MICROEXUDATES FROM CELLS GROWN IN TISSUE CULTURE

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ABSTRACT Cellular substrata of known molecular structure and measurable dimensions can be constructed as transferred films from Langmuir troughs or as adsorbed films. In addition, large molecules in culture media form measurable adsorbates. With the techniques of ellipsometry and surface chemistry it is possible to characterize and measure (within ± 3A) as a function of several parameters a microexudate of molecular dimensions deposited when tissue cultured cells contact certain substrata. The selective attraction of substratum and cell for microexudate has been determined, and the time course of deposition in Eagle's medium is characterized by a rapid initial accretion of material. During this period, microexudate can diffuse several cell diameters and cannot be detected in the culture medium. In Eagle's medium the cells cannot be detached from glass surfaces by versene or trypsin unless the surface of cell or substratum is coated with certain molecules. Trypsin becomes adsorbed to cell surfaces, continues to be enzymatically active on the surface, and digests protein components of microexudate and substratum. Microexudate appears to be a complex mosaic of molecules (including protein) synthesized within or on the surfaces of cells and secreted by cells or transferred from their surfaces to specific substrata. It is proposed that this mosaic plays, on the molecular level, a significant role in cell-to-cell interactions, cell locomotion and adhesion, and the selective application and spreading of cells on various surfaces.

INTRODUCTION

The experimental study of the development and activity of cells *in vitro* requires consideration of several parameters such as cell strain, internal and surface condition of the cell, the culture medium, cellular contacts, and the substratum upon which the cell rests. In addition, the experimenter must be cognizant of the complex interplay between these parameters, as well as the modifications introduced when the behavior of cells is subjected to laboratory investigations.

An early example of the interdependency between the above parameters was presented by Weiss (1945). He demonstrated that an explant of cells in liquid culture produced a microscopic colloidal exudate or "ground mat" whose fine fibrillar network determined the orientation and arrangement of the cells growing outward

from the explant. Thus, the cells in conjunction with their growth medium modified their environment in such a way that their pattern of locomotion was affected. Another example of this interdependency among the parameters which bear upon cellular behavior are the alterations by cellular products of the growth medium and substratum, which in turn modify the surfaces and activities of the cells (changes in pH, accumulation of toxic products, etc.). Thus, a cell and its environs are generally in a state of dynamic equilibrium, one modifying the other in complex ways.

The investigations described in this report were undertaken to determine whether, in view of the presence of the aforementioned exudate of microscopic dimensions, the cell can alter its substratum through the deposition of a microexudate of molecular dimensions, nondetectable by light microscopy (see Rosenberg (1960)). By adapting to biological systems several of the techniques commonly used in the construction and measurement of monomolecular films, it was possible to demonstrate that cells cultured *in vitro* can produce upon contact with a substratum a microexudate that alters the molecular carpet upon which the cell rests. The characteristics of this microexudate are the subject of this paper.

The first section describes the techniques used to observe this microexudate as well as the methods used to prepare the cells and control the substrata for the cells. Later sections on the results are divided into two parts: part I deals primarily with observations and measurements of physical characteristics of the microexudate; part II deals with the chemical composition and source of this material. In a final section the role of this molecular material is discussed, and it is proposed that the microexudate is composed, in part, of molecules previously adsorbed to the surface of cells.

To clarify the presentation, the following definitions of terms are presented.

Colloidal exudate: An exudate of microscopic dimensions (greater than 0.1 micron) produced by cells cultured *in vitro*.

Microexudate: A substance of submicroscopic dimensions (roughly 20 to 40A) given off by cells cultured *in vitro*.

Cell population surface density (also designated by the symbol CPSD): The average of several counts of the number of cells covering a surface area of 1 mm^2.

Relative cellular cross section (also designated by the symbol RCS): The product of the average cross-sectional area of the spherical, unspread cell (in square millimeters) and the cell population surface density (CPSD).

Mean microexudate thickness: The thickness (in angstroms) of the microexudate averaged over an area of 1 mm^2.

MATERIAL AND METHODS

The basal medium (with no serum components) described by Eagle (1955), and henceforth referred to as Eagle's medium, was used in these investigations. The cells for the experiments were obtained from stock cultures of several strains reared in

Eagle's medium supplemented with 10 per cent horse serum or from freshly dissociated tissues of chick embryos. The stock cultures, prior to use, were washed with calcium- and magnesium-free Earle's solution. They were detached either enzymatically with 0.05 per cent trypsin or with 0.05 per cent sodium versenate (disodium ethylenediaminetetracetate) at 37°C (pH approximately 7.8) for 3 to 5 min. The loosened cells were centrifuged for 5 min at 600 R.P.M., the supernatant was discarded, and the pellet of cells was washed carefully with Eagle's medium. For immediate use the pellet was resuspended by gentle pipetting in the test medium, or suspended in the standard culture medium and maintained for several hours in a roller tube for later use. Cells resuspended in Eagle's medium must be used without delay or kept in a roller tube since they rapidly adhere to glass.

The fresh embryonic tissue was dissociated enzymatically with 0.5 per cent trypsin-pancreatin for 10 to 15 min at 37°C, centrifuged, and used as above.

The test slides are made of glass with a vacuum-deposited chrome coating that is nontoxic to the growth and spreading of cells. Each slide is cleaned with cerium oxide, dried with a jet of filtered air, and sterilized under ultraviolet light for 10 min. No adsorption of ozone has been detected. Base readings on all slides are obtained with an ellipsometer.

The ellipsometer as developed by Rothen[1] (1945) can be used to measure the overall thickness of molecular films with a precision of a fraction of 1 A. The instrument is designed on the principle that when light, linearly polarized in a plane making an angle other than 0 or 90° with the plane of incidence, impinges upon a metallic surface, the reflected light is elliptically polarized. The shape and orientation of the ellipse depend upon the angle of incidence and the optical constants of the metal. The parameters of the ellipse will be altered if the metallic surface is covered with a thin transparent film, and these alterations can be measured and calibrated in terms of the thickness of the transparent film. The error due to changes in the index of refraction of the transparent film will in general be very small. The field of view of the optical system is 1 mm^2, and measurements of film thickness, therefore, represent an average or mean over this area. Hence, the measured thickness of a surface film will be determined by the configuration, orientation, dimensions, and surface concentration of the molecules of which the film is composed.

0.5 ml of the cellular suspension to be studied is placed on the slide. The material is kept in a saturated atmosphere of 5 per cent CO_2, 95 per cent air to maintain a pH of 7.4 at the test temperature. During each experiment a duplicate set of control slides is covered with the same amount of cell-free test medium. As additional controls, samples of the supernatant medium that bathes the cells resting upon the slide, are transferred to clean chrome-plated test slides to determine the presence of adsorbable molecules.

To measure the CPSD, the test slide is examined microscopically, and cell counts are made over several regions of the slide and then averaged.

At designated times the cells are removed mechanically from the slides by means of a jet of isotonic saline and/or doubly distilled water. This procedure is fully effective in removing the attached cells. Many cells thus removed from the surface can be recultured.

Upon removal of the cells, the slides are air-dried and their surface is studied in the ellipsometer to determine the presence and mean thickness of any thin films. Slides upon which films of microexudate have been deposited then can be used for additional experiments.

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[1] The author is highly indebted to Dr. A. Rothen of The Rockefeller Institute for the loan of the equipment required for the study of molecular films.

Precautions. Several precautions are essential to ensure reproducible measurements. (1) All slides must be carefully cleaned with a mild abrasive such as cerium oxide. (2) The slides must be used shortly after cleaning. (3) Experiments should be conducted in an air-conditioned, sterile atmosphere to reduce contaminants. (4) Cells suspended in protein-free media must be mildly agitated since they adhere very rapidly to the surfaces of containers. (5) The cells must be prepared with a minimum of centrifugation and pipetting to reduce the possibility of mechanical damage to the cells, resulting in the leakage of materials that could then adsorb to the surface. If these precautions are adopted, measurements can be repeated with an accuracy of $\pm$ 3 A.

RESULTS—PART I

A. *Observations.* By means of the aforementioned procedures it has been possible to detect on the test slides a microexudate given off by cells. The surface of the slide, as viewed through the ellipsometer, appears to be covered with a relatively uniform film dotted with small "high spots" representing the locations from which the cells were detached. Thus, the microexudate can be divided into two surface regions; a subcellular one and an intercellular one of varying molecular concentrations. If the density of cells on the surface is small such that the mean intercellular distance is greater than four or five cell diameters, it is possible to measure the *average* thickness of the intercellular microexudate; with greater densities of cells, the measured thickness is an *average* of that of the subcellular and intercellular microexudates. This instrumental averaging must be taken into account in evaluating the measurements discussed in the next section.

Besides the above instrumental limitation, one must also be mindful that the culture medium may modify the surface that is examined for the presence of molecular material. The common growth media for the *in vitro* culture of cells usually contain components of serum or other large molecules which readily adsorb to surfaces. Thus, the medium itself may rapidly alter the substratum for the cells, and it is likely that in many situations the cell surface might be similarly modified as a result of adsorption. These alterations of the substratum are measurable with the ellipsometer, but make the detection of microexudates from cells far more difficult and ambiguous. To prevent this difficulty and to obtain reproducible and meaningful measurements, it was necessary to use for relatively short periods a synthetic culture medium containing no adsorbable molecules, namely, Eagle's medium.

The cells used in the experiments described below, namely, fresh embryonic chick skin and retina, and tissue culture strains of rat kidney, human epidermoid carcinoma, and human conjunctiva, during the experimental time interval (4 hrs.) exhibited in Eagle's medium none of the morphological changes associated with cellular degeneration such as withdrawal of processes, increased refractiveness of granules and nucleoli, vacuolization, or pyknosis of nuclei. Except for chick retina, the cells showed good attachment, spreading, and process formation on glass surfaces.

In addition, the human conjunctiva cells (grown in our laboratory for the past 2 years) could be maintained and subcultured in Eagle's medium for several days with excellent spreading and process formation on glass surfaces but little, if any, multiplication. Moreover, if these cells were then transferred to a standard growth medium (Eagle's mixture supplemented with 10 per cent calf serum), they again resumed proliferation. For these reasons, human conjunctiva cells in Eagle's medium were used for the longer time intervals (up to 29 hrs.).

The methods used to detach or dissociate these cells (see section on methods) can result in certain morphological changes. Under phase microscopy the surfaces of trypsin-treated cells can be seen to have both fine and coarse corrugations whereas the surfaces of cells treated with versene (as described above) appear considerably smoother. In addition, the versene-treated cells appear to spread more rapidly, although no actual quantitative tests have been carried out to verify this impression.

Cells in Eagle's medium allowed to attach and spread on a clean glass surface can no longer be detached by digestion of protein with trypsin or removal of the cations, magnesium and calcium, with versene, although the application of trypsin often results in the withdrawal of processes. On the other hand, if either the cells or the substratum are first washed with a 1 per cent solution of bovine gamma globulin or horse serum so as to form an adsorbed molecular film on the surface of the cell or the glass, and the cells are then resuspended in Eagle's medium and allowed to spread, they become detachable with either trypsin or versene treatment. The relationship between this observation and the presence of a microexudate is obscure, although the experiments to be described suggest certain explanations.

B. *Measurements*

1. *Time Course of the Deposition of Microexudates.* As exemplified in Fig. 1, the average thickness of microexudate increases as a function of time, the time course varying for different types of cells. The cells had previously been detached or dissociated with trypsin (see methods section), and then suspended in Eagle's medium. Since each cell type differs in its mean diameter and mean cross-sectional area, the CPSD was selected so that the RCS is roughly equal for all curves. For this figure an approximate value of 5 per cent was chosen; a value at which the cells are sufficiently distant (roughly four cell diameters) from one another, prior to spreading upon the chromed surface, for ellipsometer measurements to include essentially intercellular regions. Thus, the initial rapid rise in average thickness most likely results from an accumulation of molecules intercellularly rather than a change in dimensions, orientation, configuration, or stacking of molecules. The mean diameters of the cell types in Fig. 1 are as follows: embryonic skin (curve A), 10 microns; human conjunctiva (curve B), 14 microns; human epidermoid carcinoma (curve C), 15 microns; embryonic chick retina (curve D), 7 microns. It has been possible to reproduce curve B for human conjunctiva cells on three separate occasions with an accuracy of ± 2 A. The other curves have not been

studied for reproducibility. It is difficult to assess accurately the detailed differences between these curves, that is, whether the time course of the deposition of microexudate of a given cell type or strain is specific for that particular material. An appraisal of specificity must await the results of more detailed studies currently in progress. However, a major point of interest is that the cells upon contact with the indicated substratum very rapidly produce material of molecular dimensions. The time curve for this intercellular material has a high initial slope, and the rate of increase of its average thickness then decreases with time, the maximum rate of change of curvature occurring at 1 to 2 hrs.

If the RCS is markedly increased such that the ellipsometer measurement then averages the thicknesses of both the subcellular and intercellular microexudates, an interesting, as yet unexplained, phenomenon results. This is illustrated in Fig. 2, where the RCS is roughly greater than 15 per cent (intercellular distance approximately two cell diameters), and the cell types and their mean diameters are: embryonic chick skin (curve A), 10 microns; rat kidney (curve B), 14 microns; embryonic chick retina (curve C), 7 microns. These time curves show an initial peak followed by a temporary dip. Although the spreading and interactions of cells in the case of high surface concentrations, may lead to considerable modifications in the microexudate, these factors, in themselves, have not as yet been adequate to explain the bizarre configuration of these curves.

TABLE I

ADSORBABLE MATERIAL IN SUPERNATANT OF HUMAN CONJUNCTIVA CELLS

Time (hours)	0.25	0.5	1.0	2.0	3.0	4.0	5.0	20.0	23.0	25.5	29.0
Average thickness (angstroms)*	4 5	4 5 1	4 5 1	4 6	4 4 1	3 4	2 2	0	2	6	1

* Multiple determinations.

Finally, curve E of Fig. 1 and curve D of Fig. 2 illustrate control readings, obtained when cell-free Eagle's medium is allowed to remain on the test slide for designated periods of time. The small variations are less than 3 A and demonstrate how errors due to contaminants in the atmosphere or in the medium may be minimized if great care is taken. In addition, as described in the methods section, the medium supernatant to the cells was studied for the presence of adsorbable molecules. The results of this study, illustrated in Table I, indicate that the adsorbate so obtained is essentially negligible.

2. *Microexudates in Different Culture Media.* With certain alterations in the culture medium the time curve of average microexudate thickness undergoes the changes illustrated in Fig. 3. If Eagle's medium is supplemented with horse serum, the large serum molecules are very rapidly adsorbed to the substratum, forming a layer roughly 23 A thick. This alteration of the cellular substratum will occur when-

ever adsorbable molecules are present in the medium, and there are good indications, presented in a later section, that these molecules adsorb to and thus modify also the surface of the cell. The problem then arises as to whether a microexudate can still be detected as an overlay on the adsorbed layer.

Curve B of Fig. 3 is the typical time curve for trypsinized human conjunctiva cells cultured in Eagle's medium (375 cells/mm^2). If the same cell strain after trypsin treatment is cultured in Eagle's medium supplemented with 5 per cent horse serum (370 cells/mm^2), curve A results. The almost instantaneous adsorbate, 23 A in thickness, pictures the adsorption of molecules from the medium, but above this layer there seems to be a gradual overlay of material, possibly of microexudate. In any case, the appearance of microexudate as a result of cellular contact with the modified substratum is markedly retarded.

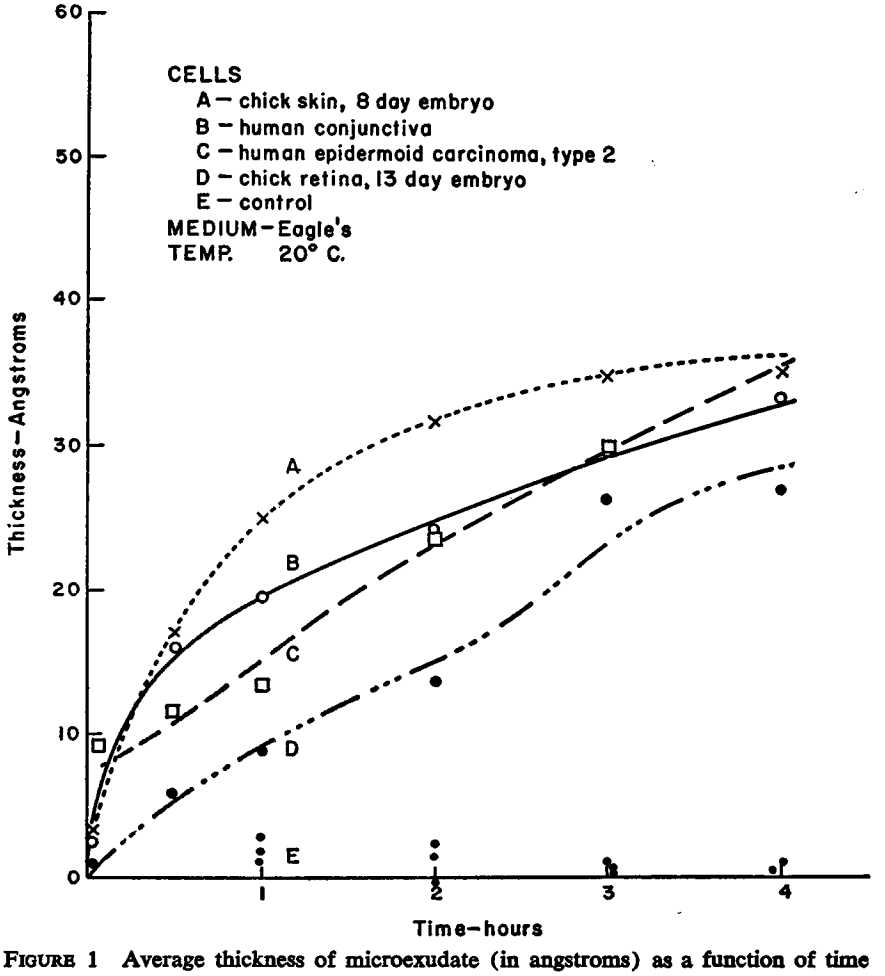

FIGURE 1 Average thickness of microexudate (in angstroms) as a function of time for various cell strains. (Low concentration of cells.)

Curve C depicts, on the other hand, microexudate production when the same strain of cells is cultured in Eagle's medium supplemented with 5 per cent bovine ultrafiltrate. This ultrafiltrate consists of small nonadsorbable molecules; that is, if a cell-free sample of the ultrafiltrate is allowed to remain on a test slide for a 15-min. period and is then washed off, no change in the surface of the slide can be detected by means of ellipsometry. For both curve B and curve C, the cells contact the same substratum, but it is not possible to determine whether the slight differences in configuration of the curves are in response to the changes in the culture medium. Far more elaborate studies are required. However, the striking decrease in microexudate that occurs when serum in the medium adsorbs to surfaces led to a study of the effects of controlled substrata.

3. *Formation of Microexudates on Different Substrata.* Several tech-

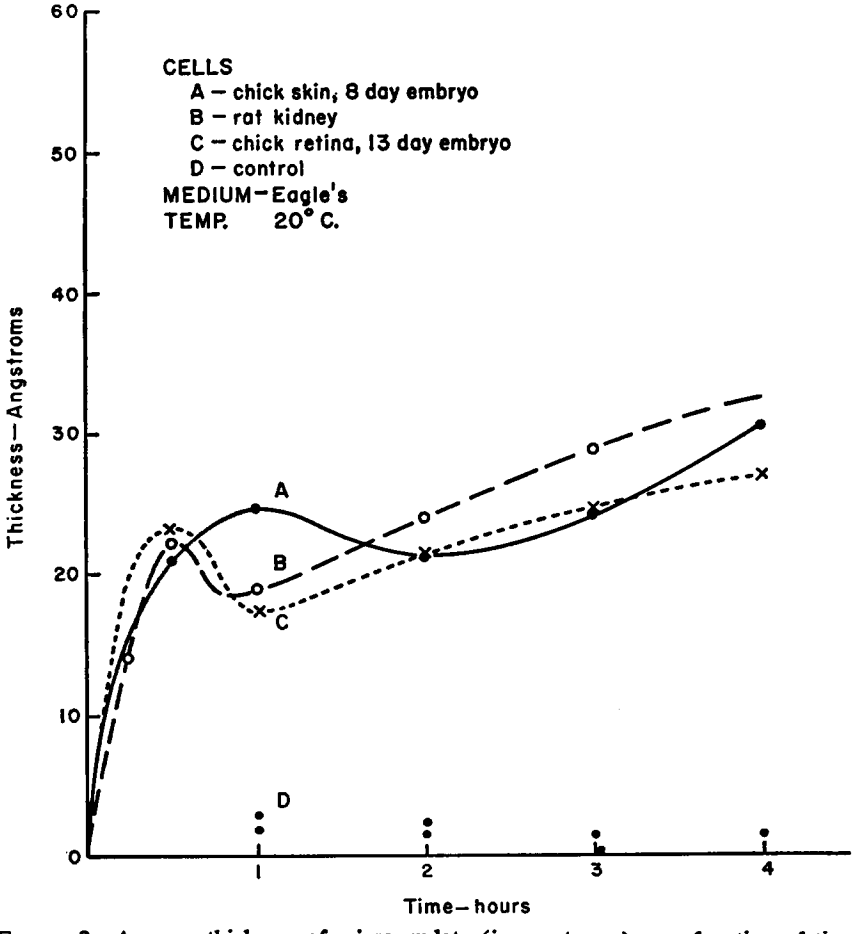

FIGURE 2 Average thickness of microexudate (in angstroms) as a function of time for various cell strains. (High concentration of cells.)

niques are commonly used in the field of surface chemistry to prepare thin films of known molecular structure and thickness. One technique is to adsorb molecules directly to surfaces; another is to use a Langmuir trough, containing a buffered solution upon whose surface is placed a small amount of the material to be studied. Many materials form a monomolecular film on the surface of the buffer, and by application of lateral pressure along the surface, the monomolecular film can be transferred to clean surfaces. By means of repeated transfers, a transferred film can be prepared with a thickness varying from one to several thousand molecular layers. For a review of these techniques, see Rothen (1956).

Such adsorbed or transferred films can then serve as specific substrata of known molecular structure, thickness, and orientation for the study of surface reactions of cells. The presence and average thickness of the films prior to the additions of

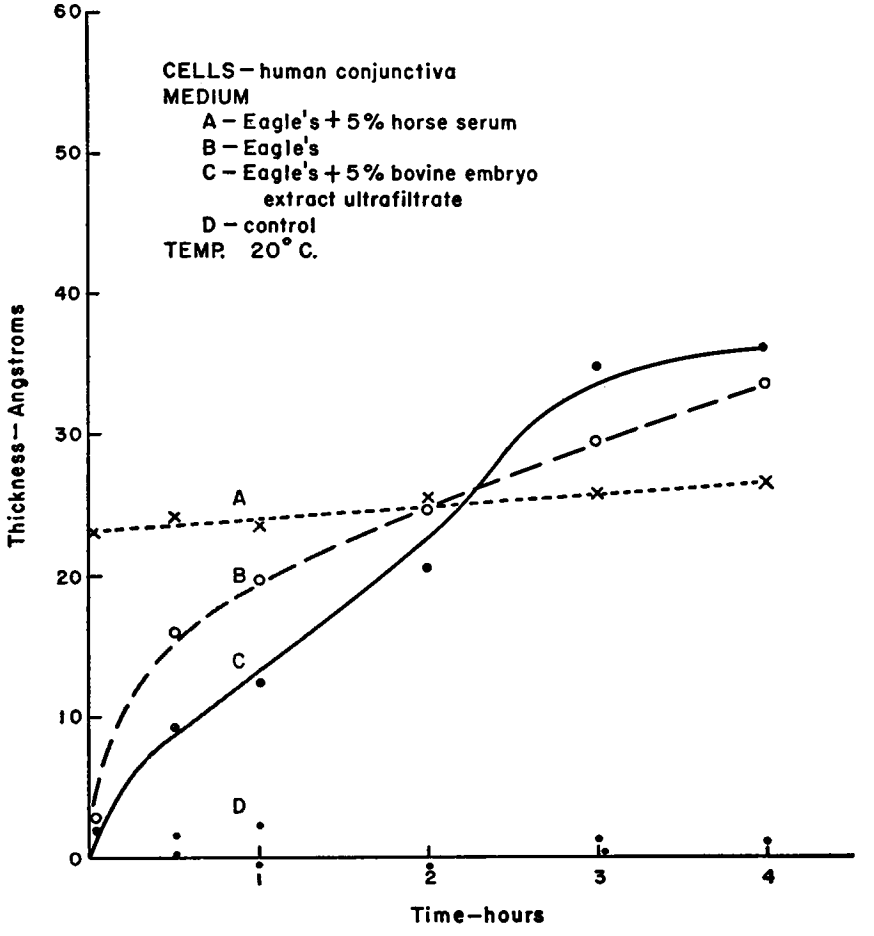

FIGURE 3 Average thickness of microexudate (in angstroms) as a function of time for various growth media.

cells and medium can be readily ascertained with the ellipsometer. Any subsequent changes in average thickness after the addition of cells are then attributable to cellular effects. Fig. 4 portrays the changes in average thickness when human conjunctiva cells, detached from tissue culture flasks by trypsin, are allowed to grow in Eagle's medium on specified substrata for 2 hours and then removed.

The average thicknesses of transferred and adsorbed monolayers are given in the lower portion of the figure with glass as the reference substratum. The second-

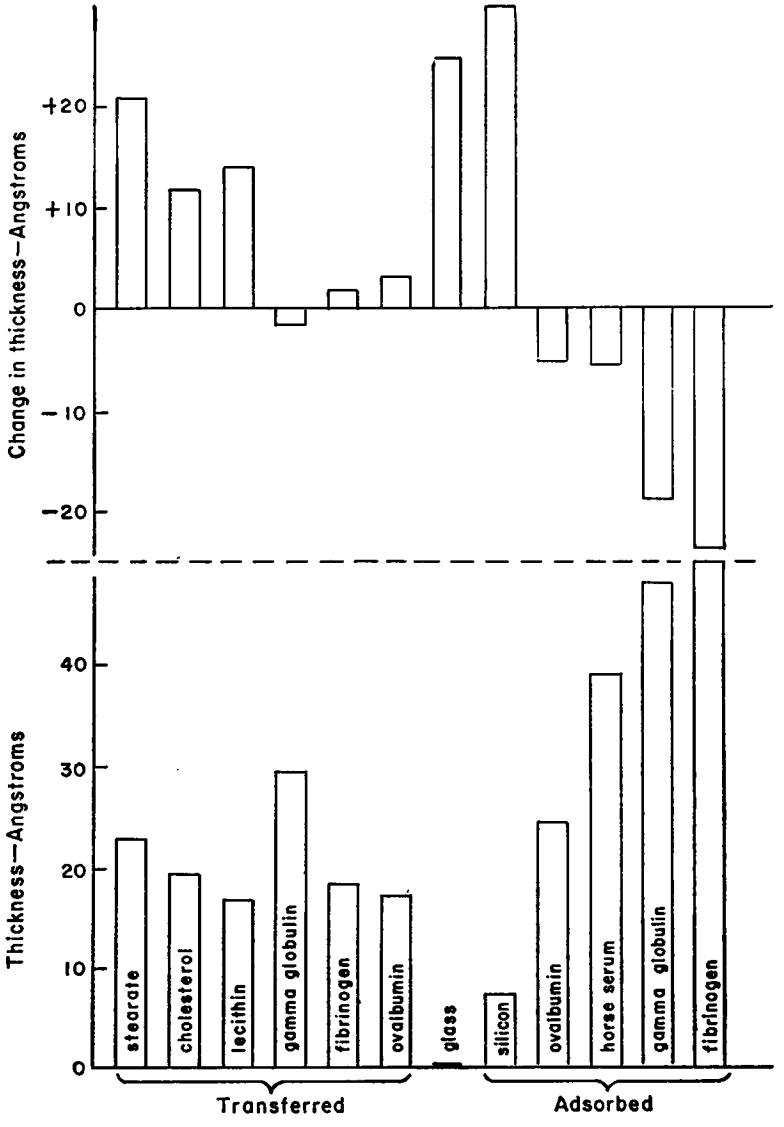

FIGURE 4 Change in average thickness (in angstroms) of various transferred or adsorbed substrates after growth of trypsin-dissociated cells for 2 hrs.

ary changes in average thickness after the addition of cells are given in the upper portion of the figure. After 2 hrs. the cells have deposited on the hydrophilic glass the previously described microexudate, of an average thickness of 24 A. A similar deposit has occurred on the hydrophobic adsorbed layer of organic silicone. On transferred monolayers of stearate, cholesterol, and lecithin there is an increase in average thickness apparently attributable to microexudate, the magnitude of the change being somewhat less on transferred lecithin and cholesterol. However, transferred films of bovine gamma globulin, fibrinogen, and ovalbumin underwent no changes in average thickness, and this indicates that no significant amounts of microexudate had been deposited by the cells on these substrata. Finally, when the cells were grown on adsorbed protein layers of bovine gamma globulin and fibrinogen, the average thickness of the substratum underwent significant reduction.

These experimental results show that the appearance and mean thickness of the microexudate is signally dependent upon the molecular constitution of the substratum on which the cell rests, and that trypsin-treated cells are capable of reducing adsorbed protein films by substantial amounts. In addition, these observations suggest that the production and deposition of the microexudate could be a function of a selective and competitive attraction for the microexudate by the cell and the substratum, and that trypsin-treated cells retain trypsin activity.

Let us consider the latter suggestion. Northrop (1926) demonstrated that there is no uptake of trypsin by the cytoplasm of live cells. Rothen (1948) has shown that protein films, acted upon by trypsin, continue to exhibit trypsin activity even after transfer of the film. It, therefore, appears reasonable to assume that the cell retains trypsin activity as a result of adsorption of trypsin to its surface. This assumption can be tested experimentally. One would expect versene-treated cells or cells whose adsorbed trypsin has been inactivated to cause little, if any, reduction in the average thickness of an underlying adsorbed film of protein. In addition, if microexudate includes some protein components, the average thickness of material produced by trypsin-treated cells should be less than that available from versene-treated cells. The experiments described in the next section verify these predictions.

4. *Microexudate and Cell Treatment.* The adsorption to a chromed glass surface of trypsin, in contrast to versene, can be demonstrated by means of the ellipsometer. When solutions of versene or trypsin were placed upon the cleaned surface and removed after 15 min. the results tabulated in Table II were obtained. Thus trypsin is adsorbed, whereas versene is not.

By the methods described in section 3, one can investigate the interaction between trypsin- or versene-treated cells and known substrata. Trypsin-treated or versene-treated cells were cultured for 2 hrs. in Eagle's medium on controlled substrata of adsorbed layers of serum or protein. In addition, some trypsin-treated cells were washed with a 10 per cent solution of calf serum to inhibit or inactivate the trypsin on the surface of the cell, and the cells were then suspended in Eagle's medium. The striking results are detailed in Fig. 5. Three primary adsorbed films were

examined, namely, calf serum, fibrinogen, and bovine gamma globulin. The average thickness of the adsorbed films is shown in the lower portion of the figure; the secondary changes in average thickness after the addition of cells are shown in the upper portion. The results of control readings on a glass surface, consistent with previously described measurements, are shown on the far right.

This figure clearly illustrates that the presence of trypsin-treated cells has led to digestion of the underlying protein, whereas the presence of cells coated with a trypsin inhibitor or versene-treated cells have had no effect. In fact, the amount of

TABLE II

ADSORPTION OF TRYPSIN OR VERSENE TO GLASS

Material	Average thickness of adsorbed layer (angstroms)*
Trypsin	6
	7
Versene	0
	0

* Multiple determinations.

reduction in average thickness is comparable to the amount of material removed when 0.05 per cent trypsin at pH 7.6 is applied directly to an adsorbed film for 15 min. at 37°C (see Table III). These data substantiate the proposition that trypsin molecules are adsorbed to the surface of cells, continue to be enzymatically active at that site, and suggest that trypsin then creeps over the substratum.

A corollary to this finding is that if microexudate is in part composed of protein, the time curves for its average thickness should vary with the method of prior cellular treatment. This variation is detailed in Fig. 6, which shows that, as a function of time, molecular material of greater average thickness is deposited by versene-treated cells. It appears that the reduced average thickness of the microexudate

TABLE III

CHANGE IN AVERAGE THICKNESS OF ADSORBED LAYERS AS A RESULT OF TRYPSIN ACTION

Molecular film	Average thickness of film (angstroms)*	Change in average thickness after trypsin (angstroms)*
Calf serum	21	−6
	19	−4
Fibrinogen	48	−20
	46	−18
Bovine gamma globulin	77	−26

* Multiple determinations.

produced by the trypsin-treated cells is a consequence of the digestion of protein components by adsorbed trypsin.

DISCUSSION

The techniques described above clearly demonstrate how the interactions between cell, substratum, and medium, as well as the adsorption of enzymes to cell surfaces can be studied quantitatively. The data, so obtained, support the following conclusions:

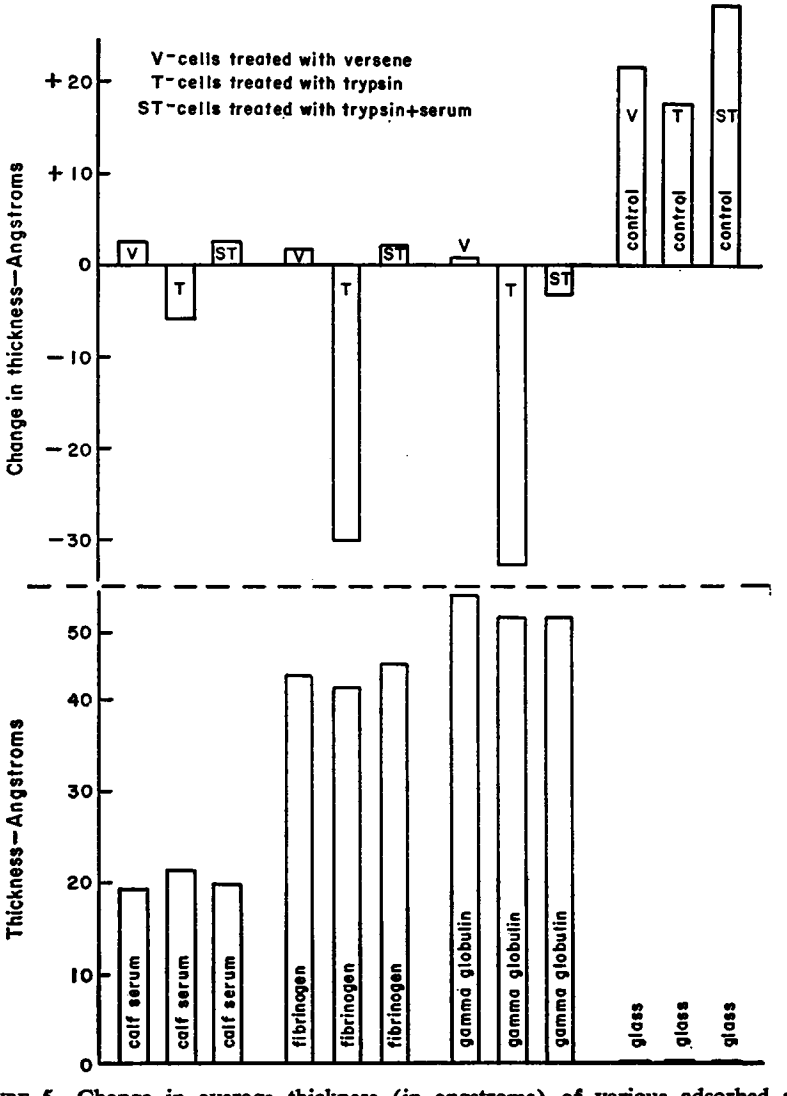

FIGURE 5 Change in average thickness (in angstroms) of various adsorbed substrates after growth of trypsin-, versene-, or trypsin-serum-dissociated cells for 2 hrs.

1. Cells can produce a microexudate or a film of molecular dimensions that coats and modifies their substratum (Figs. 1, 2).
2. The culture medium, through adsorbable components, alters both the surface and substratum of cells (Fig. 3).
3. Changes in substratum affect the deposition of microexudate, possibly as a result of the selective attractions of the substratum and cell surface for this material (Figs. 3, 4).
4. Trypsin becomes adsorbed to the surface of cells, continues to be enzymatically active on the surface, and leads to changes in the microexudate (Figs. 5, 6; Table II).

Although these results point out the significant and complex role played by molecular mechanisms when cells are maintained in tissue culture, they leave many

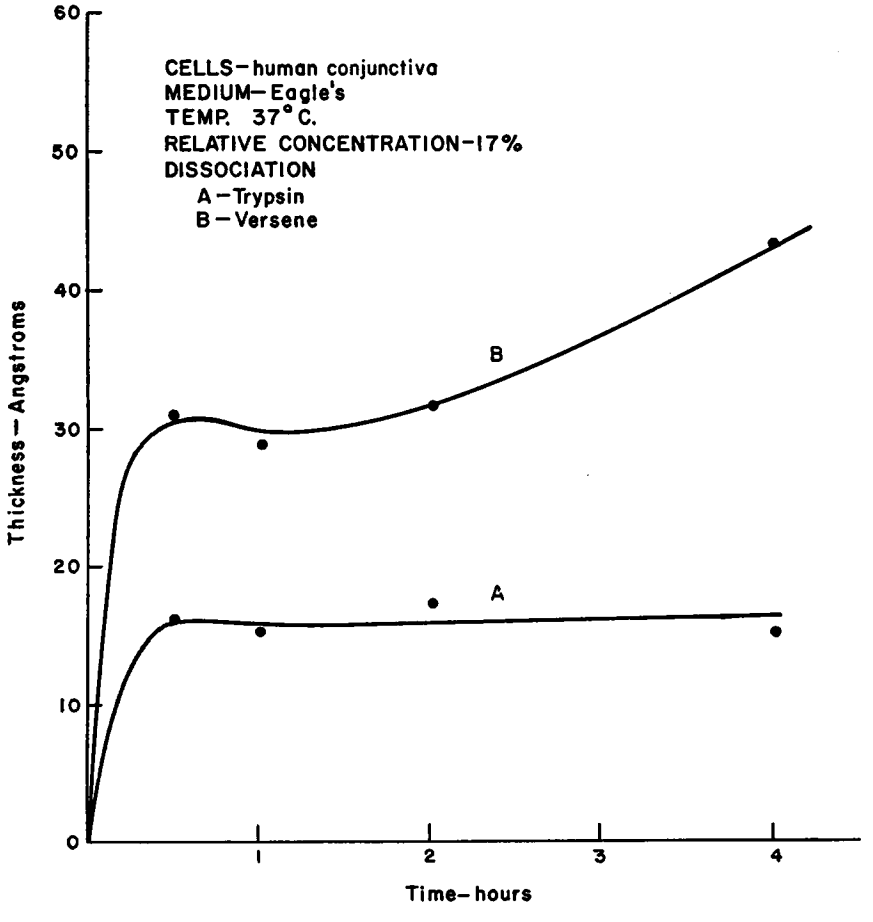

FIGURE 6 Average thickness of microexudate (in angstroms) as a function of time for trypsin- and versene-dissociated cells.

important questions unanswered. Information regarding the specificity, source, role in cellular behavior, or composition of this microexudate or molecular film is incomplete and inconclusive. For the moment several of these questions will have to remain unanswered. However, experiments have been completed which render some speculations regarding the composition and source of this material more probable than others. These results are discussed in part II below.

RESULTS—PART II

A. *Measurements*

1. *Some Chemical Characteristics of Microexudate.* (*a*) *Enzymatic tests.* Fig. 6 has shown that less molecular material is produced by trypsin-treated cells in contrast to versene-treated cells. The conclusion reached in part I has been that trypsin is adsorbed to the surface of cells and digests some protein components of microexudate. A supplementary experiment to demonstrate the presence of protein in microexudate is summarized in Table IV. The tabulated data establish that

TABLE IV

CHANGE IN AVERAGE THICKNESS OF MICROEXUDATE AS A RESULT OF TRYPSIN ACTION

Cells	Average thickness of microexudate (angstroms)*	Change after trypsin (angstroms)*
Trypsin-treated	27	1
	28	1
Versene-treated	44	−14
	49	−15

* Multiple determinations.

trypsin (0.05 per cent, pH 7.6) can lead to a reduction in average thickness of microexudate from versene-treated cells, but none in that from trypsin-treated cells. In fact, the effect of trypsin is to reduce the average thickness to a level similar to that obtained with trypsin-treated cells. Thus, the action of trypsin in digesting some protein components of microexudate is apparently similar whether the enzyme is adsorbed to the cellular surface or applied directly to the microexudate.

On the other hand, applications of solutions (0.05 per cent) of the enzymes lipase and hyaluronidase at 37°C, pH 7.4, led to no significant change in average thickness of the molecular material.

(*b*) *Tests by acids and bases.* The average thickness of material composing the microexudate remains essentially unaltered by the action of 0.01 N to 1.0 N HCl, NaOH, and $NaHCO_3$ at 23°C for 45 min. 1 N NaOH or HCl at 100°C readily removes a major portion of material in 15 min.

(*c*) *Isolation if microexudate.* In view of the action of strong acids and bases at 100°C, an effort was made to hydrolyze some components of the material. Human

conjunctiva cells were grown for 4 hrs. in Eagle's medium on 150 clean glass slides, and after removal of the cells, the glass was washed and subjected to the hydrolytic action of 1 N HCl at 100°C. The hydrolysate was then evaporated to dryness, and both water and chloroform-methanol-soluble fractions of the residue were chromatographed. In general the results of these experiments were poor. Some amino acids and lipid material were detected, but it was not possible to maintain fully satisfactory controls. More effective experiments are now underway, and perhaps the sole conclusion to be drawn at present is that the microexudate is most likely a complex molecular mosaic that includes protein.

2. *Dependence of Microexudate upon Other Physical Variables.* The determination of sources for the microexudate poses several problems. The deposition may result from a stripping off of a molecular coat from the cell surface or may consist of cellular secretions, excretions, cell debris, or cytoplasmic fragments; extracellular synthesis may also be involved. Because of the difficulty of isolating each of these possible sources, it was decided to measure the average thickness of microexudate deposited during longer time intervals, and to determine its functional dependence on additional physical variables such as CPSD and temperature. It was hoped that these studies together with the aforementioned data would provide indications of the source of the molecular deposit. The results of these tests are presented in the subsequent paragraphs.

(*a*) *Time course of the deposition of microexudate.* The average thickness of molecular material deposited on test slides by human conjunctiva cells in either pure Eagle's medium or in Eagle's medium supplemented with 10 per cent calf serum is tabulated in Table V (for the latter medium, the number of angstroms in excess of the 20 A adsorbed layer of calf serum is given). As noted in the section on observations, part I, these cells have been maintained successfully in Eagle's medium for periods in excess of 29 hrs.

In a standard growth medium (row 3) the over-all measurement was only slightly greater than the average thickness of an adsorbed layer of calf serum in the

TABLE V

AVERAGE THICKNESS OF MICROEXUDATE *VERSUS* TIME (HUMAN CONJUNCTIVA CELLS; TEMPERATURE 37°C; VERSENE TREATMENT; RELATIVE CELLULAR CROSS SECTION 20 PER CENT)

Time (hours)	0.5	1.0	2.0	3.0	4.0
Average thickness in Eagle's medium (angstroms)*	25 24 20	31 27 29	—	40 37 37	—
Average thickness in Eagle's medium supplemented with 10 per cent calf serum (angstroms)*	6 7	10 10	4 6	9 11	8 8

* Multiple determinations.

TABLE VI

AVERAGE THICKNESS OF MICROEXUDATE *VERSUS* RELATIVE CELLULAR CROSS SECTION (HUMAN CONJUNCTIVA CELLS; TEMPERATURE 37°C; VERSENE TREATMENT; GROWTH PERIOD 29 HRS.)

Relative cellular cross section (per cent)	0	0.25	0.5	1	4	11	19	47
Average thickness (angstroms)*	1	5	15	23	51	54	58	73
	0	7	15	23	54	61	67	81

* Multiple determinations.

absence of cells. In contrast, cells in pure Eagle's medium apparently yield the microexudate almost immediately upon contact with the clean glass surface, and the maximum average thickness of the film is reached in 1 to 3 hrs.

(*b*) *Microexudates at various relative cellular cross sections* (*RCS*). The functional dependence of the average thickness of microexudate upon the RCS is of interest in that the surface coverage by the deposited material seems to be complete even when intercellular distances are considerable. Table VI presents data to substantiate this statement.

At fairly low values of RCS (5 to 10 per cent), when the intercellular distance is approximately three to four cell diameters, the average thickness is close to its peak. At these concentrations the measured average thickness (see observations, part I) is that of the film of intercellular material. With increasing concentration, the subcellular area increases, and the fact that the readings represent instrumental averages of both subcellular and intercellular material is no longer negligible. For the highest concentration tabulated, the spread cells cover essentially the entire surface of the test slide, so that the average thickness is that of subcellular material. Prior to speculating on the significance of these results, some additional experiments are considered.

(*c*) *Microexudate versus temperature.* Fig. 7 illustrates how the average microexudate thickness varies with temperature. Only a slight increase with increasing temperature indicates that the rate of deposition of this material is not a function of cellular metabolic activity.

TABLE V—*concluded*

6.0	20.0	23.0	25.0	29.0	45.0	53.0	69.0	77.0
—	39	47	53	47	—	—	—	—
	42	51	56	47				
	41							
4	11	5	—	8	11	5	7	13
6	9	11		8	8	6	9	15

(*d*) *Adsorbable material in supernatants.* Table I, in an earlier section of this paper, contains data showing that Eagle's medium, supernatant to human conjunctiva cells spread upon test slides, contains few, if any, adsorbable molecules. It is presumed that if adsorbable molecules are given off by cells, they are in sufficiently small quantity so as to be present only on surfaces, rather than in the bulk of the medium. The results of an additional experiment, supporting this contention, are presented in Table VII. Human conjunctiva cells were maintained in suspension

TABLE VII

AVERAGE THICKNESS OF MOLECULAR LAYER ABSORBED FROM EAGLE'S MEDIUM USED FOR ROLLER TUBE CULTURE (HUMAN CONJUNCTIVA CELLS; TEMPERATURE 37°C; VERSENE-TREATED)

Time in roller tube (hours)	2	26	2†
Average thickness	5	8	3
(angstroms)*	3	7	4
	3	9	4

* Multiple determinations.
† High concentration of cells.

in Eagle's medium in roller tube cultures. The total cross-sectional area of cells in suspension was far in excess of the total surface area of glass in contact with the suspension. At designated periods, aliquots were centrifuged, and the supernatant tested for the presence of adsorbable molecular material. Few, if any, molecules were adsorbed from the supernant medium unless the cells had been in the roller tube for at least 26 hrs. The small amount of adsorbable material after 26 hrs. is possibly a result of cellular damage due to agitation.

The question that must now be examined is whether the foregoing empirical results weave a consistent pattern suggestive of the source and composition of microexudate. The following discussion concentrates on this question.

DISCUSSION

As noted above, the microexudate is most likely a complex mosaic of molecules (including protein) which is synthesized within or on the surface of cells and secreted or transferred such that it can be detected as a molecular film on the cellular substratum. The most likely sources for this material are cellular secretions or the transfer of molecules from the cellular surface to the surface upon which the cell rests. Both of these hypotheses are supported in varying degrees by the experiments described above and analyzed in the following summary.

1. The microexudate appears only if cells contact certain kinds of substrata, such as clean glass, silicone, or transferred stearate films. Little if any material is given off when cells rest upon transferred or adsorbed films of protein or

serum (Figs. 4, 5). The selective attraction of various substrata, therefore, appears to play an important role in the spreading of this material.

2. One would expect secreted or transferred material to appear rapidly beneath the cell and to diffuse gradually outward from the cell. In fact, an approximate calculation based upon an RCS of 10 per cent (intercellular distance of approximately three cell diameters) and a diffusion coefficient of 10^{-7} $cm^2/sec.$ leads to the prediction that the molecular fabric of the microexudate should rapidly spread intercellularly during the first 15 min. (This estimate is based solely upon physical adsorption; no chemadsorption is assumed.) The results of this calculation are certainly of the correct order of magnitude and partly in agreement with the data illustrated in Figs. 1 and 2 and Table V, although for high concentrations of cells the initial rate of microexudate appearance is not as rapid as might be expected.

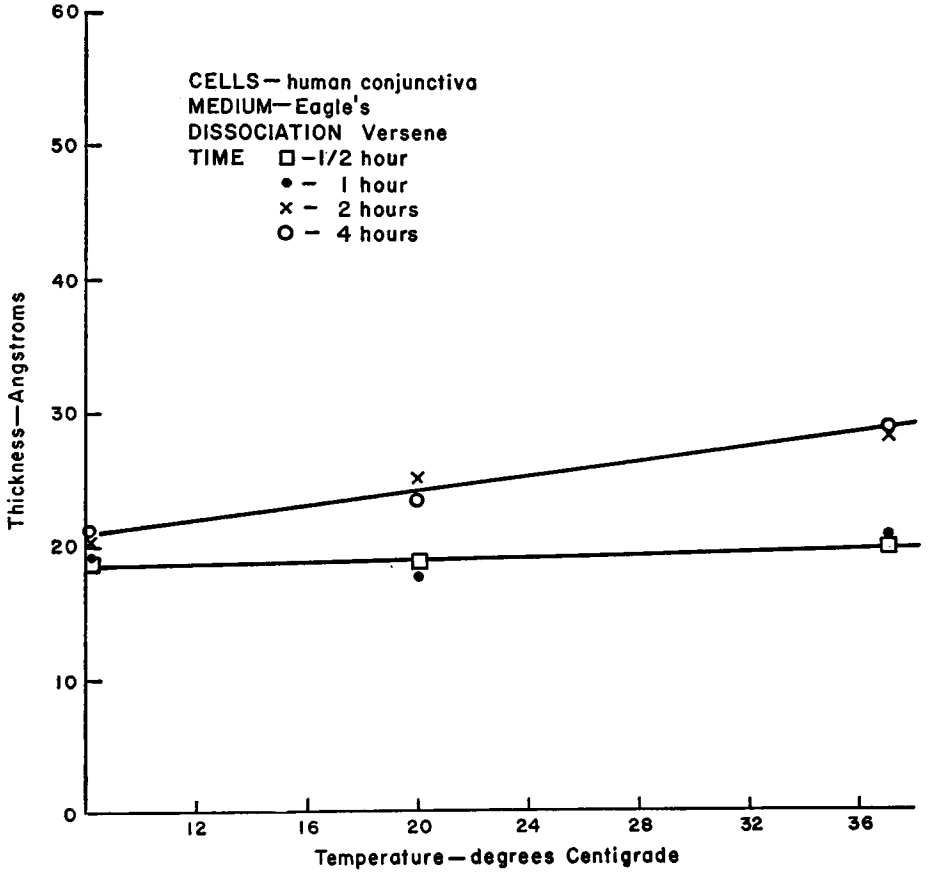

FIGURE 7 Average thickness of microexudate (in angstroms) as a function of temperature.

3. The maximum average thickness of intercellular microexudate is attained already at low values of the RCS (Table VI). Assuming a maximum film thickness of 40 A and assuming further that at the equilibrium point 30 to 40 per cent of the intercellular region has been covered with a molecular fabric, the cells would have had to give off only 0.2 to 0.6 per cent of their total weight to cover this area. This amount of material is in no way excessive and is consonant with the hypothesis that microexudate results from cellular secretion. Similarly, inasmuch as the surface area of a spherical cell is four times its cross-sectional area, a sufficient number of molecules could be transferred from the surface of the cell to bring about reasonable coverage of the intercellular spaces. Thus, the hypothesis of displacement or transfer of molecules from the surface of the cell is also in accord with these data.
4. The supernatants of attached or suspended human conjunctiva cells contain very little adsorbable material (Table I, VII). One would not expect molecular transfer from surfaces of cells to be followed by the appearance of adsorbable molecules in the medium. On the other hand, cellular secretions are more likely to add adsorbable material to the suspending medium, unless the quantity of secreted material is so minimal as to be completely adsorbed.
5. Both molecular transfer between surfaces and the physical liberation of secretions could be relatively independent of temperature, consistent with the data diagrammed in Fig. 7.

In summary, the experiments performed up to now suggest two possible sources for the molecular fabric that comprises the microexudate. Either molecules normally present on cellular surfaces as a result of adsorption, secretion, or synthesis are transferred to the substrate; or the material is directly secreted by the cell onto the substratum. The relative shares of these two sources in the total deposits cannot be assessed pending the development of additional experimental techniques.

GENERAL DISCUSSION

The experiments described in this report show that cells upon contact with a surface can deposit a complex molecular carpet on surfaces that have strong attractive forces for this material. It appears likely that this molecular film or microexudate of cellular origin consists of a combination of cellular secretions and molecules transferred from the surfaces of the cells.

In view of the predominant function of cellular secretions in tissue repair, wound healing, and cell growth and development, one would expect secretory products to contribute to the composition of microexudate. On the other hand, the likelihood that molecules adsorbed to cellular surfaces also contribute to the formation of microexudate is an unexpected yet highly significant outcome from these investigations. If microexudate is composed, in part, of molecules whose prior region of activity was the cell surface, a determination of its physical and chemical properties

should reveal significant information about the surfaces of cells, regions of poor accessibility. It then may be possible to quantitate chemically and immunologically the complex problem of specificity with respect to cell surfaces.

The suggestion has been made often that protein is adsorbed to the lipoid components of the cell membrane. Danielli (1958) contended that in addition to a primary adsorbed denatured monomolecular layer of protein, there is an overlying secondary layer of globular native protein. Weiss (1960) recently proposed that the cell surface is occupied by a network of filiform macromolecules in essentially planar array. Allison and Valentine (1960) have shown that virus particles adsorb to cell surfaces at a rate that is less than expected on the basis of Brownian collisions. The investigations described in this paper show how one protein, trypsin, is adsorbed to the surface of cells and remains enzymatically active on the surface. One exception seems to be the surface of erythrocytes which possess envelopes unlike tissue-cultured cells. According to Abramson, Moyer, and Gorin (1942), erythrocytes do not appear to adsorb protein, a property which may be necessary for their biological survival in the circulatory system (see Bangham, Pethica, and Seaman (1958)). It appears very likely, however, that the surfaces of most cells have configurations and charge distributions such that molecular adsorption can take place. The investigations described in this paper reemphasize the significance of these adsorbed materials, and suggest for the first time that these molecules actively participate in the formation of molecular films, termed microexudates, on cellular substrata.

In evaluating possible roles for this microexudate, one must view it as both a possible representative of molecular substances previously adsorbed to cell surfaces, and as a molecular film that can modify surfaces in contact with the cells. The effects of molecular adsorption to cell membranes on the chemical and physical properties of the surface are obscure. Bangham, Pethica, and Seaman (1958) discussed the functional relationship between electrophoretic mobility and the charged group at the interfaces of some blood cells. Schmitt (1941) proposed that an adhesive lining mediates cell-to-cell interactions. Weiss (1941, 1950) described the selective surface stickiness ("fasciculation") appearing in pioneering nerve fibers after making peripheral connections. The investigations on microexudate indicate that adsorbed materials can be involved in cell-to-cell interactions and cell contacts. The further study of microexudate as a specimen of materials previously adsorbed to cellular surfaces, as well as additional studies on the interactions between cell and microexudate may help clarify some aspects of the complex phenomena of cell contact.

In addition, microexudate can result in alterations of the cellular substratum. In this capacity it may act as an ultrafine "ground mat," several orders of magnitude smaller than the colloidal exudate described by Weiss (1945) and discussed in the introduction to this report. This molecular carpet could then directly influence cell locomotion, adhesion to surfaces, and the selective application and spreading of cells on various surfaces. Some recent data of Taylor (1960) on the attachment and

spreading of cells partly corroborate this assumption. Crisp and Ryland (1960) have shown how films affect the settlement of marine organisms, and Zvyegintsev (1959) has considered their influence on the adsorption of microorganisms. It seems, therefore, that molecular films or microexudates can assume the role of a ubiquitous mediator of interactions on the molecular level.

Several investigators (Steinberg (1958), Lieberman and Ove (1958)) have proposed that a surface protein and polyvalent cation are intimately related to the processes of attachment and flattening of cells on surfaces. Lieberman and Ove (1958) also suggested that attachment with basic peptides does not appear to involve a polyvalent cation, since such bonds can be released with trypsin but not with versene. The bridge of microexudate, however, between the cell and glass differs in that it is resistant to both trypsin and versene, even though microexudate contains some trypsin-digestible components. Thus, it appears that microexudate could act as an ionic bridge, or what is more likely, that the bonding mechanism involves London-van der Waals forces (see Curtis (1960)) or modifications of these forces described by Yos, Bade, Jehle (1957), Lifshitz (1955), Derjaguin, Abrikossova, Lifshitz (1956), and Overbeek (1952).

Undoubtedly several mechanisms are at play on the molecular level. Steinberg (1958), Weiss (1958), and Curtis (1960) have reviewed many of them, such as the steric interactions of complementary sites, short-range electrostatic forces, calcium bonding, intercellular bridges, long-range London-van der Waals forces, mechanical linkages of surface microfibrils, and some specific cellular exudates. The experiments described in this report are limited to the study of molecular materials produced by cells contacting specified surfaces. Major questions regarding the chemical characteristics of microexudate, its role in biological systems, its specificity and structure are left open and await clarification. These investigations, however, exemplify and establish the usefulness of surface chemistry techniques in studying these questions. It is hoped that answers can be provided by further research to supplant any premature formulations.

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