 15 000 rev./min in a Sorvall RC2-B refrigerated centrifuge (Rotor SS-34) for 30 min, and the supernatant was then dialyzed.

Scintillation counting

All samples were counted as 1 ml aqueous volumes in 10 ml of toluene–Triton X-100 (2 : 1, v/v) containing 0.4 % (w/v) PPO. Counting was carried out in a Beckman LS-150 scintillation counter.

Muscle cells

Primary cells were obtained from leg muscle of day 11–12 chick embryos [13]. Cells were grown on collagen-treated 100-mm plastic culture dishes in 6.0 ml of medium. Cultures were fed every other day with fresh medium. On Day 5 confluent primary cultures were dissociated with collagenase (0.05 % in Ca^{2+} and Mg^{2+}-free Saline G). The muscle fibers were filtered out through si k and the single cells were replated in secondary culture.

Similar treatment of secondary cultures yielded tertiary cultures which exhibited little, if any, tendency to form muscle fibers. The cells which predominate in tertiary cultures will be referred to as "fibroblasts".

RESULTS

(1) *Binding of radioactivity from commercial [^{14}C]glucosamine to medium proteins*

In order to determine the extent to which radioactivity from commercially prepared [^{14}C]glucosamine associates with macromolecular components of culture medium, [^{14}C]glucosamine (99 % pure by manufacturer's specifications) and culture medium were incubated together and then precipitated with trichloroacetic acid. After 24 h of incubation with complete medium, 0.1–0.2 % of the total radioactivity was precipitable. This percentage was approximately the same for all concentrations

of [^{14}C]glucosamine tested. Analogous experiments with medium containing either horse serum or embryo extract indicated that 90 % of the precipitable radioactivity from whole medium was associated with horse serum components and 10 % was associated with embryo extract components (Table I).

TABLE I

ACID-PRECIPITABLE BINDING OF [^{14}C]GLUCOSAMINE TO MEDIUM PROTEINS

Acid-precipitable counts were normalized to cpm/ml incubation mixture per μCi [^{14}C]glucosamine (50 Ci/mole). Media were incubated at 37 °C with 2 μCi/ml [^{14}C]glucosamine (50 Ci/mole). After 22 h, 1 ml was precipitated with trichloroacetic acid; the pellet was washed, solubilized and then counted (cf. Materials and Methods).

Sample	Acid-precipitable counts	cpm per mg protein added
Complete culture medium (10 % horse serum, 5 % embryo extract (9.1 mg protein/ml)	3900	430
10 % horse serum (8.4 mg protein/ml)	3500	417
5 % embryo extract (0.7 mg protein/ml)	500	714
Bovine serum albumin (10 mg protein/ml)	3800	380

Although the acid-precipitable radioactivity was not removed with seven 3-ml trichloroacetic acid washes, it was conceivable that the radioactivity was non-specifically "trapped" during the initial acid precipitation. Since the amount of non-specific trapping should not be affected by the time of incubation, whereas the binding of radioactivity to medium components should be time dependent, the number of precipitable counts was studied as a function of incubation time. Fig. 1 demonstrates that the amount of radioactivity precipitable from medium containing either horse serum or embryo extract increases with the time of incubation. The time-dependent increase in precipitable radioactivity suggests that more than 95 % of the radioactivity in the acid precipitates is attributable to binding during the incubation period rather than to non-specific entrapment during acid precipitation. In a second series of experiments, the dependence of precipitable radioactivity upon protein concentration was determined. Purified [^{14}C]glucosamine (cf. Dowex separation below) was incubated with increasing concentrations of dialyzed horse serum and the reaction terminated after 2 or 24 h (Fig. 2). Results from these experiments indicate that the amount of acid-precipitable radioactivity is directly related to serum concentration.

The proteinaceous nature of the binding components in culture medium is suggested by their acid precipitability. Direct evidence that protein can bind radioactive material from commercial preparations of [^{14}C]glucosamine was obtained by incubating the isotope with medium containing highly purified bovine serum albumin in the absence of other proteins. After a 24 h incubation, the specific radioactivity of acid-precipitable albumin was comparable to that of precipitable horse serum com-

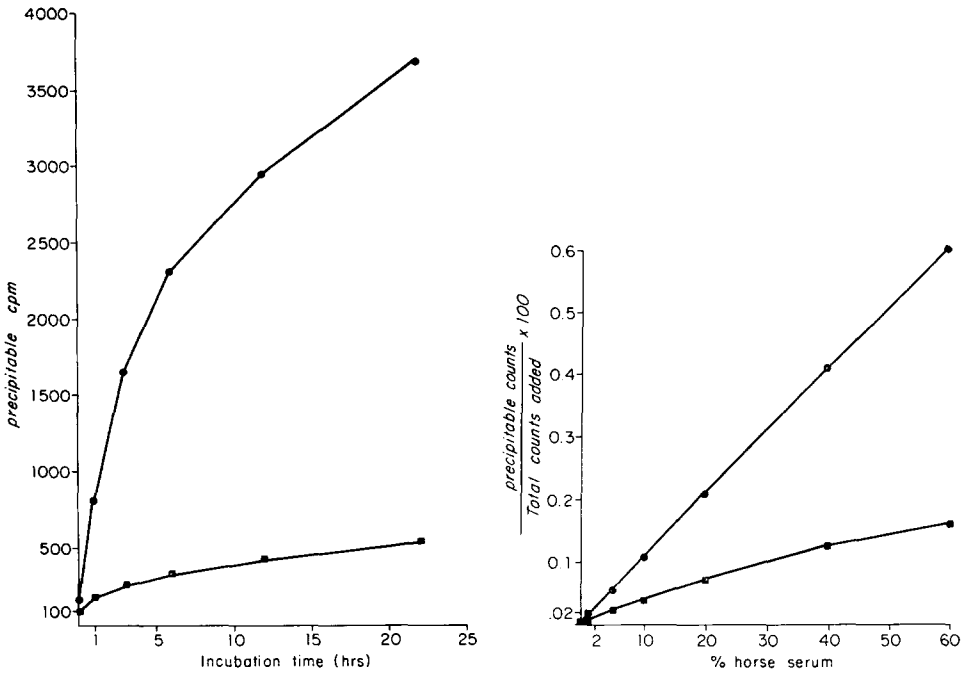

Fig. 1. Time-dependent increase of [^{14}C]glucosamine binding to serum protein. Media were incubated at 37 °C with 1 μCi/ml [^{14}C]glucosamine. At times indicated in the figure, 1.0-ml aliquots were removed, adjusted to 1 mM unlabeled glucosamine and then precipitated with trichloroacetic acid; the pellets were washed, solubilized and then counted. ●–●, medium containing 10 % horse serum and no embryo extract; ■–■, medium containing only 5 % embryo extract.

Fig. 2. [^{14}C]Glucosamine binding as a function of serum concentration. Purified [^{14}C]glucosamine was incubated at 37 °C with increasing concentrations of horse serum. [^{14}C]Glucosamine was purified by Dowex (H^+) chromatography (cf. Fig. 4, Fractions 78–87). The pooled fractions were neutralized with NaOH. Horse serum was exhaustively dialyzed against 0.1 M NaH_2PO_4 containing 0.15 M NaCl, pH 7.4. 1-ml reaction volumes contained: $1.8 \cdot 10^6$ cpm of [^{14}C]glucosamine in 400 μl; 0–600 μl of horse serum, and 600–0 μl of buffer, respectively. After 2 h and 24 h of incubation, 0.5-ml aliquots were adjusted to approx. 50 mg/ml protein with bovine serum albumin and precipitated with trichloroacetic acid. The pellets were washed, solubilized and counted. ■–■, 2 h incubation; ●–●, 24 h incubation.

ponents when these two reaction mixtures were incubated at equivalent protein concentrations (Table I). Further evidence for the proteinaceous nature of the binding components was obtained by pronase digestion. When anomalously labeled serum proteins were treated for 3 days with pronase and then dialyzed, less than 5 % of the initial non-dialyzable radioactivity was retained.

The stability of the association between protein and radioactive material was examined further in a series of dialysis experiments. When culture medium which had been incubated with [^{14}C]glucosamine for 24 h was dialyzed sequentially against unlabeled glucosamine (10^{-2} M), NaCl (1 M), guanidine · HCl (6 M) and finally against water the number of precipitable counts was decreased by only 10 %. In a second set of experiments, separate serum and albumin incubation mixtures were first

precipitated with trichloroacetic acid. The pellets were washed with acid, resolubilized with 5 % sodium dodecylsulfate, and then dialyzed against 1 % sodium dodecylsulfate. Only a small fraction (approx. 20 %) of the counts initially associated with the precipitates were dialyzable under either set of experimental conditions (Table II). The failure of dialysis against either guanidine · HCl or detergent to dilute the number of protein-

TABLE II

NON-DIALYZABILITY OF ACID-PRECIPITABLE RADIOACTIVITY

Acid-precipitable counts were normalized to cpm/ml incubation mixture per μCi[^{14}C]glucosamine added. Incubation mixture was sequentially dialyzed vs cold glucosamine (10^{-2} M); NaCl (1 M); guanidine · HCl (6 M); and water. Washed trichloroacetic acid pellets were resolubilized with 5 % sodium dodecylsulfate and dialyzed vs 1 % sodium dodecylsulfate. Media were incubated at 37 °C with [^{14}C]glucosamine: 1 μCi/ml (Expt 1), and 0.5 μCi/ml (Expt 2). After 23 h, (Column A), 1 ml samples were made 10^{-3} M in unlabeled glucosamine and precipitated with trichloroacetic acid. The pellets were washed with trichloroacetic acid, solubilized with 1 ml sodium dodecylsulfate (5 %) and counted, or (Column B, Expt 2) dialyzed vs four changes of 1 % detergent (300 ml each) and then counted. Alternatively (Column B, Expt 1), the incubation mixture was dialyzed as outlined above, reprecipitated, resolubilized and counted.

Expt number	Sample	Acid-precipitable counts (A)	Non-dialyzable acid-precipitable counts (%) (B)
1	Complete culture medium 10 % horse serum, 5 % embryo extract (9.1 mg protein/ml)	3900	90
2	10 % horse serum (8.4 mg protein/ml)	4550	86
	Bovine serum albumin (10 mg protein/ml)	3950	76

TABLE III

BINDING OF [^{14}C]GLUCOSAMINE TO SERUM PROTEINS UNDER "NON-PHYSIOLOGICAL" CONDITIONS

Control conditions, 0.1 M phosphate buffer (pH 7.4)–0.1 M NaCl. Horse or fetal calf serum (10 %, v/v) was incubated at 37 °C with 1 μCi/ml [^{14}C]glucosamine under various conditions described in the Table. 0.1 ml Fetal calf serum was incubated 5 min in a boiling water-bath, then 0.9 ml 2 % sodium dodecylsulfate containing 1 μCi [^{14}C]glucosamine was added.

Serum	Conditions	Non-dialyzable cpm	Percent of control
Horse	Control	3346	100
Horse	5 M NaCl	2465	73
Horse	2 % sodium dodecylsulfate	2832	83
Fetal calf	Control	3072	100
Fetal calf	2 % sodium dodecylsulfate	1939	63
Fetal calf	Boiled, solubilized and incubated in 2 % sodium dodecylsulfate	2156	70

bound counts suggests that the association between protein and radioactive material is stable not only to extensive dialysis but also to dialysis under conditions of protein denaturation.

The fact that radioactivity became firmly associated with purified bovine serum albumin indicates that the formation of the sugar–protein complex does not necessarily require the participation of glycosyltransferases or specific glucosamine receptor proteins which might be constituents of unfractionated sera. Additional evidence that the association between horse serum proteins and "glucosamine" is not enzyme dependent was obtained by incubating serum with commercial [^{14}C]glucosamine under conditions expected to inhibit enzyme activity. As indicated in Table III the presence of 5 M NaCl or 2 % sodium dodecylsulfate in the incubation mixture had only a minimal effect on the degree of association.

(2) *Competition by unlabeled glucosamine*

To establish whether the radioactivity binding to serum proteins was, in fact, associated with glucosamine, horse serum (10 %) was incubated with commerical [^{14}C]glucosamine (1 μCi/ml) in the presence of increasing amounts of unlabeled glucosamine. Fig. 3 demonstrates that a 60 % inhibition of acid-precipitable counts is

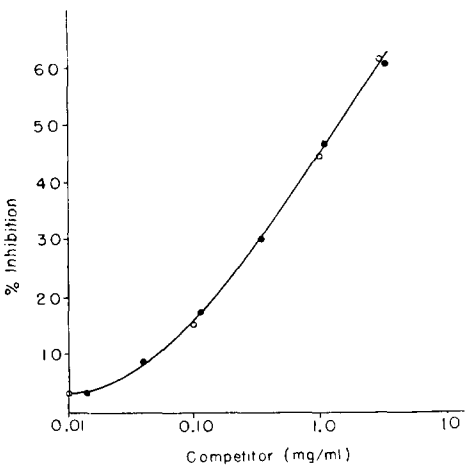

Fig. 3. Competition of [^{14}C]glucosamine binding to serum proteins by unlabeled glucosamine. 1 μCi/ml of [^{14}C]glucosamine was incubated with 10 % (v/v) horse serum in 0.1 M NaH_2PO_4 (pH 7.4) containing 0.1 M NaCl and 0–3 mg/ml of unlabeled glucosamine (stock solution in phosphate buffer previously adjusted to pH 7.4). After 28 h of incubation at 37 °C, 1-ml incubation mixtures were precipitated with trichloroacetic acid. The pellets were washed, solubilized and counted. The average values from two experiments, each performed in duplicate, are plotted (○–○) and (●–●).

achieved at a competitor concentration of 3 mg/ml. This represents a competitor to [^{14}C]glucosamine ratio of 750 : 1 ([^{14}C]glucosamine at 250 μCi/mg = 1 μCi/4 · 10^{-3} mg, therefore, 3 mg competitor = 750 : 1). Higher concentrations of competitor result in observable browning and yield data which is complicated by these side reactions. Nevertheless, the competition at 3 mg/ml competitor and below indicates that the major radioactive binding species is either glucosamine itself, a glucosamine-

related decomposition product which is formed during incubation, or an impurity which is present in both [^{14}C]glucosamine and unlabeled glucosamine.

(3) *Purity of commercial* [^{14}C]*glucosamine*

To ascertain the purity of the commercial glucosamine preparation, several batches of [^{14}C]glucosamine were analyzed by Dowex column chromatography [11]. 99 % of the radioactivity applied to the column was eluted with 0.3 M HCl (Fig. 4),

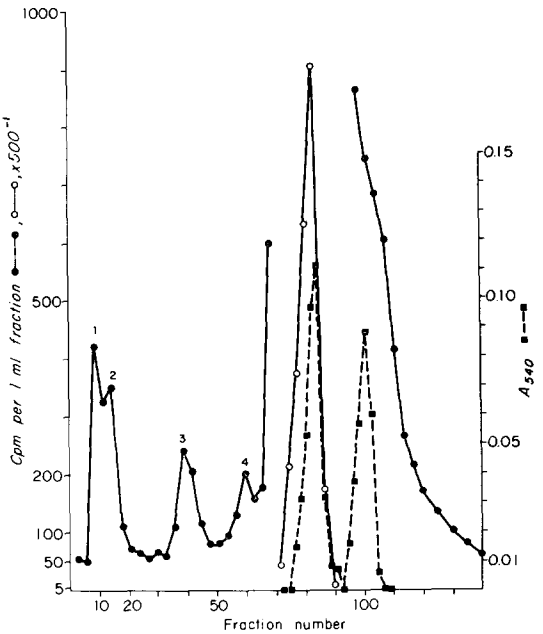

Fig. 4. Radioactive non-glucosamine species in commercial [^{14}C]glucosamine resolved by Dowex (H^+) chromatography. 2 μCi of [^{14}C]glucosamine (containing 1 mM glucosamine and 1 mM galactosamine markers) was chromatographed on Dowex 50 W-X12 (H^+) in 0.3 M HCl. Column size was 0.9 cm × 40 cm and flow rate was 4 ml/h. 1-ml fractions were counted directly (●–●); ordinate expanded by 500 (○–○); or tested for hexosamine by the Elson-Morgan procedure (■- - -■). Glucosamine and galactosamine standards eluted at Fractions 75–88 and 94–105, respectively.

and most of the remaining counts were resolved as one peak upon subsequent 1.0 M HCl elution. At least 98 % of the total radioactivity co-eluted with unlabeled glucosamine carrier, as detected by a modification of the Elson-Morgan reaction [12]. This value concurs with the manufacturer's specifications. However, in addition to glucosamine, four distinct peaks of unidentified radioactivity were observed in the 0.3 M HCl elution profile as well as the possibility of a galactosamine contaminant. Different batches of [^{14}C]glucosamine contained the same relative amounts of non-glucosamine Peaks 3 and 4, whereas the size of Peaks 1 and 2 varied greatly among [^{14}C]-glucosamine preparations. In the limited number of cases observed, Peak 1 seemed to be more prevalent in older batches of [^{14}C]glucosamine.

(4) *Binding activity of radioactive components resolved by Dowex chromatography*

To determine the binding capacity of the various radioactive species resolved

by Dowex chromatography, the radioactive profile obtained by chromatography of commercial [^{14}C]glucosamine was assayed for binding activity with serum proteins (Fig. 5). Radioactivity from the glucosamine region accounted for nearly 98 % of the total acid-precipitable counts; but in addition, Fig. 5 illustrates that radioactive non-glucosamine components may also be rendered acid precipitable when incubated with

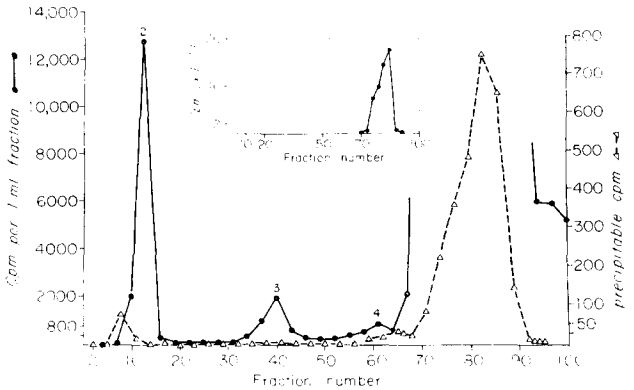

Fig. 5. Binding of radioactive glucosamine and radioactive non-glucosamine species to serum protein. [^{14}C]Glucosamine (10 μCi) was chromatographed on Dowex (H^+) as described in Fig. 4. 1-ml fractions were: (a) counted directly (●–●); (b) neutralized, incubated with horse serum (10 %) in 0.1 M NaH_2PO_4 buffer, pH 7.2, for 30 h and then precipitated with trichloroacetic acid (△–△). The precipitates were washed, solubilized and counted. Insert, profile of Dowex chromatogram showing the entire glucosamine region.

serum proteins. The most significant case of non-glucosamine binding involves Peak 1 material. Although Peak 1 is obscured in the Dowex chromatography profile due to the large quantity of radioactive material in Peak 2, the presence of a separate Peak 1 component is strongly suggested by the result of binding experiments. While the

TABLE IV

RELATIVE BINDING OF PURIFIED [^{14}C]GLUCOSAMINE AND RADIOACTIVE CONTAMINANTS TO HORSE SERUM

Dowex fractions of commercial [^{14}C]glucosamine were pooled from the peak areas (see for example, Fig. 4) and neutralized with NaOH. Sodium phosphate was added (to 0.1 M) and then titrated to pH 7.4. The radioactive solution was brought to a concentration of 10 % horse serum with serum which had been dialyzed against sodium phosphate buffer containing 0.15 M NaCl. After incubation at 37 °C for 25 h, 1-ml aliquots were acid precipitated. The pellets were washed, solubilized in detergent and counted.

Sample	^{14}C cpm/ml at 0 h	Acid-precipitable cpm/ml at 25 h (as % of counts at 0 h)
Peak No. 1	39 000	2.7
Peak No. 3	7 300	2.3
Peak No. 4	19 000	2.7
Glucosamine region	780 000	0.1

radioactive contaminants present in Peaks 1–4 account for only a small fraction of the total precipitable radioactivity obtained after 30 h of incubation with commercial preparations of [^{14}C]glucosamine, when expressed as a percent of the initially added counts, the radioactive material from Peaks 1, 3 and 4 exhibits 25 times more binding to acid-precipitable serum components than does material isolated from the glucosamine region of the Dowex chromatogram (Table IV). Therefore, the non-glucosamine species are apparently more reactive in the binding assay than is glucosamine.

(5) *Chromatographic similarity of trypsin-released ^{14}C cell surface material and trypsin-treated conditioned medium to trypsin-treated culture medium which has been incubated with [^{14}C]glucosamine in the absence of cells*

The ability of culture medium proteins to bind radioactivity upon incubation with labeled glucosamine is important to consider when one is using this radioactive precursor to study cell surface glycoproteins because any such anomalously labeled

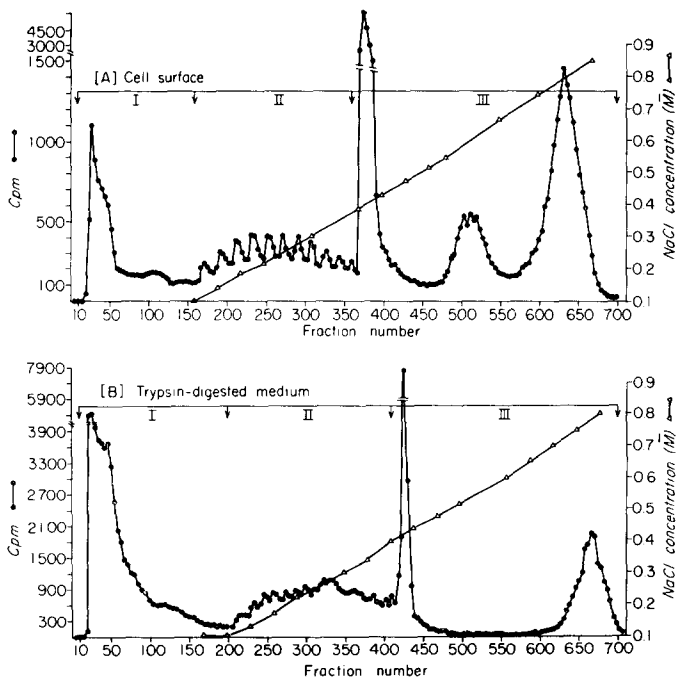

Fig. 6. DEAE–Sephadex A-25 chromatography of [^{14}C]glucosamine-labeled cell surface material (A) and trypsin-digested medium from the same confluent "fibroblast" cultures (B). To label the cells, cultures were incubated for 20 h at 37 °C with 3 ml of complete medium containing 8 μCi/ml of [^{14}C]glucosamine. After incubation, the medium was removed (cf. below) and the cultures were digested briefly with trypsin (cf. Materials and Methods). Cells and debris were removed by centrifugation and the supernatant was dialyzed. The sample was eluted with a 600 ml linear NaCl gradient from 0.09–1.0 M at a constant flow rate of 15 ml/h. 1-ml fractions were collected and counted directly (A). Resolvable non-mucopolysaccharide counts (Regions I and II) accounted for 0.1 % of the counts initially added to the medium. The homologous medium was also dialyzed and digested with trypsin; then redialyzed and chromatographed as described for the "cell surface" fraction (B). Regions I and II contained 0.4 % of the initially added counts.

protein which also binds to the cell surface may be mistaken for a cell-specific product. For example, when third-passage muscle cultures ("fibroblasts") are grown in medium containing [^{14}C]glucosamine, and "cell surface material" is subsequently released from these washed, intact cells by mild trypsin digestion, a spectrum of labeled macromolecules can be resolved by DEAE column chromatography (Fig. 6a). Region III of Fig. 6a contains three distinct peaks of mucopolysaccharide material ([3]; Angello, J.C., unpublished). Regions I and II of Fig. 6a describe other putative carbohydrate-containing material (glycopeptides?) from the surfaces of third-passage muscle cells. A similar profile is obtained when the conditioned medium from the [^{14}C]glucosamine-labeled cultures is trypsin digested, dialyzed and chromatographed (Fig. 6b).

In order to determine whether any anomalously labeled medium components were resolvable by DEAE column chromatography, culture medium and [^{14}C]glucosamine were incubated together in the absence of cells for 20 h and subsequently dialyzed. After trypsin digestion and further dialysis of the incubated medium, the non-dialyzable material was chromatographed on DEAE-Sephadex (Fig. 7). Material labeled under cell-free conditions elutes in the same general non-mucopolysaccharide areas as that obtained from the "cell surface" and from conditioned medium (Fig. 6).

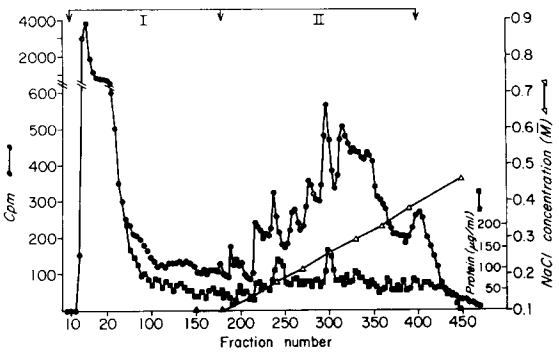

Fig. 7. DEAE Sephadex A-25 chromatography of trypsin-digested medium components which had been labeled in the absence of cells. 8 ml of complete medium was incubated with 5 μCi/ml of [^{14}C]-glucosamine for 22 h at 37 °C. The medium was then dialyzed, trypsin digested, and redialyzed (see Materials and Methods). Chromatography was carried out as described in Fig. 6. 0.16 % of the initially added counts were resolved. This value is equivalent to at least 25 % of the Region I and II radioactivity obtained from both the cell surface and conditioned medium of third-passage chick embryo fibroblasts (compare to Figs 6A and B). At NaCl concentrations higher than 0.5 M no further radioactivity was eluted. ●–●, radioactivity; ■–■, protein concentration (μg/ml); △–△, NaCl concentration (M).

All the results obtained with [^{14}C]glucosamine were reproducible with [^{3}H]-glucosamine; but in this regard it is worth noting that [^{3}H]glucosamine was considerably more labile than [^{14}C]glucosamine during refrigerated storage of the stock solution. Although Dowex-purified [^{3}H]glucosamine yielded only 0.15–0.30 % trichloroacetic acid precipitable counts after incubation for 20 h with 10 % horse serum, older unfractionated batches of [^{3}H]glucosamine (approx. 10 months storage at 4 °C) were found to yield up to five times more precipitable label. Ovbiously, reactive breakdown products had accumulated with prolonged storage.

(6) *Binding of radioactive glucosamine and radioactive fucose to other serum types*

The above studies have concentrated upon the binding of glucosamine to protein mixtures commonly used for growth of chick muscle cells in culture (horse serum and embryo extract). Other tissue culture systems require different sera for optimal cell growth and differentiation. To determine whether commercial [^{14}C]-glucosamine preparations contained radioactive material which bound to macromolecules in other kinds of serum, radioactive glucosamine was incubated with human, fetal calf and newborn calf serum (all 10 mg/ml) for 28 h. The reaction mixtures were dialyzed sequentially against unlabeled glucosamine (10^{-3} M), NaCl (1 M) and water. As in the experiments with horse serum, 0.1–0.2 % of the total added counts were non-dialyzable and acid precipitable (Table V). Thus [^{14}C]glucosamine-related radioactivity appears to bind to proteins in most commonly used sera.

TABLE V

NON-DIALYZABLE, ACID-PRECIPITABLE BINDING OF [^{14}C]GLUCOSAMINE TO VARIOUS SERUM TYPES

Non-dialyzable acid-precipitable cpm were normalized to cpm/ml incubation mixture per μCi[^{14}C]-glucosamine added. 10 mg/ml solutions of various sera in 0.1 M NaH_2PO_4 buffer containing 0.1 M NaCl, pH 7.2, were incubated with 0.5 μCi/ml of [^{14}C]glucosamine at 37 °C for 28 h. 6 ml aliquots were sequentially dialyzed against 3 l volumes of: 0.1 M NaH_2PO_4 containing 0.1 M NaCl and 10^{-3} M unlabeled glucosamine, pH 7.2; 1.0 M NaCl; and water. 1-ml aliquots of the dialyzed solutions were precipitated with trichloroacetic acid. The pellets were washed, solubilized and counted.

Sample	Non-dialyzable acid-precipitable cpm
Human serum (10 mg protein/ml)	1530
Fetal calf serum (10 mg protein/ml)	2370
Newborn calf serum (10 mg protein/ml)	1830

In addition, the binding to fetal calf serum was at least 60 % active in the presence of 2 % sodium dodecylsulfate, or when the serum had been heat denatured (5 min boiling water-bath) and resolubilized with 2 % sodium dodecylsulfate prior to incubation (Table III).

TABLE VI

ACID-PRECIPITABLE BINDING OF [^{3}H]FUCOSE TO MEDIUM PROTEINS

Acid-precipitable cpm were normalized to cpm/ml incubation mixture per μCi[^{3}H]fucose added. 10 mg/ml solutions of horse serum or purified bovine serum albumin in 0.1 M NaH_2PO_4 containing 0.1 M NaCl, pH 7.4, were incubated with 3.3 μCi/ml of [^{3}H]fucose at 37 °C. At 0 h and after 26 h of incubation, 1-ml aliquots were precipitated with trichloroacetic acid. The pellets were washed, solubilized and counted.

Sample	Acid-precipitable cpm	
	0 h	26 h
Horse serum (10 mg protein/ml)	20	390
Bovine serum albumin (10 mg protein/ml)	30	610

In an additional set of experiments we examined the possibility that fucose, another sugar which is commonly employed for cell surface studies, may bind to serum proteins. When horse serum or bovine serum albumin is incubated at 37 °C with commercial prepartions of [^{3}H]fucose, a 20-fold increase in the amount of acid-precipitable radioactivity occurs over a 26 h period (Table VI).

DISCUSSION

The following observations have been described in this report: (1) Commercial preparations of radioactive glucosamine contain material that binds to acid-precipitable culture medium macromolecules. Less than 25 % of the total bound radioactivity is removed by extensive dialysis; and 50 % of the counts remain non-dialyzable after trypsin digestion.

The radioactivity bound to components of medium containing 10% horse serum is equivalent to 0.1–0.2 % of the added label. This is significant when compared to the total non-mucopolysaccharide macromolecular label routinely recovered from cell surfaces (up to 0.35 % of the added label, depending on the cell type and culture density) or from medium in which cells have been labeled for 24 h (up to 0.8 % of the added label.) In a typical experiment in which 3 ml of culture medium was incubated with 2 μCi/ml of [^{14}C]glucosamine, the total precipitable radioactivity would amount to approximately 12 000 cpm.

(2) Dowex column chromatography of commercial [^{14}C]glucosamine reveals, in addition to glucosamine, low levels of non-glucosamine impurities. Binding experiments with Dowex column fractions show that radioactive material which chromatographs as glucosamine, as well as several of the observable impurities, can associate with acid-precipitable horse serum macromolecules.

(3) Excess unlabeled glucosamine competes with radioactive glucosamine for binding to horse serum macromolecules. The high level of competitor needed for 60 % competition is not a consequence of endogenous competitor (e.g. glucosamine) in the serum since both dialyzed and non-dialyzed serum components bind radioactive glucosamine equally well.

(4) The chromatographic profile of trypsinized culture medium which had been exposed to [^{14}C]glucosamine in the absence of cells was similar to the profile of trypsin-released cell surface material from cell cultures exposed to[^{14}C]glucosamine, as well as to the profile of medium which had been exposed to cells for 24 h and then trypsin digested.

The mechanism by which medium components are labeled upon incubation with radioactive glucosamine in the absence of cells is unresolved. Although glucosamine itself is the primary source of the label (Fig. 5), a decomposition product of radioactive glucosamine may be the species which binds to protein .Previous investigators have shown that glucosamine decomposes under conditions similar to those used in our studies and that one of the many resulting products is hydroxymethylfurfural [14]. As an example of reactive decomposition products, the aldehyde function of hydroxymethylfurfural derived from C_1 of the hexose would presumably be reactive toward amino groups and, thereby, could label medium proteins via a Schiff-base mechanism. These same authors reported that the decomposition of glucosamine is also accompanied by the formation of NH_3. We have detected the production of NH_3

from glucosamine (0.5 M) in the absence of protein under our conditions of incubation by means of an ammonia electrode (see Materials and Methods). It is thus possible that some of the anomalous labeling of medium proteins by glucosamine is due to such decomposition products.

Another possible mechanism by which medium proteins may be extracellularly labeled by glucosamine is by condensation of protein amino groups with the carbonyl function present in the open-chain aldehyde conformation of glucosamine. The availability of protein amino groups for Schiff-base formation with glucose under physiological conditions has recently been proposed for hemoglobin [15]. This type of reaction has been extensively studied in many systems [9, 16]. In particular, glucosamine has been shown to condense with certain amino acids to yield N-glycosidic compounds [17, 18]. Previous investigators have also reported that glucosamine binds to proteins such as horse serum globulin, horse serum albumin and bovine serum albumin in a manner which is resistant to dialysis [19]. The earlier studies were not, however, done with radioactive glucosamine and were not interpreted with respect to their possible relevance to in vitro studies of glycoprotein and mucopolysaccharide synthesis. Our observation that at least 60 % of the radioactivity which binds to serum proteins is competible by excess unlabeled glucosamine does not distinguish between these two possibilities.

We have presented evidence that glucosamine and fucose, or related breakdown products, associate with macromolecular serum components. In a series of experiments designed to detect serum proteins with recognition sites for specific sugars, Chipowsky et al. [20] found that medium containing 10 % calf serum did not contain proteins which bind galactose, glucose, or *N*-acetylglucosamine. The suggestion that serum does not contain proteins which recognize specific sugars is consistent with our interpretation that the anomalous labeling of serum proteins by glucosamine is a function of chemically reactive groups on essentially all proteins rather than interaction with specific receptor proteins.

The observation of glucosamine binding to culture medium protein in the absence of cells would appear to have considerable relevance to current studies of cell surfaces. In studies of this type [1–5] radioactive glucosamine is added to the medium in which the cells are growing. Following an incorporation period of up to 72 h, "cell surface" material is removed from washed, intact cells. Although much of this labeled material is undoubtedly of cellular origin, the contribution of medium proteins to these preparations is not usually considered. Hamburger et al. [7], provided clear evidence nearly a decade ago that exogenously added serum proteins bind tenaciously to the surface of cells grown in vitro. These authors further demonstrated that one of the classes of horse serum proteins which binds to the HeLa cell surface is γ-globulin. More recent experiments by Bischoff and Lowe [8] suggest that cultured chick muscle cells adsorb serum protein from the growth medium onto their surfaces. Furthermore, in our own laboratory, we have shown that rabbit antiserum prepared against cultured chick muscle cells contains antibodies which precipitate several horse serum components when examined on Ouchterlony diffusion plates (unpublished). From these experiments it would seem that cell surfaces share an intimate relationship with serum proteins. Thus it is possible that some proteins or glycopeptides, defined as cell surface constituents on the basis of their release following brief trypsinization, may actually be proteins of medium origin which have adsorbed

to the cell surface. Since results from the present study indicate that medium proteins may become labeled by incubation with radioactive glucosamine in the absence of cells, the mere association of radioactive glucosamine with cell surface proteins and glycopeptides should not be accepted as sufficient evidence that they result from cellular biosynthesis.

In a preliminary experiment to determine the extent to which anomalously labeled medium proteins bind to the cell surface, prelabeled proteins were incubated with unlabeled cells for 20 h and the trypsin-released, "cell-surface" radioactivity was then measured. Since only a small fraction of the added radioactive protein became bound, it is possible that the direct binding of anomalously labeled medium proteins may not be a major contributor to cell surface radioactivity. However, it remains possible that serum proteins which have already bound to the cell surface may become labeled when radioactive sugar precursors are added to the culture medium.

In addition to artifactual cell surface labeling due to anomalously labeled medium proteins, it is also possible that legitimate cell surface proteins could become anomalously labeled via the direct binding of radioactive sugar precursors. In this instance the degree to which individual cell surface proteins became anomalously labeled could depend upon quantitative and qualitative differences between surface proteins per se as well as upon the extent to which their reactive groups were exposed. Errors introduced at this level could azect the results of chromatographic analysis as well as measurements of the rate at which total surface glycoproteins were being synthesized.

The extent to which anomalously labeled medium proteins might confuse the interpretation of cell surface data is probably variable from system to system and depends on the reactivity of the serum proteins toward the particular radioactive sugar precursor, on the serum protein binding capacity of the cells in question, and on the overall metabolism of these cells (i.e. transport of the precursor, macromolecular synthesis and cell surface turnover). Perhaps the most practical way for investigators to contend with anomalous protein labeling is simply to be aware that it could be a problem and should be controlled for at the level of studying specific cell surface components.

An equally confusing situation may be anticipated in the analysis of radioactive macromolecular cell "secretory" products and "conditioned media". We have found, for example, that when confluent cell cultures are incubated with medium containing [^{14}C]glucosamine, the amount of radioactive material obtained from medium which resolves in the Region II area of a DEAE chromatogram is only 5–10 times greater (based on initially added counts) than when cells are not present during the incubation period. This alone suggests that an overall error of at least 10–20 % may be expected in experiments which measure gross "synthetic" capacities. The potential error increases with cells which are less active, or with subconfluent cultures.

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