Lawrence Berkeley National Laboratory
Recent Work

Title
AN ALTERED SURFACE HYALURONIC ACID COMPOSITION UPON TRANSFORMATION OF CHICHEN EMBRYO FIBROBLASTS: ITS INFLUENCE ON THE ACCESSIBILITY OF CELL COMPONENTS TO AN AMINE SPECIFIC PROBE

Permalink
https://escholarship.org/uc/item/7dn6b6sh

Author
Parry, G.

Publication Date
1979-02-01

AN ALTERED SURFACE HYALURONIC ACID COMPOSITION UPON TRANSFORMATION OF CHICKEN EMBRYO FIBROBLASTS: ITS INFLUENCE ON THE ACCESSIBILITY OF CELL COMPONENTS TO AN AMINE SPECIFIC PROBE

Gordon Parry, John Blenis and Susan P. Hawkes

February 1979

DISCLAIMER

AN ALTERED SURFACE HYALURONIC ACID COMPOSITION UPON TRANSFORMATION

OF CHICKEN EMBRYO FIBROBLASTS: Its influence on the accessibility of

cell components to an amine specific probe

Gordon Parry, John Blenis[†] and Susan P. Hawkes[*]

Laboratory of Chemical Biodynamics

Lawrence Berkeley Laboratory
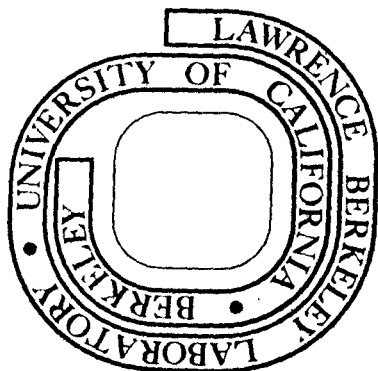
University of California

Berkeley, California 94720

Present Address:

  [†]Department of Biochemistry
  Michigan State University
  East Lansing, MI 48824

  [*]Midland Macromolecular Institute
  1910 West St. Andrews Drive
  Midland, MI 48640

ABSTRACT

Surface characteristics of chicken embryo fibroblasts transformed by Rous sarcoma virus have been examined with regard to the organisation of hyaluronic acid at the cell surface. Earlier work using an amine specific probe, fluorescamine, demonstrated that non-transformed cells exhibited 2-3 times more fluorescence from bound fluorescamine than transformed cells when monolayers were reacted with this probe. We have suggested that this is due to the presence of a barrier on the surface of transformed cells that reduces the accessibility of cell components to fluorescamine. We present evidence to substantiate this hypothesis and further identify the surface barrier as being at least partly composed of hyaluronic acid. This was demonstrated by the ability of a purified and specific hyaluronidase (ex _Streptomyces_ _hyalurolyticus_) to modulate the response of transformed cells to fluorescamine. Treatment of transformed cells with this enzyme causes them to react in a similar manner to non-transformed cells in subsequent reactions with fluorescamine, both in terms of the nature of components labeled and their fluorescence intensity.

Kinetic experiments using cells infected with a temperature sensitive mutant virus demonstrate that this alteration in surface composition is an early event in the transformation process.

The significance of alterations in the organisation or content of surface hyaluronic acid upon transformation is discussed in the light of known physical properties of this macromolecule.

INTRODUCTION

In higher organisms mucopolysaccharides have generally been considered to be extracellular matrix components[1]. There is, however, an increasing weight of evidence that they are also found on the surface of many animal cells. Kraemer[2] has reported that mucopolysaccharides can be found on the surfaces of cells grown in both monolayer and suspension cultures, while Culp has found that mucopolysaccharides constitute part of the attachment site anchoring a cell to its substrate[3]. Studies on the milk fat globule membranes, derived from the plasma membrane of epithelial cells, have also demonstrated the presence of mucopolysaccharides associated with the plasma membrane[4].

We have recently examined the surface of chicken embryo fibroblasts and have defined some alterations that occur following transformation with Rous sarcoma virus[5,6]. Our approach has been to use an amine specific probe, fluorescamine, that can react with various cell components to form fluorescent derivatives[7,8,9]. Reaction of fluorescamine with cell monolayers results in normal cells yielding 2-3-fold greater fluorescence than transformed cells[5]. We have presented evidence which suggests that this is due to the presence of a barrier on the surface of transformed cells that reduces the accessibility of cell components to fluorescamine[5,6]. In this paper we present evidence that this barrier is composed, at least partly, of the mucopolysaccharide, hyaluronic acid, located on the outer surface of the plasma membrane.

Our experimental approach has been to study the transformation of chicken embryo fibroblasts (CEF's) with a temperature sensitive mutant of RSV, Prague A strain, as reported previously[5]. We have characterised

detailed aspects of the interaction of fluorescamine with both normal and trans-formed cells and have obtained information on the nature of the surface barrier on transformed cells by examining treatments that can modulate the reaction of monolayers with fluorescamine.

EXPERIMENTAL PROCEDURE

Cell culture. Chicken embryo fibroblasts were prepared as previously described[12]. Unless otherwise mentioned, tertiary cultures were seeded at a density of 5 x 10^5 cells in each 60 mm culture plate in medium 199 (5 ml) supplemented with 0.5% chicken serum, 2% tryptose phosphate broth and 0.1% glucose. Cells were kept at 41° for 16 hours after trypsinisation and then some plates were shifted to 35°. A supplement (0.5 ml) of tryptose phosphate broth (70%), glucose (2%) and sodium bicarbonate (0.75%) was added to all the plates at the time of temperature shift. For most experiments cells were used 22-24 hours later.

Labeling of cells with fluorescamine and analysis of fluorescent products by SDS polyacrylamide gel electrophoresis. This was carried out as previously described[5]. Briefly, 2 ml of labeling solution (500 µg fluorescamine in 0.2 M sodium borate, pH 9.0) was added to monolayers, the plates shaken gently for 30 sec, and the label rapidly removed. Monolayers were solubilized in 67 mM sodium borate pH 9.0/2% sodium dodocyl sulfate for fluorescence determinations. While the exact value of the fluorescence obtained varied slightly from one experiment to the next, qualitatively similar results were obtained in all experiments. However, for this reason, the data reported is for single representative experiments. Fluorescent gels were photographed under high intensity UV lighting using Kodak Process Contrast Ortho film. In general exposures of 2-5 minutes were used. If the gel was exposed to such illumination for periods of 30 to 45 minutes, unlabeled proteins became slightly fluorescent, presumably as a result of photo-degradation reactions involving aromatic amino acids.

Sub-cellular fractionation procedures. Ribosomal RNA in CEF's was radioactively labeled with ^{3}H uridine (20 Ci/mmol, 50 µl in 5 ml medium) for 24 hours. CEF's were then fractionated using a

modification of the method of Wirth et al[11]. Briefly, after reaction with

fluorescamine monolayers were rinsed twice with low ionic strength buffer (SS) composed

of Hepes (10 mM) pH 7.4 at 25°, potassium chloride (10 mM) and magnesium

acetate (1.5 mM). Cells were removed from the plates using a rubber police-

man (approximately 5 plates in 2 ml) and homogenised in SS buffer using a

Dounce homogeniser (20 strokes) followed by a Potter homogeniser (50 strokes)

operating at approximately 1000 rpm. Cell disruption was monitored

using phase contrast microscopy and also by determining the proportion of

radioactively labeled ribosomes that could be released by treating the

homogenate with puromycin (0.5 mM) and potassium chloride (0.5 M) and

then centrifuging the suspension at 100,000 g for 30 minutes. Such quanti-

tation demonstrated cell disruption to be at least 93% efficient (Table 1).

For isolation of "soluble fraction" components the homogenate was centrifuged

at 100,000 g for 2 hours in a SW27 rotor and the supernatant then removed.

This fraction was taken to represent the intracellular soluble components.

For isolation of the rough endoplasmic reticulum (RER) the homogenate

was centrifuged at 800 g for 2 minutes and the supernatant carefully removed.

This was loaded onto a continuous gradient of 15%-40% sucrose (30 ml)

in Hepes (25 mM), potassium chloride (25 mM) and magnesium acetate (5.0 mM)

and underlayered with a 55% sucrose solution (5 ml) of the same ionic composi-

tion. This gradient was then centrifuged at 100,000 g for 4.5 hours using a

Beckman SW27 rotor.

Samples treated with puromycin and potassium chloride were processed

in the following way. The supernatant obtained by centrifuging the homogenate

at 800 g x 2 minutes was adjusted to 0.5 mM puromycin and 0.5 M potassium

chloride. This was incubated at 25° for 15 minutes and at 4° for 60 minutes be-

fore being loaded on a sucrose gradient with the same composition as described

previously, except that the potassium chloride concentration was adjusted to

0.5 M[2]. Fractions were collected from the gradient and analysed for trichloroacetic

acid precipitable radioactivity and also for fluoresccence from bound fluorescamine.

RER was isolated from 4the 40%/55% sucrose interface. Radioactive samples were counted in Permafluor 150 scintillation fluid.

Protein estimation. Protein was estimated by the method of Lowry et al.[13].

MATERIALS

Hyaluronidase preparations from Streptomyces were obtained from Calbiochem and Miles Biochemicals. Similar results were obtained with enzymes from either source. Different batches were also reproducible in their effects.

Fluorescamine was obtained from Hoffman La Roche. Materials for gel electrophoresis were obtained from Bio-Rad Laboratories. All other chemicals used were A.R. grade and obtained from either Mallinckrodt or Baker Companies.

RESULTS

To define the molecular basis for differential reactions of normal and transformed CEF's with fluorescamine, a detailed investigation of the subcellular distribution of fluorescamine derivatives was carried out. Early work had suggested that fluorescamine reacted primarily with cell surface components[7,8,9] Our recent studies using a modified labeling procedure and a more sensitive method of detecting fluorescence have demonstrated reaction with many cell components[5]. Since a knowledge of the subcellular specificity of fluorescamine reactions was fundamental to understanding the molecular basis for the difference in responses of normal and transformed cells, initial experiments addressed this particular point in detail.

Analysis of fluorescamine components in LA-24 infected cells using SDS polyacrylamide gel electrophoresis. Chicken embryo fibroblasts

infected with LA-24 were carried to the tertiary stage at a non-permissive
temperature (41°). Some plates were then incubated at 35° for 22 hours
and subsequently reacted with fluorescamine. The components labeled at 41°
and at 35° were then resolved by SDS polyacrylamide gel electrophoresis and
detected in the gel by observing their fluorescence under UV light. As
seen in Fig. 1, and also as previously reported[5], transformation results
is significantly lower fluorescence from most of the labeled components.
The fluorescence of a component migrating with the mobility of fibronectin
is reduced to a lesser extent than that of most of the other components.
We have previously reported that for cells cultured at 41°, the fluorescent
components migrate with the same mobilities as proteins stained with Coomassie
Blue. This observation suggests that fluorescamine reacts with many cellular
components other than surface components.

An analysis of the subcellular distribution of fluorescamine binding
components. To further investigate whether fluorescamine reacted with
both internal components as well as surface components, cells were labeled
with fluorescamine and the proportion of fluorescence in the soluble fraction
and the rough endoplasmic reticulum was determined. Subcellular fractionation
was carried out as described in the "Experimental Procedures" section.
When normal chicken embryo fibroblasts were reacted with fluorescamine, it
was found that approximately 25% of the total fluorescence was located
in the soluble fraction (Table II). This was true for cells that were
reacted with fluorescamine and subsequently fractionated. Cells cultured at 41°
had a slightly higher proportion of their total fluorescence bound to
soluble components than did cells cultured at 35°.

Rough endoplasmic reticulum (RER) was isolated from a homogenate
of normal chicken embryo fibroblasts by a modification of the procedure of
Wirth et al.[11]. The homogenate was first centrifuged at 800 g for 2

minutes and the supernatant then removed. The RER was separated from soluble components and other membrane components, including plasma membranes and smooth membranes in this supernatant, by density gradient centrifugation. The location of the RER on the gradient was determined using cells which had been incubated with ^{3}H uridine for 24 hours to radioactively label ribosomal RNA. After fractionating the gradient, the position of the RER was identified by determining the position of TCA precipitable ^{3}H counts along the gradient (Fig. 2). Both the RER and the free polysome positions were located by this method. The peak of radioactivity at the 55%/40% sucrose interface was taken to be that of the RER. When the distribution of fluorescamine along the gradient was examined, two peaks were detected. One corresponded exactly to the position of the RER and the other was located at the top of the gradient. The fluorescence at the density of the RER membranes was demonstrated to be located in the RER, and not in any fraction banding at a similar density by its sensitivity to puromycin and KCl. When the RER is treated with these reagents, ribosomes are displaced from the membrane and the resulting ribosome-free membrane has a lower equilibrium density than the RER. When 800 g supernatant from fluorescamine labeled cells was treated with puromycin and KCl and centrifuged on the sucrose gradient, the fluorescent peak at the position of the RER was not detectable, and fluorescence was observed only in the upper region of the gradient.

The proportion of the total fluorescence that could be detected in the RER was determined, taking into account both the RER in the microsomal fraction and also that which sedimented in the initial low speed centri-fugation. The quantity of RER in this pellet was determined by estimating TCA precipitable ^{3}H uridine in the fraction and assuming the same fluorescence per unit of radioactivity as measured in the pure microsomal RER. These calculations revealed that approximately 25% of the total fluorescence was

located in the RER (Table III).

The sensitivity of fluorescamine labeled components to chymotrypsin.
To assess which fluorescamine labeled components were located at the cell
surface, normal cells that had been reacted with fluorescamine were treated
with chymotrypsin (5 µg/ml) for 10 minutes. Enzyme activity was inhibited
after this time by adding to sylphenylalanyl chloromethyl ketone to the
incubation. Fluorescent components were then resolved using SDS polyacrylamide
gel electrophoresis. Chymotrypsin treatment led to the loss of a major
fluorescent band that migrated with the same mobility as purified fibronectin,
and also to decreased fluorescence of bands with slightly lower molecular
weights (Fig. 3). However, most fluorescent bands were not removed by
this treatment, which/consistent with an intracellular location of these
components. The enzyme treatment did not affect cell viability but was
sufficient to degrade many surface components labeled with ^{125}I using
lactoperoxidase (data not shown).

The effect of agitation on the reaction of fluorescamine with monolayers.
The reaction of fluorescamine with monolayers showed a surprising dependence
on agitation of plates during the labeling procedure. If plates were
not agitated, the fluorescence, expressed per mg protein, increased very
significantly. LA-24 infected cells cultured at 41° (non-transformed) and
35° (transformed) were approximately threefold and fivefold more fluorescent,
respectively, than the relative controls labeled with agitation (Table
IV). Furthermore, under non-agitating labeling conditions the difference
between 41° and 35° cells, with respect to fluorescence yield, was virtually
eliminated. The two effects of agitation, i) a decrease in absolute values
of fluorescence and ii) the differential reaction of normal and transformed
cells with fluorescamine, were only observed if the plates were agitated

during the labeling procedure. Agitation of the monolayers either before
or after the labeling procedure did not have a significant effect on
fluorescence values. The analysis of fluorescent components by SDS polyacryl-
amide gel electrophoresis demonstrated that the fluorescence of all bands
was considerably increased in cells labeled without agitation (Fig. 4).
Moreover, both the fluorescent bands and those observed after Coomassie
Blue staining were significantly broadened (data not shown). These data are
consistent with the reaction of considerable numbers of fluorescamine
molecules with single protein and glycoprotein molecules such that the
molecular weights of the resulting complexes were significantly different
from those of the proteins or glycoproteins themselves.

The similarity of cells grown at either 35° or 41° with respect to
their reaction with fluorescamine under non-agitating conditions is consistent
with the idea that fluorescamine does not monitor the loss of specific
surface components but instead monitors the formation of a barrier on
the surface of cells cultured at 35°. When monolayers are labeled without
agitation it would seem that this barrier can be overcome.

The effect of Hyaluronidase on the reaction of fluorescamine with
monolayers. The previous set of experiments supported our earlier data which
demonstrated a general decrease in the extent of reaction of transformed
cell components with fluorescamine. This supported the hypothesis
that a barrier on the surface of transformed cells reduced the accessibility
of cellular macromolecules to fluorescamine. The possible nature of such
a barrier was examined by considering factors that could influence the
reaction of fluorescamine with cell components in general. A macromolecule
that could behave in this way is hyaluronic acid. The following properties
of the molecule are consistent with such a possibility.

a) It has been reported that transformed chicken embryo fibroblasts
synthesize hyaluronic acid more rapidly than do normal fibroblasts(17).

b) When located in cartilage, hyaluronic acid is associated with other mucopolysaccharides and proteins to form a complex that has very high viscosity in water[14]. Moreover, hyaluronic acid itself forms viscous aqueous solutions and has been reported to alter the physical properties of other macromolecules in aqueous mixtures. Such a property may influence the diffusion of molecules close to cell monolayers[15].

To assess whether differences in surface hyaluronic acid might be responsible for the differential reaction of fluorescamine with normal and transformed cells, monolayers were treated with a hyaluronidase preparation from Streptomyces hyalurolyticus prior to their reaction with fluorescamine, and its effect observed by determining fluorescence per mg protein of treated and control labeled monolayers. This enzyme acts exclusively on hyaluronic acid[16] and the preparations used were reported to be free of protease activities. Hyaluronidase treatment of non-transformed (41°) cells resulted in a slight increase in fluorescence after reaction with fluorescamine, whereas, the fluorescence of cells cultured at 35° was increased very significantly under similar conditions (Table V). Cells cultured at both 35° and 41° behaved very similarly with respect to their reaction with fluorescamine if they were treated with the enzyme before the labeling procedure. The effect of varying hyaluronidase concentration on the elevation of fluorescence was determined (Fig. 5) and it was found that increasing enzyme concentration from 10 to 100 turbity reducing units (TRU)/ml had no effect on the final fluorescence value. This implied that at 10 TRU/ml the enzymic reaction had reached its end point.

SDS-polyacrylamide gel analysis of CEF's treated with hyaluronidase prior to reaction with fluorescamine. To examine whether the same components were fluorescently labeled if monolayers, grown either at 35° or 41°, were

treated with hyaluronidase before reaction with fluorescamine, solubilized cell components were resolved by SDS electrophoresis and then the positions of the bands identified by photographing the gel in UV light. Results of a representative experiment are shown in Fig. 6. It is clear that after hyaluronidase treatment, the fluorescent gel profiles of cells cultured at 35° and 41° are virtually identical. Hyaluronidase treatment of cells cultured at 41° caused only small changes in the fluorescence profile. These were in the high molecular weight region of the gel at the position of a band migrating with the same mobility as fibronectin and another band of slightly lower molecular weight. The fluorescent bands in this region were significantly broader than in the controls, making a comparison of their intensities very difficult. This could have been caused by loss of the components after enzyme treatment, either due to the hyaluronidase activity itself or to the action of a minor, unrecognized contaminating enzyme. Alternatively, the more extensive reaction with fluorescamine after enzyme action may have produced protein-fluorescamine products with a very wide range of molecular weights which then migrated as a diffuse band.

Since fibronectin is very sensitive to proteases, the first possibility was investigated by examining the effect of hyaluronidase on cell surface proteins labeled with ^{125}I (data not shown). It was found that the mobility of fibronectin was unchanged after hyaluronidase treatment and that the iodination profile of ^{125}I labeled components treated with hyaluronidase was very similar to the profile of untreated cells. Moreover, the commercial preparation of hyaluronidase is reported to be free of proteases. Further, hyaluronidase was found to be active in the presence of the serine protease inhibitor, phenylmethylsulphonyl fluoride, and also in the presence of 0.5% chicken serum (Table VI). Thus it seems likely that the second possibility accounts for the broadening of the bands.

Thus in terms of both fluorescence intensity and the distribution
of
of fluorescamine, labeled components/cells cultured at 35° and at 41°

behave similarly if first treated with hyaluronidase before reaction with

fluorescamine.

Competition of hyaluronidase activity by exogeneously added hyaluronic

acid. The direct involvement of hyaluronidase activity in mediating the

observed effects was confirmed by competing out the elevation of fluorescamine

binding by including hyaluronic acid in the enzyme incubation (Fig. 7).

LA-24 infected cells were cultured at 35° and treated with hyaluronidase in

the presence of hyaluronic acid (10 mg/ml). This led to a reduction

in the effect of hyaluronidase on the reaction of fluorescamine with the

monolayer, confirming that the effective activity in the enzyme preparation

was the hyaluronidase.

Hyaluronidase action monitored after shift of LA-24 infected cells

from the non-permissive to the permissive temperature for transformation.

Our earlier data demonstrated that the alteration monitored by fluores-

camine occurred early in the development of the transformed state. To

examine whether the reaction with fluorescamine could be modulated with

hyaluronidase during early times after shift to the permissive temperature,

LA-24 infected cells were cultured at 41°, shifted to 35°, and then reacted

with fluorescamine at various times after shift. Monolayers were incubated

with hyaluronidase or buffer prior to their reaction with fluorescamine.

As shown in Fig. 8, hyaluronidase modulated the fluorescamine reaction

at both early and late times after temperature shift, such that prior

enzyme treatment allowed fluorescamine to react similarly with cells cultured

at both 41° and 35° at all times during the development of the transformed

state.

<u>The effect of hyaluronic acid on the reaction of fluorescamine with</u>

<u>LA-24 infected cells cultured at 41°.</u> As hyaluronidase treatment of

cells grown at 35° caused them to behave as cells grown at 41° in terms

of their reaction with fluorescamine, it seemed possible that elevation

of the hyaluronic acid levels of cells at 41° could result in their reacting

as cells at 35°. Thus LA-24 infected cells cultured at 41° were reacted

with fluorescamine in the presence of several concentrations of hyaluronic

acid. As seen in Fig. 9, this caused a slight reduction in the fluorescence

of the cells, but the effect was insignificant compared with the reduction

brought about by cell transformation.

DISCUSSION

In this paper we have examined our earlier hypothesis that transformed

chicken embryo fibroblasts assemble a surface barrier which can be detected

by a decreased binding of the probe, fluorescamine, to transformed cell com-

ponents[5]. The studies reported here substantiate this model. The follow-

ing specific conclusions can be drawn.

i) When fluorescamine is reacted with normal cells or LA-24 infected

cells at the non-permissive temperature it forms fluorescent derivatives

with most cell components including both intracellular and surface

components. When LA-24 infected cells at the permissive temperature

are reacted with fluorescamine there is a reduction in the extent of reaction

with most components. However, reaction with some known surface components

is affected less than reaction with intracellular components. These obser-

vations are consistent with the presence of a barrier of the surface of

transformed cells that influences the accessibility of cellular macro-

molecules to fluorescamine. It is likely that some of the surface components

of transformed cells are either outside or very close to this barrier

such that their reaction with fluorescamine is altered only to a small extent.

ii) Treatment of LA-24 infected cells with a hyaluronidase preparation causes cells grown at the permissive and non-permissive temperatures to react with fluorescamine to a similar extent. The enzyme has little effect on the distribution and intensity of fluorescent derivatives in cells grown at 41° but both increases the intensity and alters the distribution of derivatives in cells at 35°. This data is consistent with transformation causing alterations in either the levels or the organization of surface hyaluronic acid that then forms a barrier to the reaction of the cells with fluorescamine.

iii) In experiments in which LA-24 infected cells were shifted from the nonpermissive to the permissive temperature, these alterations in surface hyaluronic acid occurred early in the development of the transformed state[11].

iv) Since it is highly unlikely that specific receptors exist for a compound such as fluorescamine, it probably enters the cell by dissolving in the lipid phase of the membrane and thus enters the cell from many locations. To reduce the uptake of fluorescamine, it would therefore seem likely that changes in surface hyaluronic acid occur generally over substantial regions of the cell surface.

An increase in the rate of synthesis of hyaluronic acid upon transformation of CEF's was reported early in investigations of the cellular actions of RSV[17,18]. Certainly, most of this mucopolysaccharide is secreted into the medium. The present study, however, draws attention to surface associated hyaluronic acid in CEF's and alterations in its levels or organization upon transformation. While the distribution and quantity of this mucopolysaccharide on the surface of normal and transformed cells

have not been measured, the extent of its influence on the reaction of fluorescamine with cell components indicates that significant differences in these parameters are likely to be found. Observations relating to surface hyaluronic acid were also made by Burger and Martin in their studies of agglutination of CEF's with Concavanalin A[19]. Transformed CEF's were agglutinated by Concanavalin A only if first treated with hyaluronidase.

In other systems both increased production and alterations in the distribution of hyaluronic acid after transformation have been reported (reviewed by Kraemer[20]). Glimelius and associates have carried out an extensive analysis of transformation related alterations in mucopolysaccharides in human glial and glioma cells lines[21,22]. Glioma cell lines are characterised by increased hyaluronic acid production compared with normal lines, and further demonstrate a very significant increase in surface associated hyaluronic acid. Transformation of hamster fibroblasts by Herpes simplex virus also leads to elevated synthesis of hyaluronic acid and this is reflected in an increase in surface associated hyaluronic acid also[23].

Hopwood and Dorfman[24] have reported that transformation of human fibroblasts with SV40 also leads to elevated synthesis of hyaluronic acid. This is in conflict with earlier data of Hamerman et al.[25] in which a decrease in hyaluronic acid production was observed in transformed human and mouse fibroblasts. However, as pointed out by Hopwood and Dorfman, the level of hyaluronic acid synthesis is very significantly influenced by cell density and serum factors, and the difference in results may well have been through failure to adequately control these factors in the earlier study.

There is very little information available on the structures and organization of surface mucopolysaccharides. Detailed characterization

of extra-cellular matrix mucopolysaccharides has been carried out[1,14],

but it is not known whether similar structures exist at the cell surface.

In this respect it is difficult to define the precise surface alterations

that are being observed in this study. Thus, it is possible that the

differences between normal and transformed cells, in terms of their reaction

with fluorescamine reflect either a change in the quantity of hyaluronic

acid or a change in its organisation. Organisational changes could inculde

an altered association with other surface components such as glycoproteins

or possibly sulphated glycosaminoglycans. It is also possible that differences

in the molecular weight of surface hyaluronic acid could influence the

reaction of cells with fluorescamine. It is interesting that simple addition

of hyaluronic acid to the fluorescamine reaction mixture did not influence

its reaction with non-transformed cells. This probably reflects the importance

of the surface association of hyaluronic acid for an effect on the fluores-

camine reaction.

While the significance of these observations remains to be investigated

it is interesting that the alterations occur early in the development

of the transformed state. It is possible that transformed cells surround

themselves with a coat of hyaluronic acid that alters the response of

the cell to the external environment. In this study the altered response

to fluorescamine is, of course, not of physiological significance. However,

it is quite possible that such a coat could influence the cell's response

to other compounds that are physiologically important. In this respect,

it is significant that an altered hyaluronic acid composition would

alter both surface viscosity and charge[15], and thus could affect

the local surface concentration of many compounds that may influence the

cell's behavior. By modulating surface hyaluronic acid levels as described

here, it should be possible to determine which transformation parameters are affected by such alterations in the surface composition. Recent work by Underhill and Dorfman[26] have demonstrated a role for surface hyaluronic acid in intercellular adhesions in mouse cells. It is conceivable that it could play a similar role in chicken fibroblasts.

Abbreviations: CEF's. chicken embryo fibroblasts; RER: rough endoplasmic reticulum; TCA: trichloroacetic acid; SS buffer: Hepes (10mM), potassium chloride (10 mM), magnesium acetate (2.5 mM) pH 7.6 at 22°. HA: hyaluronic acid; Hase: hyaluronidase.

ACKNOWLEDGEMENTS

REFERENCES

1. Muir, H. and Hardingham. T.E. (1975) in "Biochemistry of Carbohydrates" (Whelan, W.J., ed.) Biochemistry Series One 5, 153-222.

2. Kraemer, P.M. (1971) Biochemistry 10, 1445-1451.

3. Culp, L.A. (1976) Biochemistry 15, 4094-4104.

4. Lis, D. and Monis B. (1978) J. Supramol. Str. 8, 173-176.

5. Parry, G. and Hawkes, S.P. (1978) Proc. Natl. Acad. Sci. USA, 75, 3703-3707.

6. Parry, G. and Hawkes, S.P. (1978) J. Cell Biol. 79, 48a.

7. Hawkes, S.P., Meehan, T.D. and Bissell, M.J. (1976) Biochem. Biophys. Res. Commun. 68, 1226-1233.

8. Hawkes, S.P. (1976) "Membranes and Neoplasia: New Approaches and Strategies" (Marchesi, V.T., ed.) Prog. Clinical and Biolgoical Res. 9, 149-158.

9. Hawkes, S.P. and Bartholomew, J.C. (1977) Proc. Natl. Acad. Sci. USA, 74, 1626-1630.

10. Bissell, M.J., Farson, D. and Tung, A. (1977) J. Supramol Str. 6, 1-12.

11. Wirth, D.F., Katz, F., Small, B. and Lodish, H.F. (1977) Cell 10, 253-263.

12. Blobel, G. and Sabatini, D. (1971) Proc. Natl. Acad. Sci. USA 68, 390-394.

13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.

14. Hascall, V.C. (1977) J. Supramol. Str. 7, 101-120.

15. Laurent, T.C. (1970) in "Chemistry and Molecular Biology of the Intercellular Matrix" (E.A. Balazs, ed.) Ac. Press (London and New York) pp. 703-732.

16. Ohya, T. and Kaneko, Y. (1970) Biochim. Biophys. Acta 198, 607-609.

17. Temin, H.M. (1965) J. Natl. Cancer Inst. 35, 679-693.

18. Ishimoto, N., Temin, H.M. and Strominger, J.L. (1966) J. Biol. Chem. 241, 2052-2057.

19. Burger, M.M. and Martin, G.S. (1972) Nature 237, 9-12.

20. Kraemer, P. (1978) in "Surfaces of Normal and Malignant Cells" R. Hynes, ed.) Wiley Int. (in press).

21. Glimelius, B., Norling, B., Westermark, B. and Wasteson, A. (1978) Biochem. J. 172, 443-456.

22. Glimelius, B. Norling, B., Westemark, B. and Wasteson, A. (1978) J. Cell Physiol. (in press).

23. Satoh, C., Duff, R., Rapp, F. and Davidson, E.A. (1973) Proc. Natl. Acad. Sci. USA 70, 54-56.

24. Hopwood, J.J. and Dorfman, A. (1977) J. Biol. Chem. 252, 4777-4785

25. Hamerman, D., Todaro, G.T. and Green, H. (1965) Biochem. Biophys. Acta 101, 343-351.

26. Underhill, C. and Dorfman, A. (1978) Exp. Cell Res. 117, 155-164.

FIGURE LEGENDS

Fig. 1.   An analysis of fluorescamine labeled components resolved by SDS
polyacrylamide gel electrophoresis. Left track: LA 24 infected
cells cultured at 35°, right track: LA 24 infected cells cultured
at 41°.

Fig. 2.   An analysis of fluorescamine binding to cellular rough endo-
plasmic reticulum. Normal CEF's, labeled with ^{3}H uridine for
24 hrs, were reacted with fluorescamine, disrupted by homo-
genization and centrifuged at 800 g for 2 minutes. The supernatant
was removed and loaded onto a 15%-40% sucrose gradient and centri-
fuged at 100,000 g for 4.5 hrs. Fractions were collected from
the gradient and fluorescence and TCA precipitable radioactivity
determined in each of the fractions. a and c: Gradient buffer
contained Hepes (25 mM), KCl (25 mM) and Magnesium acetate (5.0 mM)
pH 7.6. Samples were loaded in SS buffer, (see text). b and
d: Gradient buffer was as for a,c but contained KCl (0.5 M).
Samples were adjusted to KCl (0.5 M), puromycin (0.5 mM), incubated
at 25° for 15 min, and at 4° for 60 min before being loaded
onto the gradient.

Fig. 3.   The senstiivity of fluorescamine labeled components to chymo-
trypsin. Normal CEF's were reacted with fluorescamine and some
plates then treated with chymotrypsin (5 µg/ml) for 10 minutes.
The digestion was stopped by adding tosyl lysylchloromethyl
ketone (50 µg/ml) and samples solubilized in 0.067 M sodium
borate, 2% SDS for analysis on SDS polyacrylamide gels. Right
track: untreated sample; left track: chymotrypsin treated sample.

Fig. 4. The effect of agitation on the reaction of fluorescamine with

monolayers. LA24 infected cells were reacted with fluorescamine

either with (A,C) or without agitation (B,D). Cells were then

solubilized, components separated by gel electrophoresis and

detected under u.v. light. Cells labeled under non-agitating

conditions (B,D) were significantly more fluorescent than those

labeled with agitation (A,C). Exposures were thus chosen for

tracks B,D. A.B: LA24 infected cells cultured at 35°. C,D:

LA24 infected cells cultured at 41°.

Fig. 5. The effect of increasing hyaluronidase concentration on the

reaction of fluorescamine with LA24 infected cells cultured

at 35°. Procedures were as described in the legend to Table V

except that the concentration of hyaluronidase was varied between

0 and 100 TRU/ml.

Fig. 6. SDS polyacrylamide gel analysis of CEF's treated with hyaluronidase

prior to reaction with fluorescamine. LA24 infected cells were

cultured at 41°. At 16 hrs after plating monolayers were reacted

with fluorescamine either with (B) or without (A) prior treatment

with hyaluronidase (10TRU/ml). Some plates were then shifted

to 35° for 22 hrs (C and D) and others maintained at 41° (D,F).

Cells were then reacted with fluorescamine, samples D and F

being treated with hyaluronidase prior to labeling.

Fig. 7. Competition of hyaluronidase activity with exogeneously added hyaluronic acid. LA24 infected cells were cultured at 35° and the kinetics of the hyaluronidase modulation examined. Cells were incubated in either medium 199 (control), medium 199 + 10 mg/ml hyaluronic acid (HA), medium 199 + hyaluronidase (Hase), or medium 199 + hyaluronidase + hyaluronic acid (10 mg/ml) (Hase + HA). At various times the media were removed and the cells reacted with fluorescamine. Exogenous hyaluronic acid reduced the extent of hyaluronidase modulation of fluorescamine binding.

Fig. 8. The fluorescence of LA24 infected monolayers reacted with fluorescamine at various times after shift from 41° to 35°. Some plates were treated with hyaluronidase before being reacted with fluorescamine (right block). o——o, mololayers kept at 41° throughout the experiment o-----o, monolayers shifted to 35°.

Fig. 9. The effect of hyaluronic acid on the reaction of fluorescamine with LA24 infected cells at 41°. Monolayers were labeled with fluorescamine as usual except that hyaluronic acid was included in the reaction mixture during labeling. Monolayers were then rinsed and solubilized for fluorescence determinations as usual.

Table I.   Estimation of the Efficiency of Cell Disruption

Total ^{3}H CPM*	^{3}H Released by Puromycin + KCl	% ^{3}H CPM Released
979,503	907,445	93%

*Values refer to TCA precipitable CPM.

Cells were labeled with ^{3}H uridine and homogenized as described in the "Experimental Procedures" section. The homogenate was adjusted to 0.5 M KCl and 0.5 mM puromycin, incubated at 25° for 15 minutes and at 4° for 60 minutes. The suspension was then centrifuged at 100,000 g for 30 minutes and the proportion of TCA precipitable activity remaining in the supernatant was then estimated. This was taken to represent the proportion of radioactively labeled ribosomes that were released from the cells during homogenization and hence to reflect the extent of cell disruption. Values quoted are means of duplicate estimations.

Table II. Quantitation of Fluorescamine Binding to

"Soluble Fraction" Components

	% of Total Fluorescence in Soluble Fraction	
	Experiment 1	Experiment 2
Normal Cells A) Low Density (subconfluent)	24.6	19.1
B) High Density (Highly confluent)	20.1	--
LA24 Infected Cells A) Cultured at 35°	18.8	26.6
B) Cultured at 41°	21.2	31.9

The soluble fraction of normal and LA24 infected cells was isolated as

described and the proportion of the total fluorescence in this fraction

determined. All samples were adjusted to 0.67 M sodium borate, 0.02%

SDS for determination of fluorescence. Values quoted are means of

duplicate estimations.

Table III. Quantitation of Fluorescamine Binding to Rough Endoplasmic
Reticulum Membranes of Normal CEF'S

	Fluorescence (Arb Units)
Total Homogenate	2856
Rough Endoplasmic Reticulum	
A) Microsomal Membranes 229 ⎫	723
B) Rapidly Sedimenting Membranes 494 ⎭	
% Total Fluorescence in RER	25.3%

RER was isolated as described and the proportion of fluorescamine bound
to this membrane estimated. Microsomal membranes refer to membranes
isolated from the 800 g supernatant while rapidly sedimenting membranes re-
fer to membranes sedimenting in the 800 g pellet. The proportion of RER
in this membrane pellet was estimated by resuspending the pellet in 0.5 M
KCl, 0.5 mM puromycin under the conditions described in Table I. The
proportion of radioactively labeled RNA that could be separated from
the pellet after recentrifuging at 100,000 g for 30 minutes was used to
estimate the quantity of RER membranes. By assuming uniform distribution
of fluorescamine throughout the RER, the ratio of radioactivity to
fluorescence for the pure microsomal membranes was used to estimate the
proportion of the fluorescence in the low speed pellet that could be
ascribed to fluorescamine bound to the RER. Fluorescence values quoted
are the means of duplicate estimations.

Table IV. The Effect of Gentle Agitation on Fluorescamine binding to
LA24 Infected Cells

Treatment	Relative Fluorescence	
	41°	35°
1. Agitation	105	51
2. No Agitation	284	257
3. No Agitation Followed by Agitation	206	189
Pre-Agitated Monolayers		
1. Agitation	92	56
2. No Agitation	357	238
3. No Agitation Followed by Agitation	259	183

LA24 infected cells were cultured at either 41° or 35° and reacted
with fluorescamine under the indicated conditions. Agitation involved
rotating the plates by hand during the 30 sec reaction period. In
situation 3 monolayers were reacted with fluorescamine for 30 secs,
the reaction mixture removed and the monolayers then agitated in 2 ml
sodium borate (0.2 M pH 9.0) for 30 secs. When preagitation was
carried out monolayers were agitated in this buffer before being reacted
with fluorescamine. The fluorescamine reaction was then carried out under
the indicated conditions. Fluorescence values are expressed per mg
protein, and are the means of triplicate estimations.

Table V.   The Effect of Hyaluronidase (<u>Streptomyces</u> <u>Hyalurolyticus</u>)

on Fluorescamine Reactions with LA24 Infected CEF'S

	Relative Fluorescence (Arb. Units)	
	41°	35°
Control Incubation	199	69
Hyaluronidase (100 TRU/ml)	248	317

LA24 infected cells were cultured at 41° and 35° and reacted with

fluorescamine as described in the "Experimental Procedures" section.

Prior to reaction, monolayers were washed 2 times with medium 199,

and then incubated for 15 min at 39°, either with hyaluronidase

(100 TRU/ml) in medium 199, or with medium 199 alone.   Monolayers

were washed 2 times with Hanks buffer before being reacted with

fluorescamine under the indicated conditions.   Fluorescence values

are expressed per mg protein, and are the means of duplicate.

estimation.

Table VI. The Effects of Protease Inhibitors on the Activity

of Hyaluronidase

	Fluorescence/mg Protein (Arb. units)
Untreated	53
Hyaluronidase (10 TRU/ml for 15 min)	164
Hyaluronidase (10 TRU/ml for 15 min + PMFS (2 mM)	184
Hyalyronidase (10 TRU/ml for 15 min + o.5% Chicken serum	147

LA24 infected cells were cultured at 41° and 35° , treated with hyaluronidase

in the presence of protease inhibitors and then labeled with

fluorescamine. Inhibitors were 0.5% chicken serum and phenylmethyl-

sulphonylfluoride (2 mM). Monolayers were treated as described in

the legend to Table V except that 10 TRU/ml hyaluronidase were used.

Values are the means of duplicate estimations.

XBB 7810-12916

Fig. 1
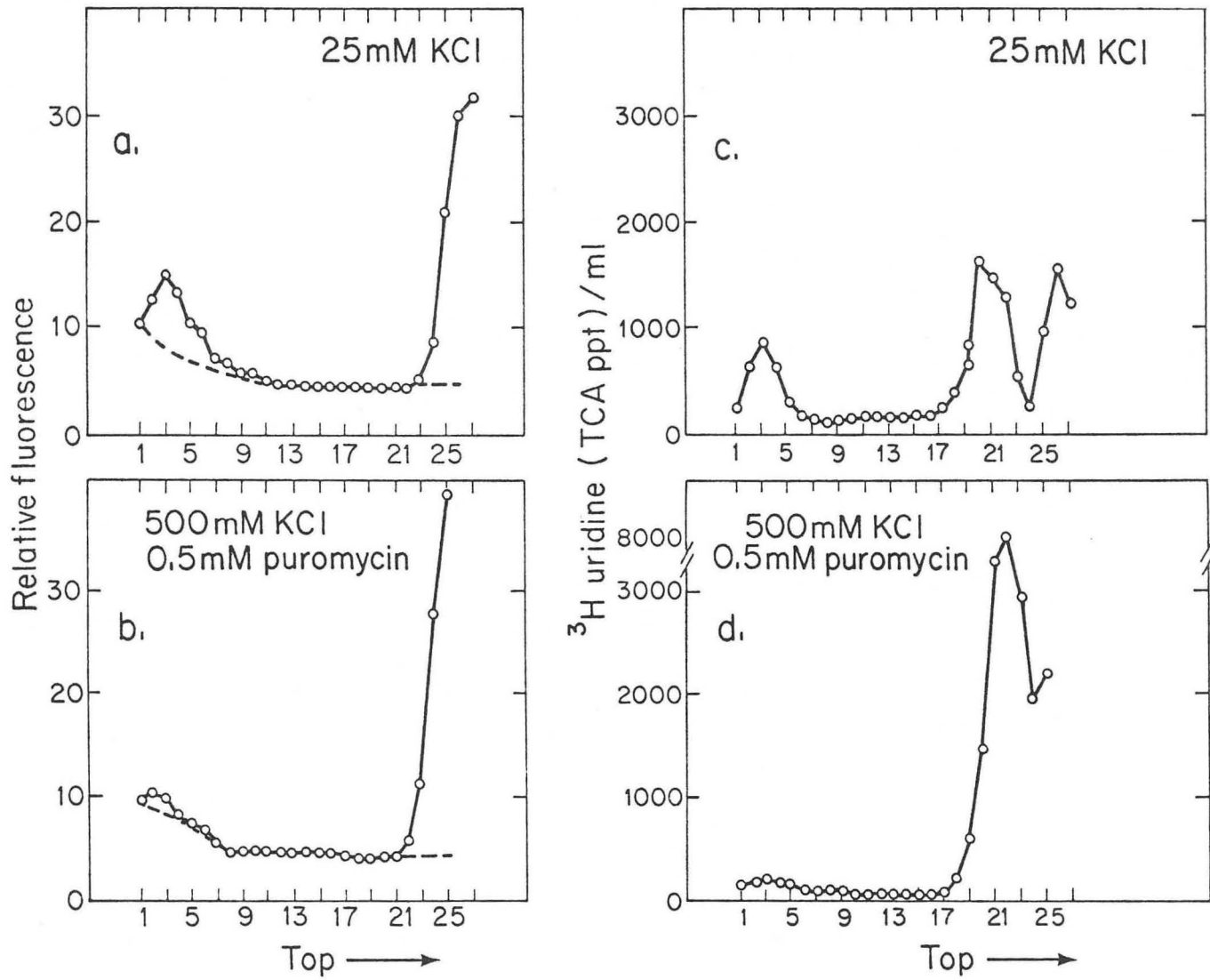
Fig. 2

XBL7810-4279

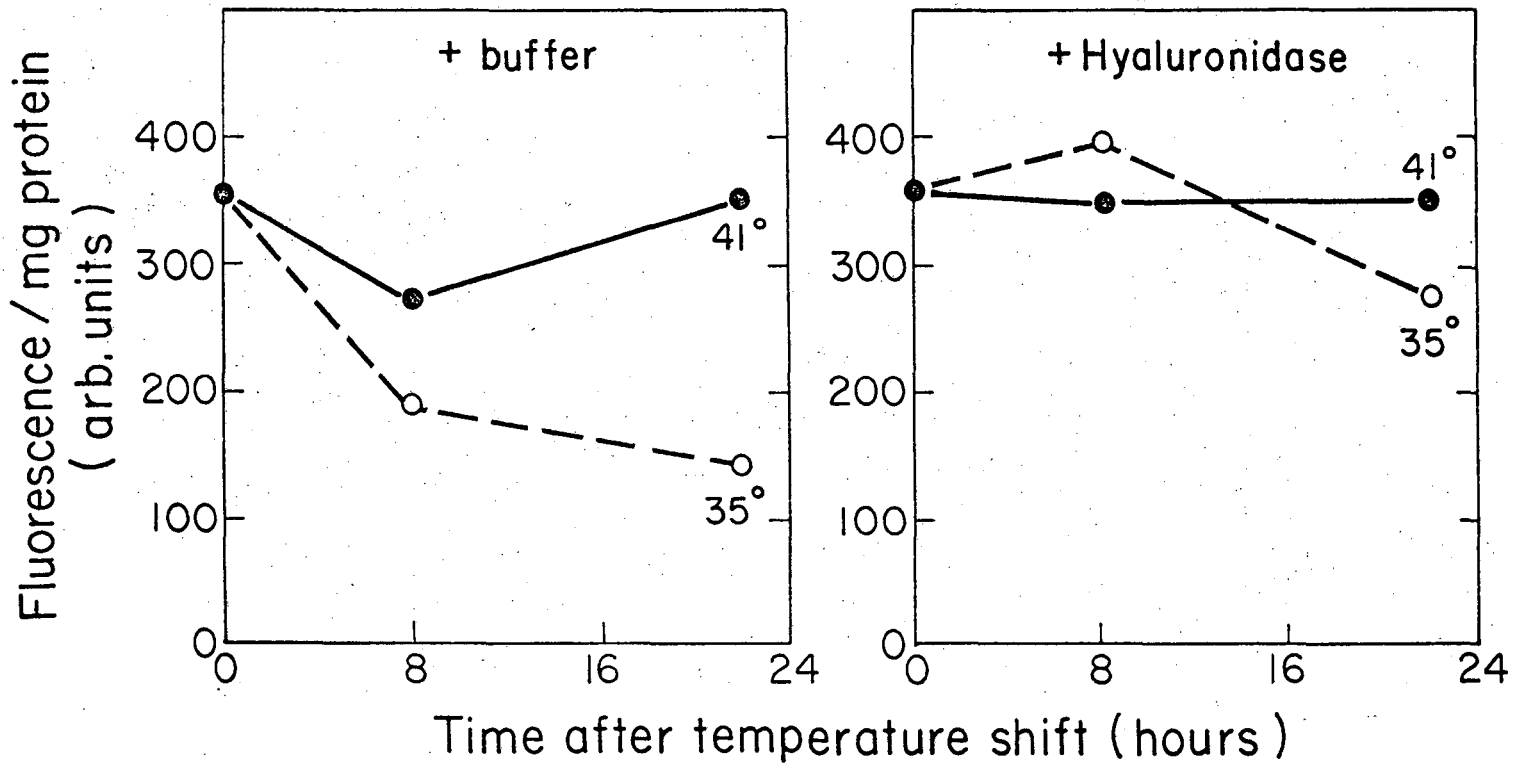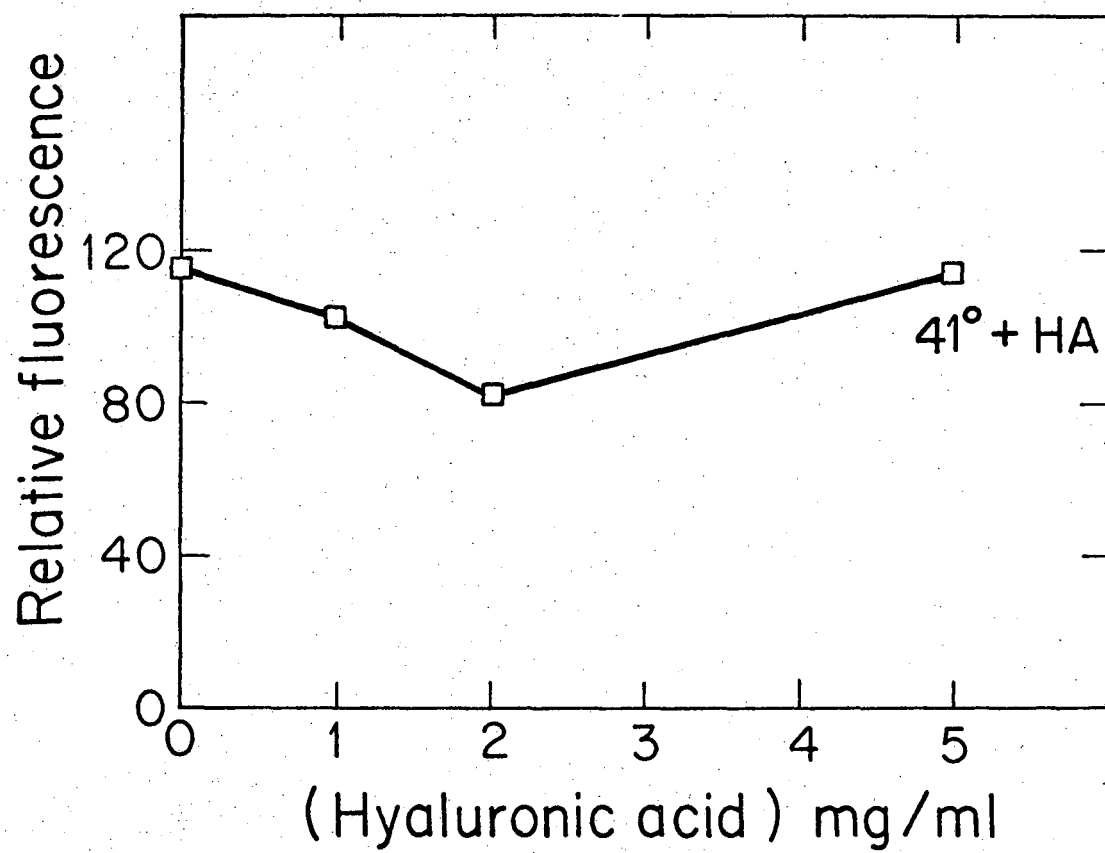32
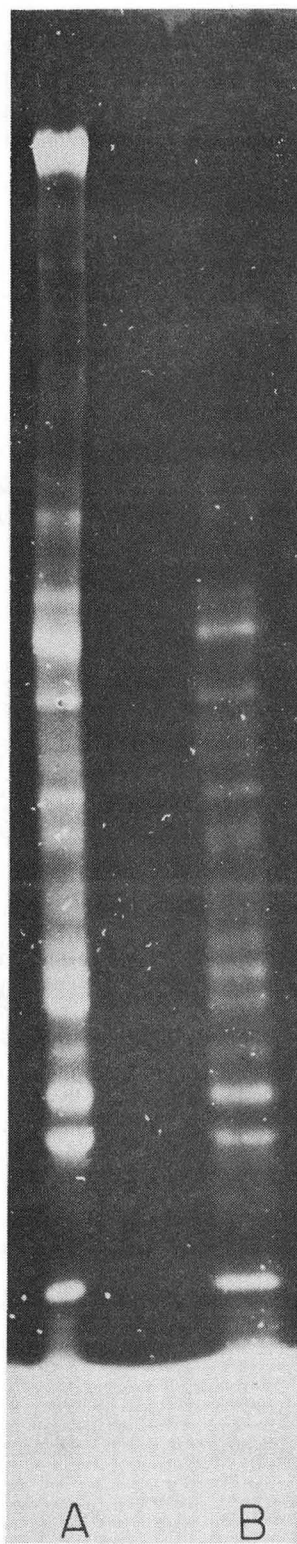
XBB 780-13345

Fig. 3
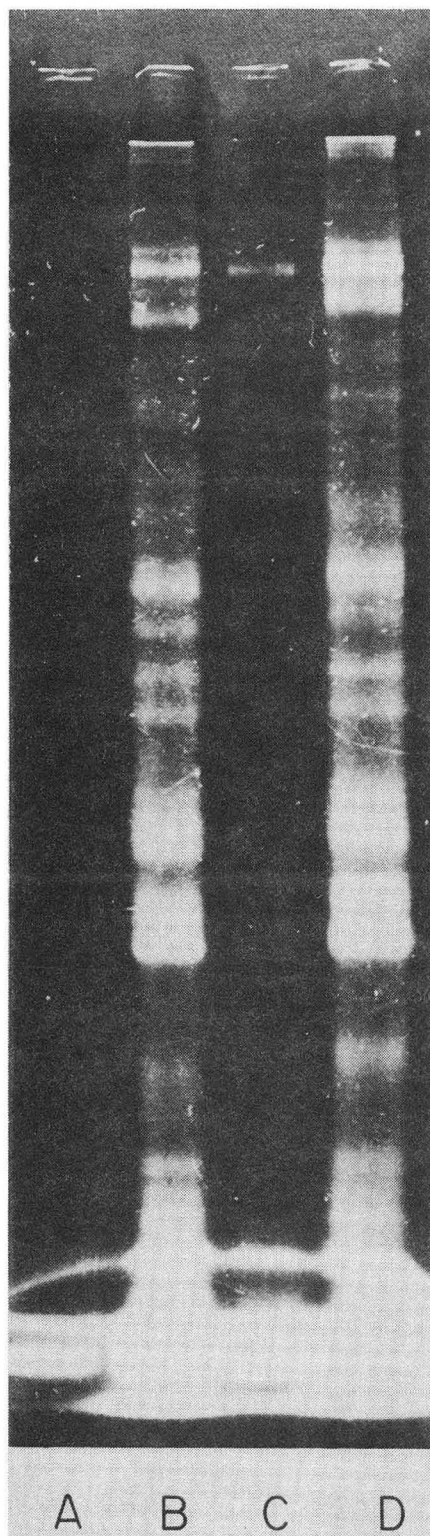
XBB 780-13346

Fig. 4

XBL 7810-4282

Fig.5

A	B	C	D	E	F
41/0	41/H	35/22	35/22H	41/22	41/22H

XBB 780-13347

Fig. 6

XBL7810-4280

Fig. 7

XBL7810-4281

Fig. 8

Fig. 9

XBL 7810-4283