

WOUND HEALING AND COLLAGEN FORMATION

V. Quantitative Electron Microscope Radioautographic Observations of Proline- H^3 Utilization by Fibroblasts

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ABSTRACT

The uptake, intracellular transport, and secretion of protein by guinea pig wound fibroblasts was studied by electron microscope radioautography using L-proline-3,4- H^3 as a tracer. Experiments were performed to determine the curve of concentration of free amino acid in the blood after intraperitoneal administration of the labeled proline. Radioautographs were quantitatively analyzed and the concentration of isotope, in grains per unit area, was determined for the following cellular and extracellular compartments: ergastoplasm, Golgi complex, peripheral cytoplasmic structures, and collagen. The concentration of label, expressed as number of grains per unit area of each subcellular system, reveals the period during which each cellular compartment is maximally labeled, and presents a clearer picture of the passage of the label through each of these compartments. The data demonstrate appearance of the label at maximum concentration in the ergastoplasm 15 minutes after injection, and this compartment remains maximally labeled for 2 hours. In the Golgi complex, concentration is not maximal until 60 minutes after injection of isotope, and appears to decrease before or at about the same rate as that of the ergastoplasm. The present experiment is consistent with previous light microscope radioautographic studies, and no storage phase was found in the fibroblasts. The findings are not simply consistent with a direct precursor-product relationship between the contents of the ergastoplasm and those of the Golgi complex. Morphologic observations of regions in the fibroblast interpretable as possible sites of communication between the ergastoplasm and the extracellular space, together with the kinetic studies, permit the suggestion of an alternate pathway of passage of at least some of the synthesized protein directly from the ergastoplasmic cisternae to the cell exterior.

INTRODUCTION

High resolution radioautography, using the electron microscope, has been employed in several systems in attempts to examine the utilization of labeled amino acids for synthesis, passage or storage, and secretion of proteins (1-7). A general pattern seems to be emerging that is similar for the

cells of the pancreas (1-3), the thyroid (4), and connective tissues (5-8). In all of these different cell types the bound, labeled amino acid first appears in the ergastoplasm and later in the Golgi apparatus. Label then appears in the zymogen granules of the pancreas, prior to secretion, or in

found in vesicles or vacuoles and then in the extracellular space; *e.g.*, the colloid of the follicle, in the case of the thyroid (4) or the cartilaginous matrix or basement lamella, as in the case of the regenerating limb bud of *Amblystoma* (6, 7).

The ease of analysis of the pathway taken by amino acids observed by radioautography is in part dependent upon the type of cell involved. In the case of the pancreas, the acinar cell is polarized and contains a characteristic pattern of location of its organelles. Because of this, it has been possible to gain a great deal of information about this cell with the light microscope and radioautography (1, 9). The fibroblast, however, has a more dispersed and probably a more plastic orientation of its several organelles, and an analysis of the events taking place in this cell presents a more complex problem making the use of electron microscope radioautography necessary to resolve the structures.

The purpose of these studies was to examine the pattern of utilization of tritium-labeled proline by the various organelles and subcellular systems of fibroblasts actively synthesizing collagen in healing wounds. Because of the lack of an evident polarized arrangement of the organelles in the fibroblast, simple impressions cannot give accurate information concerning changes in concentration of label in any site with time. Hence, the determination of concentration of label within the several cellular compartments is necessary. Furthermore, observations of the change in concentration of label in each of these compartments permit a proper kinetic analysis of possible models that might describe the passage of the label through the cell. Such a set of observations is presented and their limitations are discussed.

MATERIALS AND METHODS

Tissue Preparation

Female guinea pigs weighing 230 to 300 gm were wounded under ether anesthesia. Six linear incisions, approximately 1 cm long, were made after removing the hair from the dorsal skin of each animal. The animals were fed a diet (Nutritional Biochemicals Corporation, Cleveland, Ohio) *ad libitum* containing amounts of ascorbic acid adequate to their daily requirements.

Seven days after wounding, each animal was given, by intraperitoneal injection, L-proline-3,4- H^3 (New England Nuclear Corporation Boston, Massachusetts) (specific activity 350 mc/mole) at a dose of 20

$\mu\text{c/gm}$ body weight. A wound was removed from each animal, as previously described (10, 11), at 15 and 30 minutes, 1, 2, 4, 8, and 24 hours after the administration of proline- H^3 .

The specimens of each wound were fixed for 1 hour at 0°C in 1.5 per cent glutaraldehyde, buffered with phosphate buffer, pH 7.4. The tissues were then placed in buffered 2 per cent osmium tetroxide for 1 hour, dehydrated, and subsequently infiltrated and embedded in epoxy resin (12).

TECHNIQUES USED FOR RADIOAUTOGRAPHY

Specimen Preparation and Application of Emulsion

Thin sections (approximately 800 to 1000 Å) were placed on carbon-coated copper grids and coated with Ilford L-4 emulsion with a wire loop, according to the method described by Caro and van Tubergen (13). The radioautographs were exposed for 10 weeks. They were developed in Microdol-X for 4 minutes at 20°C, washed, fixed in Kodak acid fixer for 2 minutes, washed, and stained with a modified form of lead tartrate (Millonig, reference 14). It is important to note that the sections were stained immediately after washing and were not allowed to dry.

Another series of sections from the same blocks were treated similarly, but were stained with 1.5 per cent aqueous uranyl acetate instead of the alkaline lead stain.

All the sections represented specimens from the center of each wound. This was accomplished by examining sample 1-micron sections from each block and trimming the block to provide only the central area.

Techniques Used for Counting the Radioautographs

Electron micrographs were randomly taken of each section from each block and the pictures were printed on 11 × 14 inch paper to facilitate counting. The cells and extracellular regions were divided into the following categories:

1. Nucleus
2. Rough endoplasmic reticulum (ergastoplasm)
3. Golgi complex
4. Mitochondria
5. Peripheral cytoplasm
6. Collagen
7. Extracellular space (space other than that occupied by collagen fibrils)
8. Miscellaneous (multivesicular bodies, dense bodies, etc.)

Each micrograph was counted twice. First, the per cent area occupied by each of these compartments was determined by a procedure like that used previ-

ously (11). This is a modified Chalkley procedure (15), somewhat similar to that proposed by Loud (16) for the quantitative estimation of cytoplasmic structures in electron micrographs. A grid, scribed upon a clear plastic sheet, the size of the micrograph, containing 450 intersections, was used to count the various structures and organelles. Each structure underlying every intersection was counted and the per cent area occupied by each of these was determined separately for each micrograph. Then the silver grains overlying each compartment in each micrograph were tabulated. In marginal cases, if $\frac{2}{3}$ or more of the grain lay over the organelle, the grain was counted as related to that organelle. In cases where the amount of grain over two structures was equal, a coin was flipped. The errors inherent in this

Determination of Availability of Proline- H^3 after Intraperitoneal Administration

Three female guinea pigs (250 gm) were used for the determination of availability of free proline- H^3 after its intraperitoneal administration. The common carotid artery of each animal was cannulated with a polyethylene cannula. L-proline-3,4- H^3 (500 μ c, specific activity 5000 mc/mmmole) was administered in combination with 1 mg of unlabeled proline. Two samples of blood (4 drops each) were removed via the cannula and placed in heparinized tubes at 5, 10, 15, 20, 30, and 45 minutes and at 1, 2, 3, 4, 5, and 6 hours after intraperitoneal administration of the proline- H^3 . Forty μ l of the blood was diluted with 1 ml of distilled water to which 1 ml of 10 per cent

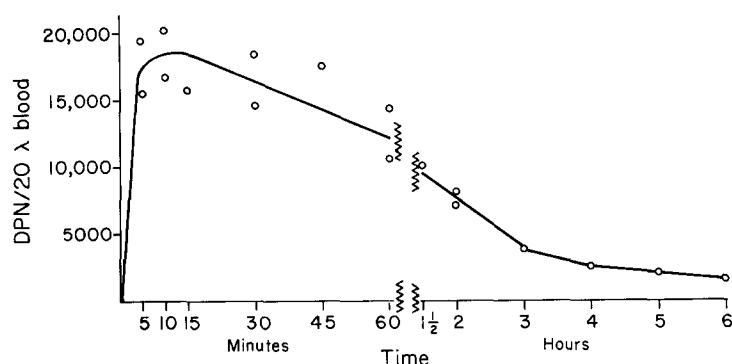


FIGURE 1 This graph demonstrates the levels of free proline, and proline present in di- and tri-peptides at various time intervals after the intraperitoneal administration of this amino acid. The values, presented from two experiments, are those for the supernatant, obtained following centrifugation of aliquots of whole blood combined with 10 per cent trichloroacetic acid, and represent the results with three different animals each of which received 2 μ c proline- H^3 per gm body weight.

approach limit the resolution of this technique (17-20), and will be discussed more fully later.

From these two counts the numbers of grains per unit area of organelle or collagen were then determined for each micrograph. The average grain count per unit area for each organelle or collagen, and the standard error of the mean for each of these, at each time period, were determined and compared (Figs. 2 and 3). The organelles to which particular attention was paid included the endoplasmic reticulum, Golgi complex, mitochondria, and peripheral cytoplasmic structures. The intracellular compartment labeled peripheral cytoplasm consists primarily of peripheral vesicles, regions where the ergastoplasm appears to contact the plasma membrane, caveolae, and peripheral aggregates of filaments. It was not always possible to be certain which of these structures lay under a silver grain and, therefore, they were grouped together into this category.

trichloroacetic acid was added. The tubes were centrifuged and 1 ml of the supernate was removed and placed in counting vials. The vials were air dried under a heat lamp, and 1 ml of hydroxide of hyamine was added to each vial. This was followed by the addition of 10 ml of scintillation mixture, and the vials were counted in a liquid scintillation counter (Packard Tricarb EX-314). The counts were corrected for background and for quenching by addition of an internal standard. The results are presented in DPM (disintegrations per minute) per 20 μ l of blood in Fig. 1.

OBSERVATIONS

Availability of Proline- H^3 after Intraperitoneal Injection

The level of free proline- H^3 rose to a maximum in the blood within ten minutes after the intra-

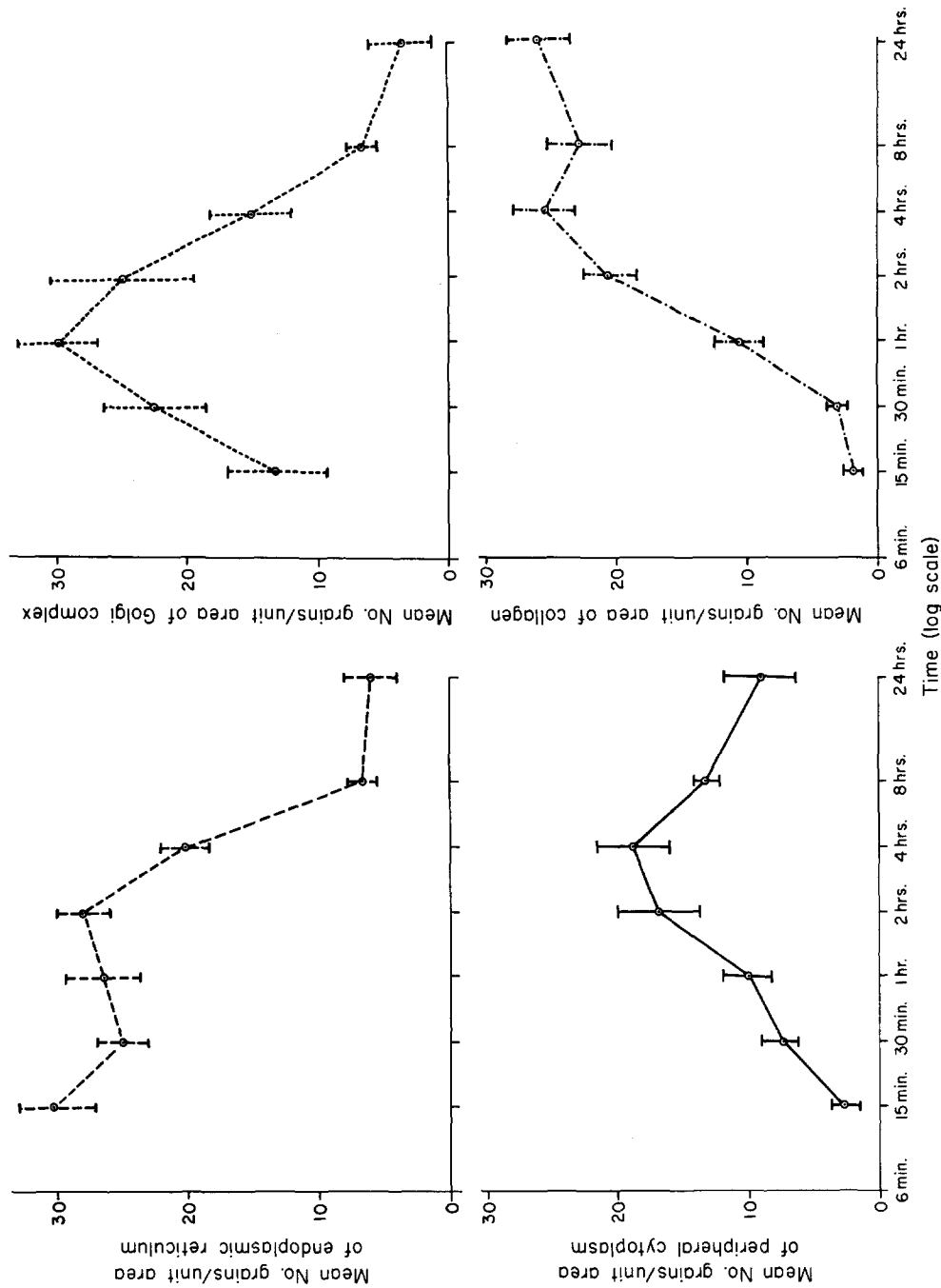


Figure 2 The four graphs demonstrate the change in concentration of activity of proline- H^3 (in mean number of grains per unit area) in the various organelles that were counted in the electron microscope radioautographs. The bars represent the standard error of the means. This is plotted on a semilog scale.

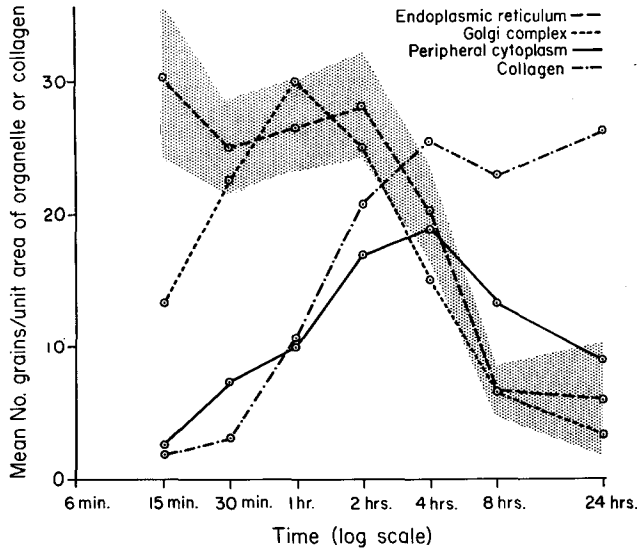


FIGURE 3 A composite of the graphs seen in Fig. 2. The shaded area represents the 95 per cent confidence limits of the values of the concentration of proline- H^3 in the endoplasmic reticulum for each of the various time periods. It can be seen that after 15 minutes there is overlapping of the values for the endoplasmic reticulum and the Golgi apparatus so that it is not possible to distinguish statistically between these two graphs.

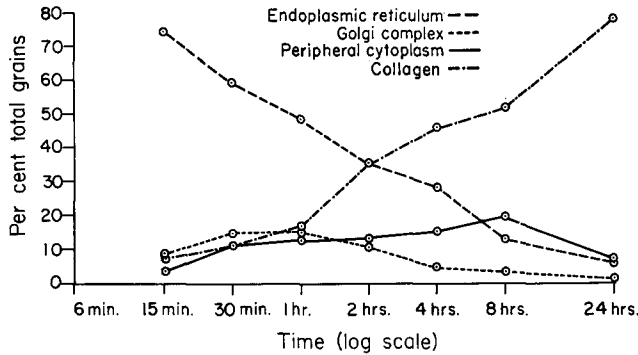


FIGURE 4 This graph contains the curve demonstrating the change in relative number of grains, measured as per cent of total grains, in the various organelles at each time period after giving proline- H^3 . This can be contrasted with the changes in concentration, and demonstrates that the concentration determinations more clearly depict the sequence of events as they occur in the cells.

peritoneal administration of this amino acid. It displayed the characteristic fall off and reached one-half of the maximum level 1½ hours after giving the isotope (Fig. 1).

Electron Microscope Radioautography

A review of the results of a quantitative analysis of the observations based on grains per unit area of organelle, followed by the results of relative grain counts, is presented below.

QUANTITATIVE OBSERVATIONS

Two types of analyses of grain counts were performed with this data. First, the concentration of grain counts (in grains/unit area) over each organelle at each time period was determined (Figs. 2 and 3). Second, the mean percentage of the total

grain counts over each organelle for each time period was plotted (Fig. 4).

The results of the concentration determinations are as follows:

1. *Endoplasmic reticulum* The concentration of label, in the ergastoplasm, had already reached a maximum within 15 minutes after intraperitoneal injection of the proline- H^3 . Beyond 15 minutes, the concentration remained approximately at its original maximum for 2 hours, after which it decreased. An area comprising the 95 per cent confidence limits for the values of the ergastoplasmic counts has been shaded in over the curve (Fig. 3). This was determined by multiplying the *P* values at the 5 per cent level by the standard error for each mean value.

2. *Golgi complex* The concentration of grains in the Golgi zone was less than half the maximum in the ergastoplasm at 15 minutes. It proceeded to rise and at 1 hour reached a maximum level equal to that seen in the endoplasmic reticulum at its maximum. After this the concentration of label in the Golgi zone decreased steadily. The time curve for the concentration of label in the Golgi region fell sooner from its peak than that in the ergastoplasm and preceded the curve for the ergastoplasm at all of the remaining time periods.
3. *Peripheral cytoplasm* The concentration of label in the peripheral cytoplasmic structures never reached as high a maximum as that of either the ergastoplasm or the Golgi complex; however, this compartment of the cell reached its peak label at 4 hours, and decreased thereafter to the end of the observations at 24 hours. The descending portion of the curve of concentration of label in the peripheral cytoplasm, after crossing the curves for both the ergastoplasm and the Golgi complex, remained significantly higher than that of both these curves.
4. *Collagen* The concentration of label in this morphological compartment was very small in the first 30 minutes. Thereafter it rose rapidly and achieved a maximum at 4 hours where it remained for the duration of the observations.

The results of the per cent determinations of the total grain counts for each compartment were similar to the impression gained from simple viewing of the radioautographs, and stand in contrast to the concentration determinations. They are presented graphically in Fig. 4 and are as follows:

1. *Endoplasmic reticulum* Fifteen minutes after the intraperitoneal administration of proline- H^3 the greatest percentage of silver grains appeared to be localized predominantly in relation to the ergastoplasm (Figs. 4 and 5). Ribosomes appeared to be

labeled in regions where tangential sections of the membranes of the ergastoplasm demonstrate the characteristic aggregates of ribosomes (21) (Figs. 5 and 6). A few developed silver grains were also present over some Golgi cisternae at this time. After 15 minutes the per cent of silver grains over the ergastoplasm decreased and continued to decrease throughout the remaining period of observation.

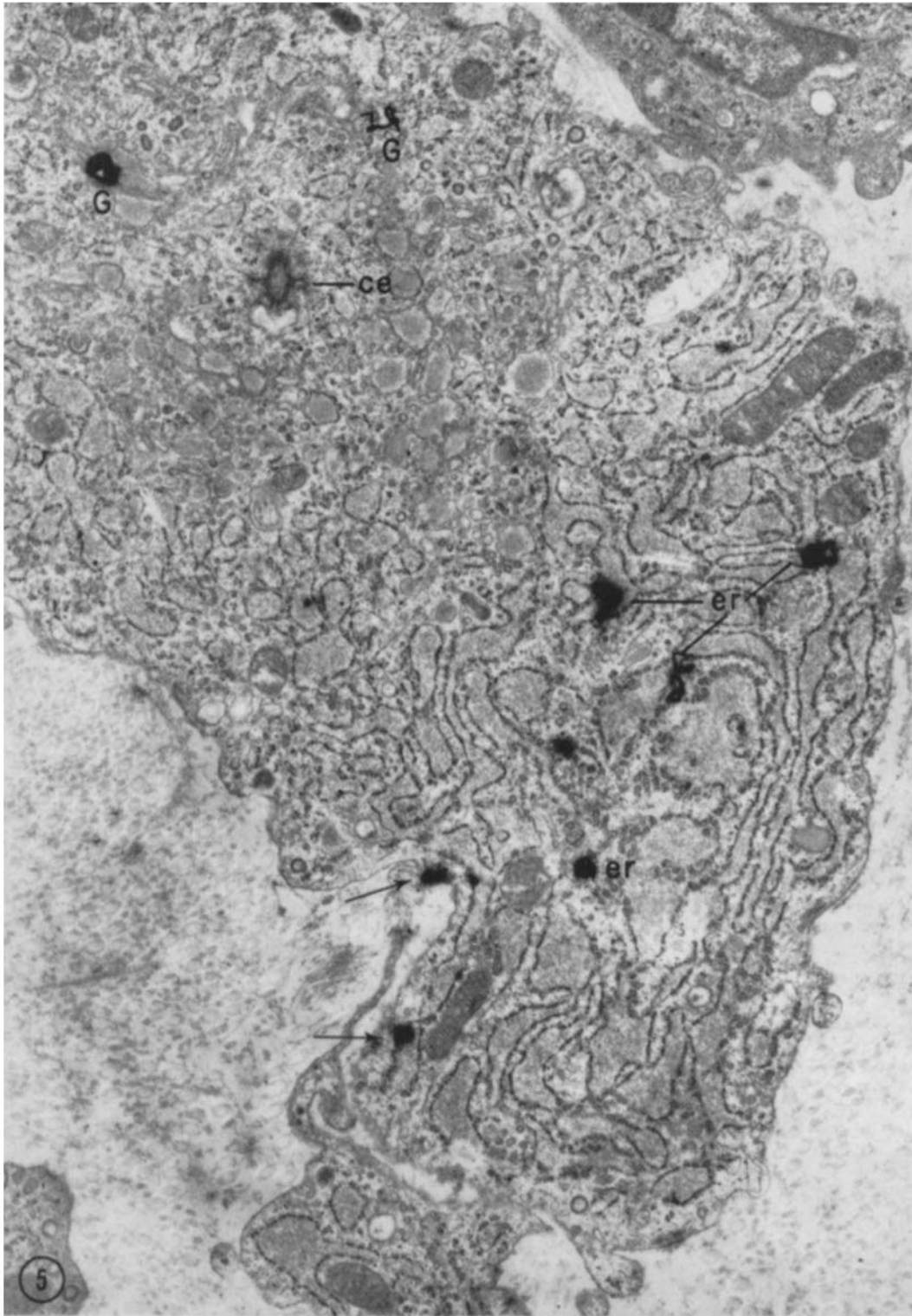
2. *Golgi Complex* The percentage of label over the Golgi vesicles and lamellae was much less than that in the ergastoplasm and rose to a maximum within 30 minutes (Figs. 4 and 6). After 1 hour the relative number of grains over the Golgi complex decreased. However, the Golgi complex contained only 14 per cent of the total grain count at its maximum, whereas the ergastoplasm contained 74 per cent of the total grains at its maximum (Fig. 4).

From 1 to 4 hours the relative content of label in the ergastoplasm and Golgi complex continued to decrease, although that of the ergastoplasm remained at a higher level than that of all the other cell compartments through this period (Figs. 4, and 7 to 9).

3. *Peripheral Cytoplasm and Collagen* From 1 to 4 hours there was an increase in the amount of isotope in both the peripheral cytoplasmic components of the wound fibroblasts and the extracellular collagen fibrils (Figs. 9 and 10).

Between 8 and 24 hours (Figs. 4, 11, and 12) the amount of isotope was decreased in all the cell compartments, although the last of these to lose the label appeared to be the peripheral cytoplasmic components whose maximum level reached only 19 per cent of the total. During this same period of time, the amount of label over the extracellular collagen increased and reached a maximum of 75 per cent.

FIGURE 5 Radioautograph of a portion of a fibroblast from a 7-day wound 15 minutes after the administration of proline- H^3 . The filamentous silver grains appear to be related to the ergastoplasm (*er*) and to the Golgi complex (*G*) in several areas of the cell. A centriole (*ce*) can be seen as well. The arrows point to two areas of increased density that do not represent silver grains. $\times 17,000$.



BACKGROUND

Fig. 13 demonstrates that the background (a fat cell) is negligible and is representative for all the radioautographs in this study.

FURTHER OBSERVATIONS OF THE ERGASTOPLASM

Most of the fibroblasts from the wounds contain regions where either the endoplasmic reticulum approximates the plasma membrane or where numerous vesicles and caveolae are located. A cell representative of this is illustrated in Fig. 14.

DISCUSSION

The present study is an extension of the previously reported light microscope investigation of proline- H^3 utilization by fibroblasts in healing wounds of guinea pigs (11). A comparison of the data from the two experiments is provided in Table I. The numerical values in the two experiments are different because of the differences in technical conditions. However, the time course of appearance and disappearance of label in cell cytoplasm and the rate of appearance and persistence of label in the extracellular collagen are quite similar in both studies.

The increased resolution of electron microscope radioautography has been used by several investigators (1-8) to permit a separation of the events occurring in the various cell organelles. For the pancreas, the data has been interpreted to indicate an obligatory passage of proteins synthesized in the ergastoplasm to the Golgi zone where "condensation" (2, 3) occurs, followed by migration as packaged granules, with temporary storage in the apical zone, followed by secretion into the lumen upon demand. Before proceeding with an analysis of the present data, it is appropriate to consider some of the general problems involved in the kinetic analysis of tracer data, and some of the special problems related to its use with radioautography.

Compartments, Specificity of Label, and Specific Activity

In any tracer study, we are dealing with movement of material between compartments. Solomon

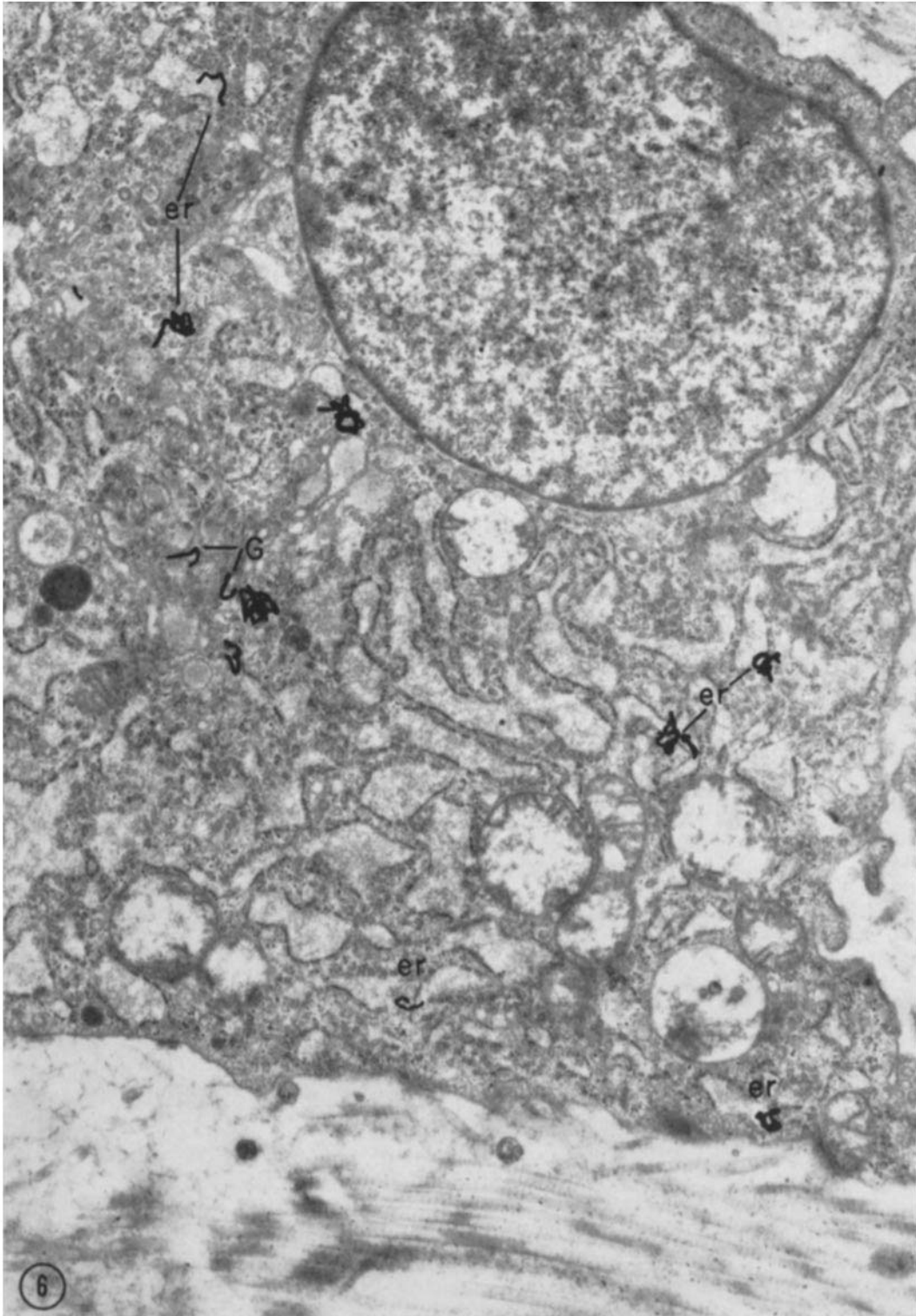
(38) points out that a compartment may be considered in the literal sense as representing a cell or any other space bounded by a membrane, or in the imaginary sense as representing reactants or products of a chemical reaction. In the present study, we are concerned with compartments in the form of organelles or subcellular systems.

Although the electron microscope has the virtue of being able to resolve the fine details of these subcellular compartments, the radioautographic procedure limits the total technique to substantially less than the maximum resolving power of the microscope. The resolution obtainable with the Ilford L-4 emulsion has been demonstrated by Caro (17) to be approximately 0.2 to 0.3 μ under the conditions used in these studies. Pelc (18), Bachmann and Salpeter (20), and Caro (17) have pointed out that the limits of resolution of any particular fine grain emulsion are related to (a) the geometric relation between the section and the emulsion, and their respective thicknesses, (b) the grain size, (c) grain dispersion, and (d) the relation of the developed filament to the original silver halide crystal within the emulsion after it has been applied.

The developed grains in the Ilford L-4 emulsion, after Microdol-X development, are filamentous and irregular in shape. It is not possible to know what relationship the latent image, that preceded this filament, has to the point source of the emitted beta particle, and, therefore, the relationship of the developed, fixed filamentous grain to the point source is dependent upon the factors listed above (20). Newer emulsions with silver grains between 0.3 and 0.7 μ offer the opportunity of some improvement in resolution (25, 26).

It is possible to calculate the mean error inherent in the use of this emulsion if certain assumptions are made. Bachmann and Salpeter (20) have demonstrated that the contribution of the photographic error inherent in emulsions is related to the grain size, the filament size, and the relation of these to each other. Assuming a filament of twice the diameter of the undeveloped silver bromide crystal (0.14 μ for Ilford L-4), this would lead to a photographic contribution to the

FIGURE 6 Radioautograph of a portion of a 7-day wound 30 minutes after the intraperitoneal administration of proline- H^3 . In this particular cell the silver grains can be seen to lie over both ergastoplasm (*er*) and Golgi complex (*G*). The collagen fibrils in this area are not labeled. $\times 17,000$.



error of about 0.15μ for Ilford L-4 emulsion. The geometric error, as stated previously, is related to section and emulsion thicknesses. Assuming a total thickness of 2000 \AA for these two, and a solid angle that would include $\frac{2}{3}$ of all of the electrons emitted from the source of radiation, the mean geometric error is calculated to be 0.24μ . The combined error contributed by these two sources represents the square root of the sums of their squares and is equal to 0.26μ , as a total mean error in any given direction. A circle with a radius of 0.26μ would, therefore, encompass a zone of possible source of beta particles located at a maximum distance from the developed silver grain. The probable error is not this great, as most of the sources of beta emission would be within, rather than at, the periphery of such a zone. (For the rationale of such calculations, see Bachmann and Salpeter, reference 20.)

Fortunately, in most instances, the structures being studied are of sufficiently large size so that it is not difficult to relate them to a particular silver grain. However, this problem is more difficult in the case of the peripheral cytoplasmic structures that range from 0.1 to 0.8μ in diameter. It is clear that grains are related to the periphery of the fibroblast, although it is not possible to be certain that they are related to a specific organelle.

It is, of course, impossible to state whether small structures such as ribosomes, membranes, etc. are specifically related to the reduced developed silver grains. As has been stated, this is generally not a problem with respect to the ergastoplasm and the Golgi complex, and it is probable that grains located over cisternae, but close to the associated membranes, represent labeled material within the lumens of these structures.

Ideally we should like to be able to follow the pathway taken by collagen or its precursors through the fibroblast, and to separate this from other entities made by the fibroblast for internal use, as well as for export. Unfortunately, there is no means to do this at present. We have chosen to use labeled proline as the tracer because of the abundance in collagen of both proline and its

product, hydroxyproline. Proline is not a specific label since it is present in concentrations of 5 per cent in many proteins, and of as much as 9 per cent in some proteins, associated with connective tissue (39). Consequently, the most one can expect to follow is the pattern of labeling of the most abundant products or macromolecules containing proline.

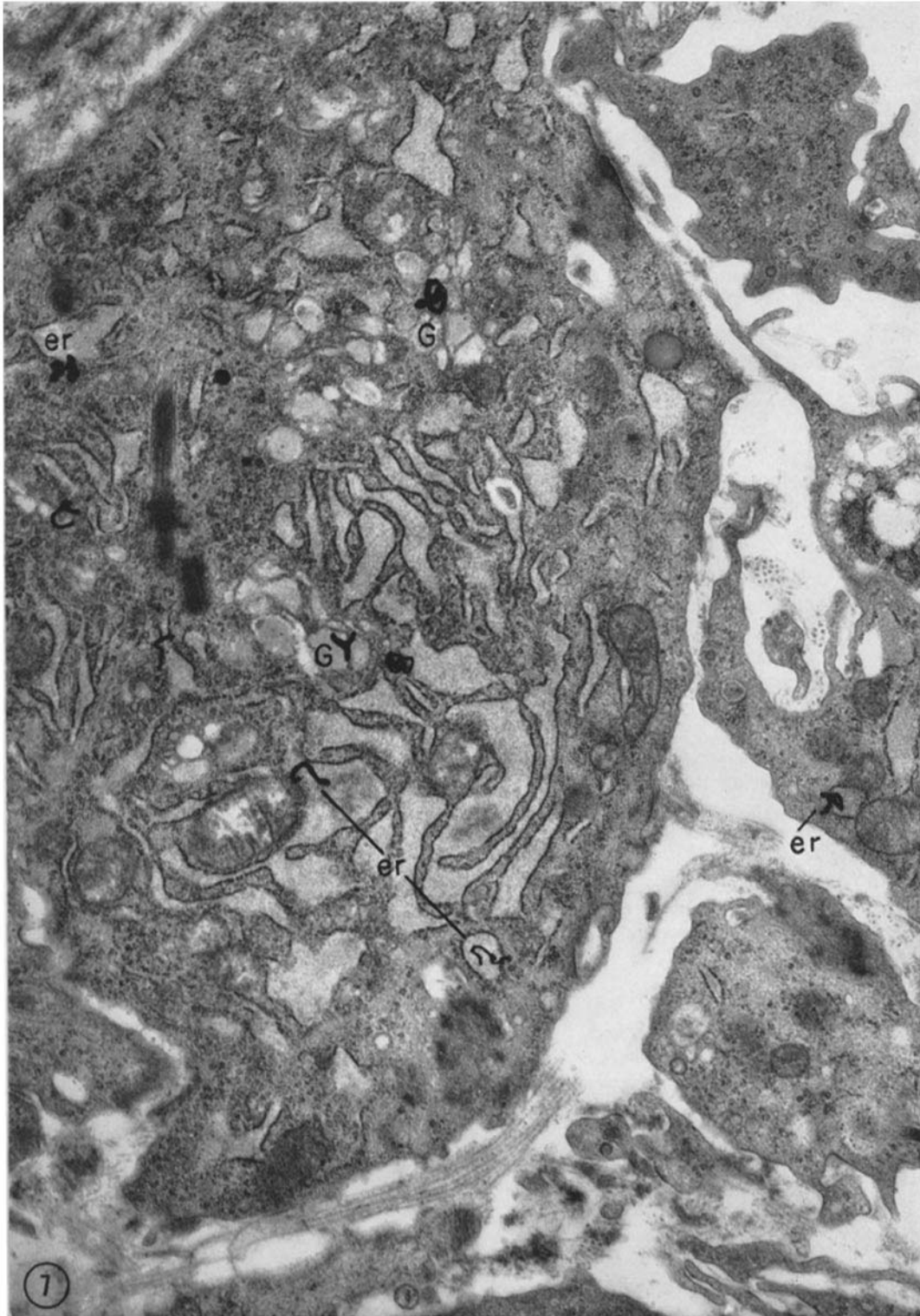
The usual method employed in tracer studies of biochemical systems is "specific activity," defined as a mole fraction or other proportional quantity per unit mass of substance (40). However, analysis is not restricted to this, but can also be in terms of total amount of label or concentration of label, whichever is appropriate (38, 40). Estimates of specific activity have been attempted in several light microscope studies (9, 41, 42); however, no practical method is presently available for measuring the concentration of proteins or other substances in the electron microscope.

The combination of these factors limits us to the third of the three useful measures for kinetic studies, namely concentration of label. This is not a serious constraint, however, since it still permits use of the already developed types of analysis worked out for other studies of compartment flow using tracers.

Kinetic Analysis

The early radioautographic studies of metabolic constituents relied on the simple observation of relative intensity of label as indicated by impressions of grain density over a particular cell or constituent. From these impressions inferences were drawn about possible sequential relationships. An improvement of this method consisted of grain counts over organelles that were then analyzed in terms of per cent of total grains attributable to a given organelle. The shift in per cent of label with time was interpreted as showing movement from one compartment to another (2, 22). It is interesting to note that no rationale has yet been given for this type of analysis. Examination of this scheme of analysis indicates that it has no single simple interpretation. Under certain cir-

FIGURE 7 Portions of several cells from a 7-day wound 1 hour after the administration of proline- H^3 . In the largest cell, most of the label can be seen to be located over the rough endoplasmic reticulum (*er*) and Golgi complex (*G*). Two centrioles can be seen in this cell, one of which appears adjacent to a ciliary projection. Single cilia are not uncommon in these cells. $\times 17,000$.



cumstances, it can be analyzed in conventional kinetic terms. Thus, if during the period of analysis the total activity remains constant, then the relative proportion of grain counts for each compartment could be taken as a measure of the quantity of label in the compartment. On the other hand, if the total activity is changing, as is usually the case, then a simple analysis does not pertain, and the conditions applicable to this must be developed. To date the simplest interpretation has been applied without qualifications (2, 22). In this instance the implicit assumption has been made that the order in which the level of label in the given compartment becomes dominant is the order of transfer. While this may be true, it is not necessarily the case. Thus if we consider two compartments, a and b, operating independently of each other and each labeling at its own rate, on a chance basis a will be labeled faster than b 50 per cent of the time, and vice versa. With three compartments, the statistics improve. Thus any given order of labeling on a chance basis will appear only one-sixth of the time.

The use of the measure of concentration, as employed here, provides data directly amenable to the more usual way of viewing tracer kinetics. With these data one can ask whether there is conformation to some particular conceptual model, and utilize, with appropriate provisos, the available kinetic formulation (38, 40). Zilversmit *et al.* (43) made the early attempts at evaluation of precursor-product relationship by tracer kinetic analysis. Others have modified and extended this. Providing certain initial assumptions are fulfilled one can use these formulations to make presumptive or probable statements about systems in which appropriate measurements can be made (40). The assumptions in the original formulation (43) are that (a) the amount of material present in the system is constant during the interval over which the measurements are made, (b) the rate of flux between the compartments is constant during the time interval of measurement, and (c) molecules are treated randomly.

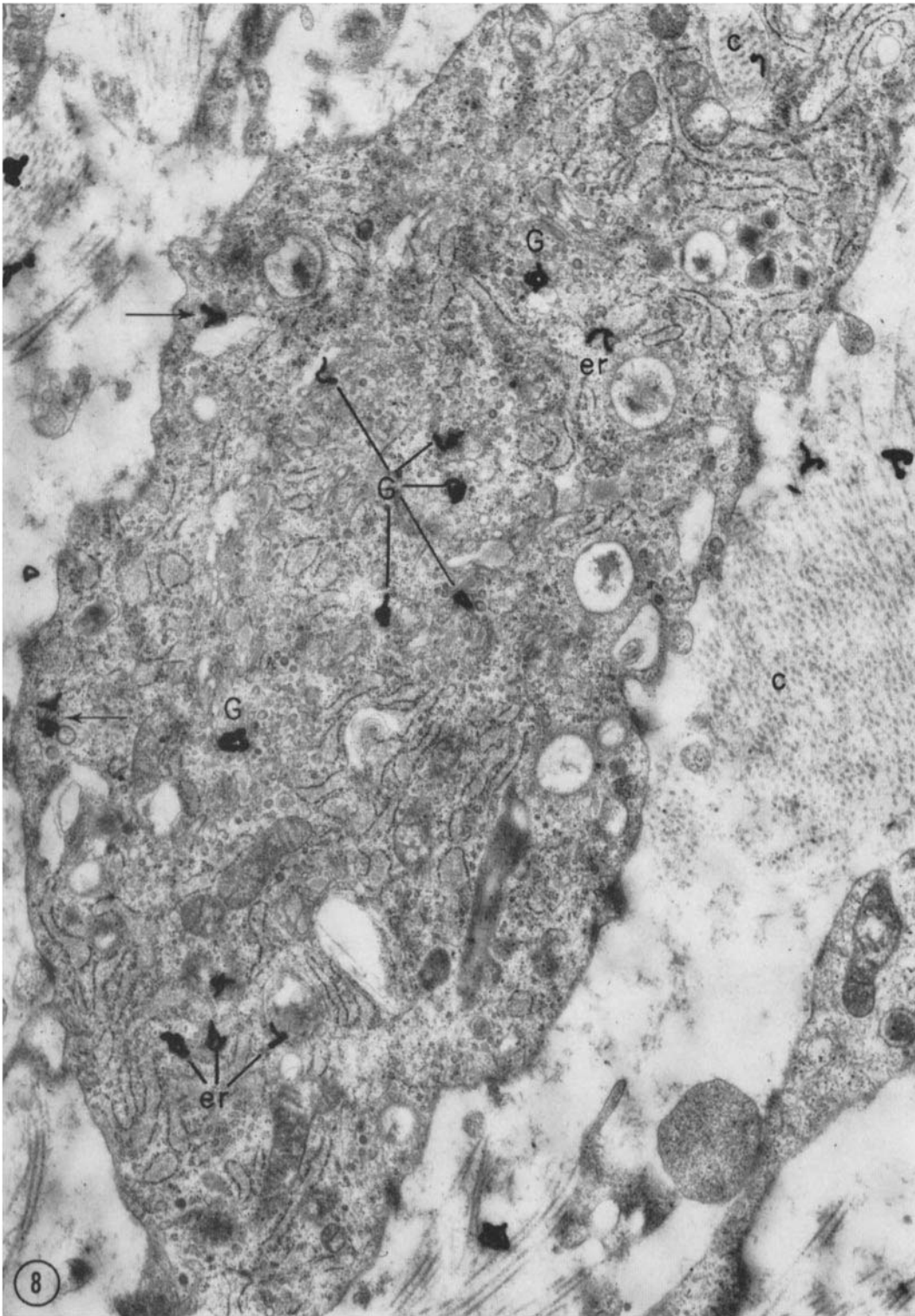
Proline Utilization by the Fibroblast

For the short period of the observations in the present experiment (only the first 8 hours need to be considered), it seems reasonable to assume that these conditions are fulfilled. This being so, the relationship we might expect to find between each of the compartments serving as a precursor for the following compartment is as follows:

1. The general form of the activity-concentration curve for each of the compartments might be expected to be similar. Thus with the curve of clearance of free proline from the blood having the form seen in Fig. 1, each curve should rise smoothly to a maximum and, after reaching the maximum, fall smoothly through the decay.
2. Any compartment that accepts its materials entirely from another compartment (38) would follow its precursor by rising to its maximum later, intersecting at its maximum with the curve for the precursor compartment, and finally, falling, but at a level of activity that exceeds that of the precursor compartment.

It can be seen that the curves in the present study do not neatly conform to this pattern. The curve for the ergastoplasm has reached its maximum at the first time-point examined. However, instead of falling smoothly away from the maximum, the curve shows a prolonged plateau that extends for 2 hours after injecting the label. The curve for the Golgi complex has a form more nearly fitting expectation. It does rise smoothly to its maximum and thereafter falls smoothly away. Moreover, it does two things not usually expected of a product curve as it relates to its precursor; namely, it reaches the same level of maximum concentration as that of the ergastoplasm, and it appears to fall faster (or at least at an equal rate in terms of the errors) than that of the supposed precursor. On the other hand, the relationship between the curves for the Golgi zone and the peripheral cytoplasm show better conformation to the pattern expected for a precursor-product relation-

FIGURE 8 Radioautograph 2 hours after administration of proline- H^3 . At this time period several silver grains can be seen to be located over extracellular collagen fibrils (c). The cell contains numerous silver grains over the Golgi complex (G) and in relation to the rough endoplasmic reticulum (er). Several filamentous silver grains can also be seen over peripheral cytoplasmic elements (arrows). $\times 13,000$.



ship. The curve for the concentration of activity in collagen is again anomalous. It rises after 30 minutes and rapidly exceeds the level of that for the peripheral cytoplasm at its peak. These differences are statistically significant, as can be seen from the standard errors shown in Fig. 2. Thus, regarding precursor-product pairs, all the appropriate conditions being met, these curves might suggest that the ergastoplasmic cisternae and the collagen fit together as one precursor-product pair, and the Golgi complex and the peripheral cytoplasm as another.

It is clear from the preliminary discussion, as well as the examination of the data, that no watertight argument can be set forth for accepting one or the other of the following two possible interpretations: The pathway followed by proline-labeled material is, as has been suggested, from ergastoplasm to Golgi complex to peripheral cytoplasm to collagen, although some necessary conditions for fitting this simple postulated relationship have not been met. The alternative is that the postulated schema does not pertain to the fibroblast and that a different relationship exists.

If we consider the first alternative, several possible sources of error could arise: (a) The "pulse" of label was long, perhaps longer than desirable for resolution of the curves for the Golgi complex and the ergastoplasm. (b) The dispersion of the grain counts and the area estimates, though reasonably small for this type of assay, adds to the lack of resolution. (c) This is further extended by the loss in resolution of grains in relation to organelles, as pointed out earlier. However, this should have least effect in relation to the ergastoplasm and Golgi complex because of their size. (d) The size of the Golgi compartment is significantly less than that of the ergastoplasm (Table II). Should all the material from the ergastoplasmic cisternae have to pass through the Golgi complex, then the transit time would be much less for this compartment than for the larger compartment (40). This would bring the curve for the Golgi complex much closer to that for the ergastoplasmic cisternae, particularly on the decay sides of the curves. The problem

of resolution would then become much more exacting.

In considering the alternative possibility, that more than one pathway exists for materials synthesized in the ergastoplasm, the following pertains: The prolonged plateau and apparently delayed decrease in concentration of label in the cisternae is compatible with the presence of synthesis of several proteins in the endoplasmic reticulum at different rates. Some of these may then be passed to the Golgi complex, and some may be dispersed elsewhere inside or outside the cell. A similar phenomenon is evident in the curves of Warshawsky *et al.* (9) and is commented upon by Nadler (44) in his analysis of the turnover rate. He attributes this to the synthesis of "sedentary" versus "exportable" protein.

While one cannot decide, on the basis of present evidence, between the alternatives, there are four pieces of evidence that argue for the possibility that two pathways for protein exist in the fibroblast:

1. Fibroblasts are known to synthesize and secrete not only collagen but also mucopolysaccharides (35, 37, 45). Other proteins may also be secreted. In the skin it has been found that the turnover of protein in polysaccharides proceeds at the same rate as that for the mucopolysaccharides (45). Furthermore the rate of turnover for the mucopolysaccharides is of the order of 1 to 2 weeks, which is much faster than the usual rate of collagen turnover (35, 36), although it may not be faster than collagen formation in wounds. The principal point, however, is that there are two quite different systems containing protein produced and secreted by fibroblasts into the extracellular spaces.
2. Carbohydrate synthesis has been demonstrated in the Golgi complex of intestinal cells (23). From the work of Godman and Lane (22) and Fewer *et al.* (24) it appears that sulfate incorporation bypasses the ergastoplasm, as indicated by the initial

FIGURE 9 Radioautograph from a portion of a 7-day wound, 4 hours after the administration of proline- H^3 . Several of the fibroblasts demonstrate developed silver grains over the rough endoplasmic reticulum (*er*) and peripheral cytoplasmic elements (arrows). A fairly large amount of label is also seen over the extracellular collagen fibrils at this time. $\times 14,000$.

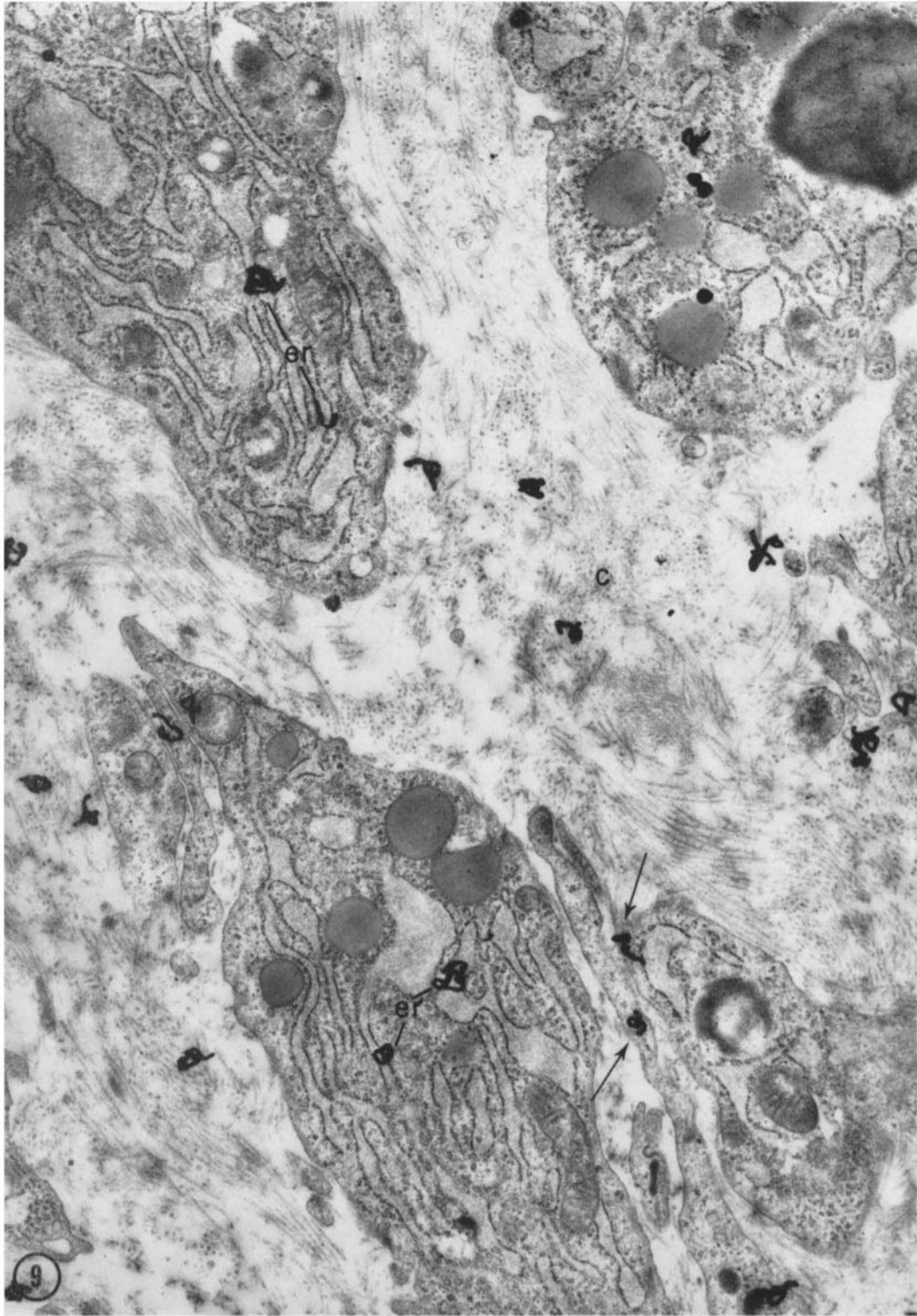


TABLE I
Comparison of Light* and Electron Microscope
Radioautographic Data on Wound Fibroblasts
and Collagen

Time after proline-H ³ administration	Cells		Collagen	
	Light Mi- croscopy*	Electron Micros- copy	Light Mi- croscopy*	Electron Micros- copy
	<i>grains/unit area</i>			
15 min.	—	49	—	2
30 min.	550	53	100	3
1 hr.	740	66	240	11
2 hrs.	—	70	—	21
4 hrs.	700	54	340	25
8 hrs.	—	26	—	23
1 day	310	18	440	26
3 days	320	—	510	—
7 days	150	—	300	—

* Derived from Ross and Benditt (11).

TABLE II
The Area in the Fibroblasts occupied by Organelles
as Determined by the Modified
Chalkley Procedure

	per cent area and sd
Endoplasmic reticulum	33 ± 4
Golgi complex	9 ± 2
Peripheral cytoplasm	20 ± 4

and rapid appearance of sulfate in the Golgi complex and subsequently in the peripheral cytoplasmic vesicles. It could be that the absence of evident label is due to a small amount of material rapidly turning over in the ergastoplasm or other part of the cell. However, if the evidence is taken as presented, then the chondroblast may well be showing the obligatory pathway for mucopolysaccharides, through the Golgi complex, where they are attached to proteins previously synthesized in the endoplasmic reticulum (22, 24, 27).

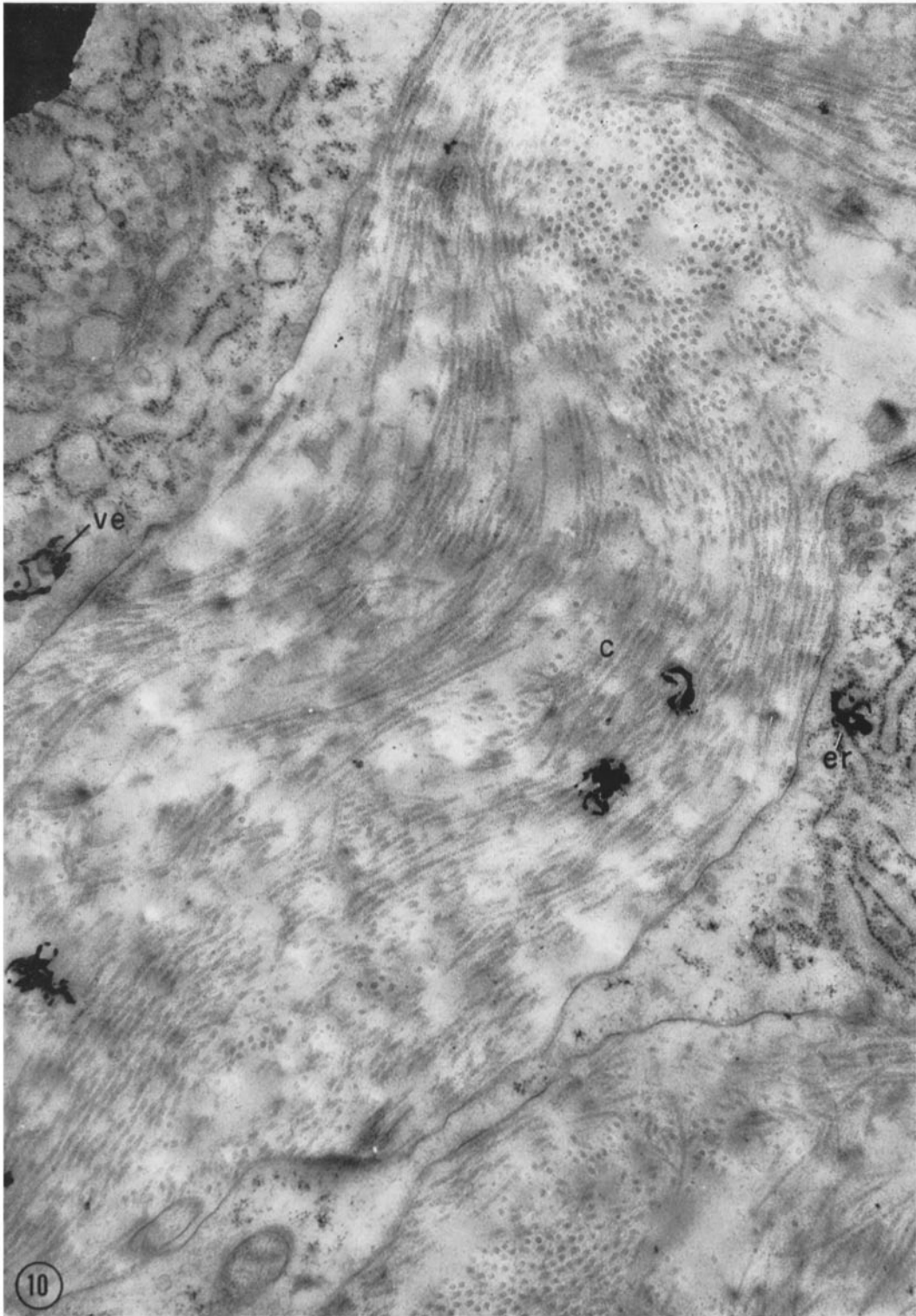
- Other evidence comes from previously reported studies of the passage of proline through the fibroblast in ascorbic acid-deficient guinea pigs (11). In those investigations it was noted that proline, given under the same circumstances as in the present study, was taken up at a moderately reduced rate when compared with the controls. The concentrations of label achieved by the cells were similar. This is curious, since collagen synthesis is very low or absent under these circumstances, as measured by hydroxyproline formation (46), and as seen by histochemical examination and electron microscopy (21).
- Finally, the presence of the apparent contact between the ergastoplasmic cisternae and the plasma membrane (Fig. 14) presents a possible pathway for protein within endoplasmic reticulum to leave the cell. Moreover, such a mechanism would be compatible with the relationship seen between the curves for concentration of label in the ergastoplasm and the collagen.

The Final Stage of Collagen Secretion by the Fibroblast

Two fundamentally different types of secretory activity have been proposed for the final stage of collagen secretion by fibroblasts. These are (a) merocrine secretory activity, and (b) apocrine secretion in the form of ecdysis (shedding of the peripheral cytoplasm), or possibly by the fusion of small vesicles under a cytoplasmic bleb (30).

Several investigators have shown regions in cells where fine filaments appear to pass directly out of the cell (29-31). However, in both the present and previous investigations (10, 11, 21) the plasma membranes of all cells are intact, and zones such as these are interpreted as regions of tangential sectioning of the cell surface. Goldberg and Green (32) have definitively shown that this is the case in their collagen-producing strain of tissue culture fibroblasts, by sectioning the cells in different planes.

FIGURE 10 Radioautograph from another area of a 7-day wound 4 hours after the administration of labeled proline. In this particular area the collagen fibrils (c) are labeled. In addition, peripheral vesicles (ve) in one cell, and the endoplasmic reticulum (er) in another cell can both be seen to have developed silver grains overlying them at this time period. × 20,000.



Stearns (33, 34) and Porter (30) suggest that cytoplasmic extrusions are pinched off, disintegrate, and become foci of fibril development. We have observed similar extrusions in wound fibroblasts, but have not seen any labeling of these cytoplasmic projections. Although these may be possible sites of collagen deposition, our observations do not support this contention.

As a result of the radioautographic studies, we suggest the possibility that collagen is released from the cells in a form of merocrine secretion, perhaps by direct, intermittent communication of the ergastoplasmic cisternae with the extracellular regions. Consistent with this possibility, in a large number of micrographs of 7-day wounds were seen regions where the endoplasmic reticulum approximates, or appears to merge with, the plasma membrane (Fig. 14). These sites were pointed out also in an earlier study of wounds (21), as well as by Karrer (28) in studies of the developing chick embryo aorta. Although frequent, these areas are not seen in every cell in each micrograph. In addition, many regions are present where a string of vesicles is located between ergastoplasmic cisternal profiles and the plasma membrane (Fig. 14). Both of these could be interpreted as possible sites

of communication between the ergastoplasm and the extracellular regions where material from the cisternae could pass directly out of the cell.

Taken together, the evidence presently available points to the need for further search for pathways of formation and secretion of proteins in different cell types. The evidence for the pancreas, which was derived from chemical, morphological, and cytochemical data, seems reasonably secure, but the evidence for other cells, including the fibroblast, is both circumstantial and incomplete.

A preliminary report of this study was made at the Second Annual Meeting of the American Society for Cell Biology held in San Francisco in 1962.

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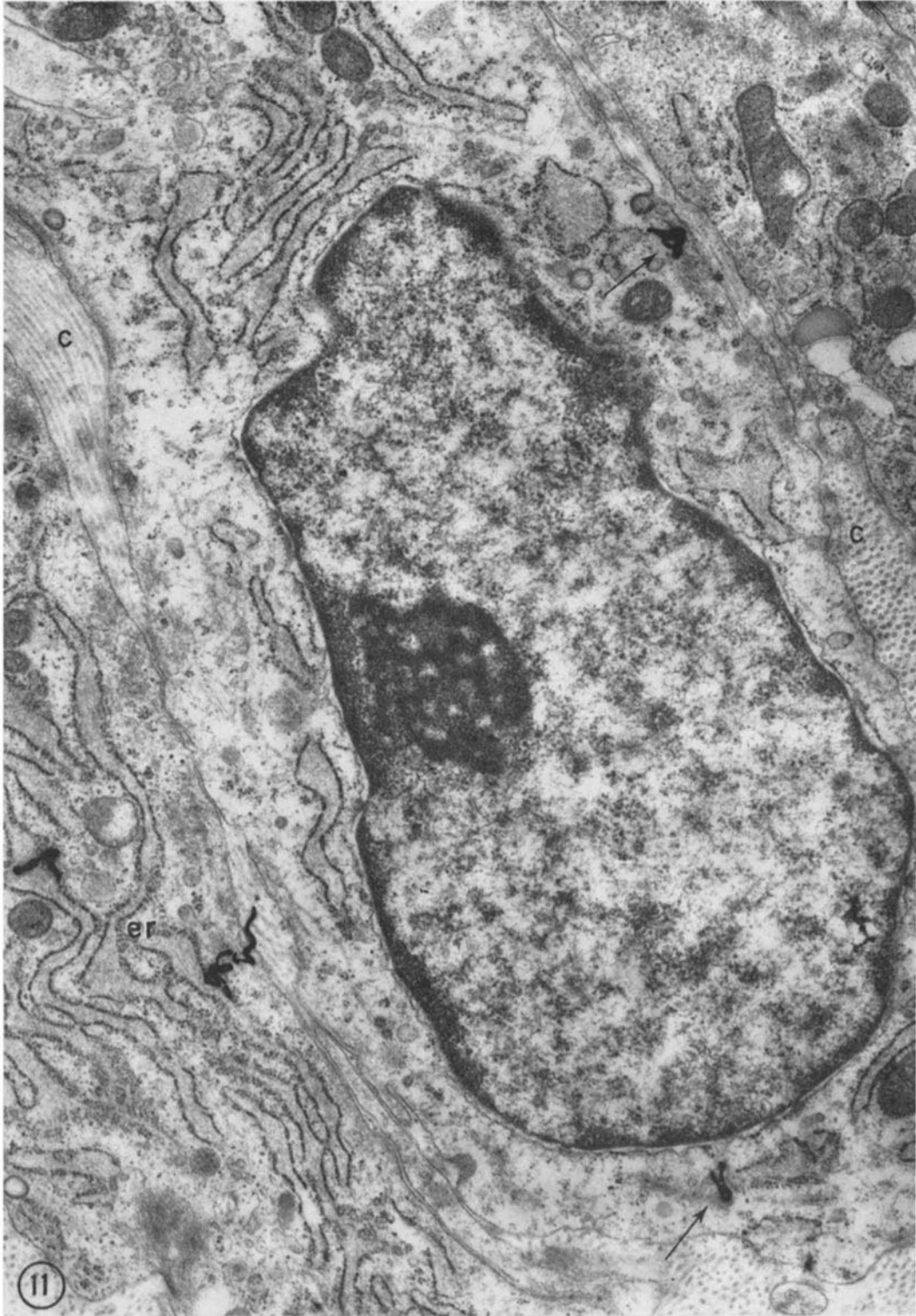
The authors would like to acknowledge the technical assistance of Mrs. Dawn Bockus, Miss Franque Remington, and Miss Leslie Caldwell. In addition, they would like to thank Dr. E. A. Smuckler for advice in the studies of blood clearance of free amino acid. We are indebted to Dr. Arnold Tamarin, Dr. Miriam Salpeter, and Dr. N. B. Everett for discussions of the manuscript.

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BIBLIOGRAPHY

1. CARO, L. G., Electron microscopic radioautography of this sections: the Golgi zone as a site of protein concentration in pancreatic acinar cells, *J. Biophysic. and Biochem. Cytol.*, 1961, **10**, 37.
2. CARO, L. G., and PALADE, G. E., Protein synthesis, storage, and discharge in the pancreatic exocrine cell. An autoradiographic study, *J. Cell Biol.*, 1964, **20**, 473.
3. VAN HEYNINGEN, H. E., Secretion of protein by the acinar cells of the rat pancreas, as studied by electron microscope autoradiography, *Anat. Rec.*, 1964, **148**, 485.
4. NADLER, N. J., YOUNG, B. A., LEBLOND, C. P., and MITMAKER, B., Elaboration of thyroglobulin in the thyroid follicle, *Endocrinology*, 1964, **74**, 333.
5. ROSS, R., Collagen formation in healing wounds, in *Advances in Biology of Skin: Wound Healing*, (W. Montagna, and R. E. Billingham, editors), Pergamon Press, London, 1964, 144.
6. REVEL, J. P., and HAY, E. D., An autoradiographic and electron microscopic study of collagen synthesis in differentiating cartilage, *Z. Zellforsch. u. Mikr. Anat.*, 1963, **61**, 110.
7. HAY, E. D., and REVEL, J. P., Autoradiographic studies of the origin of the basement lamella in *amblystoma*, *Develop. Biol.*, 1963, **7**, 152.
8. ROSS, R., and BENDITT, E. P., A comparison of the utilization of proline- H^3 in healing wounds

FIGURE 11 Micrograph of part of a 7-day wound, 8 hours after the administration of the amino acid. At this time, in these particular cells, the endoplasmic reticulum (*er*) and peripheral cytoplasmic elements (arrows) can be seen to be labeled. There is also a silver grain lying at the border between a cell and the extracellular collagen fibrils (*c*). It is not possible to determine whether this particular grain represents intra- or extracellular label. $\times 22,000$.



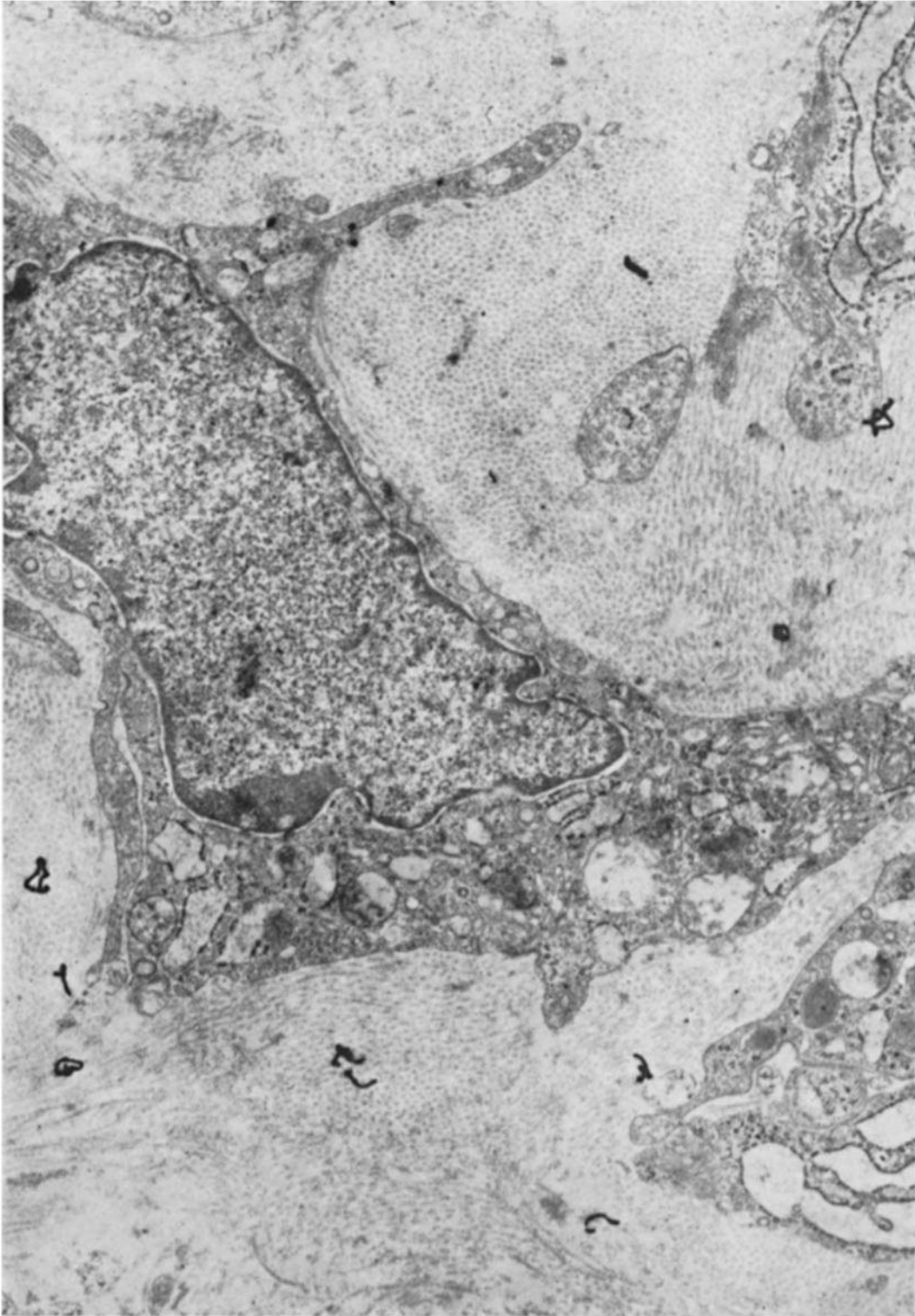


FIGURE 12 Radioautograph from a part of a 7-day wound 24 hours after the administration of proline- H^3 . In this particular area the isotope appears to be entirely extracellular. $\times 14,000$.

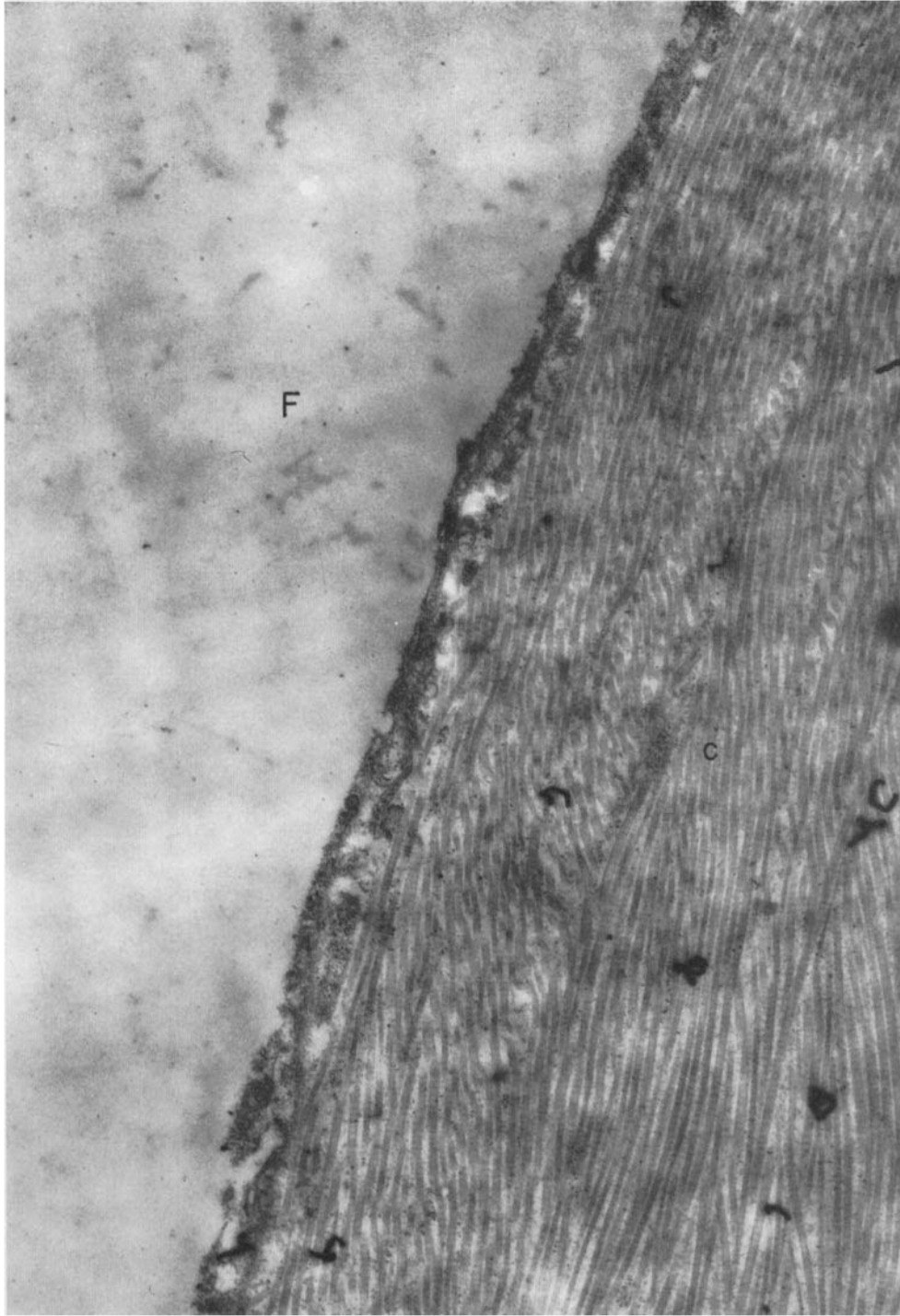
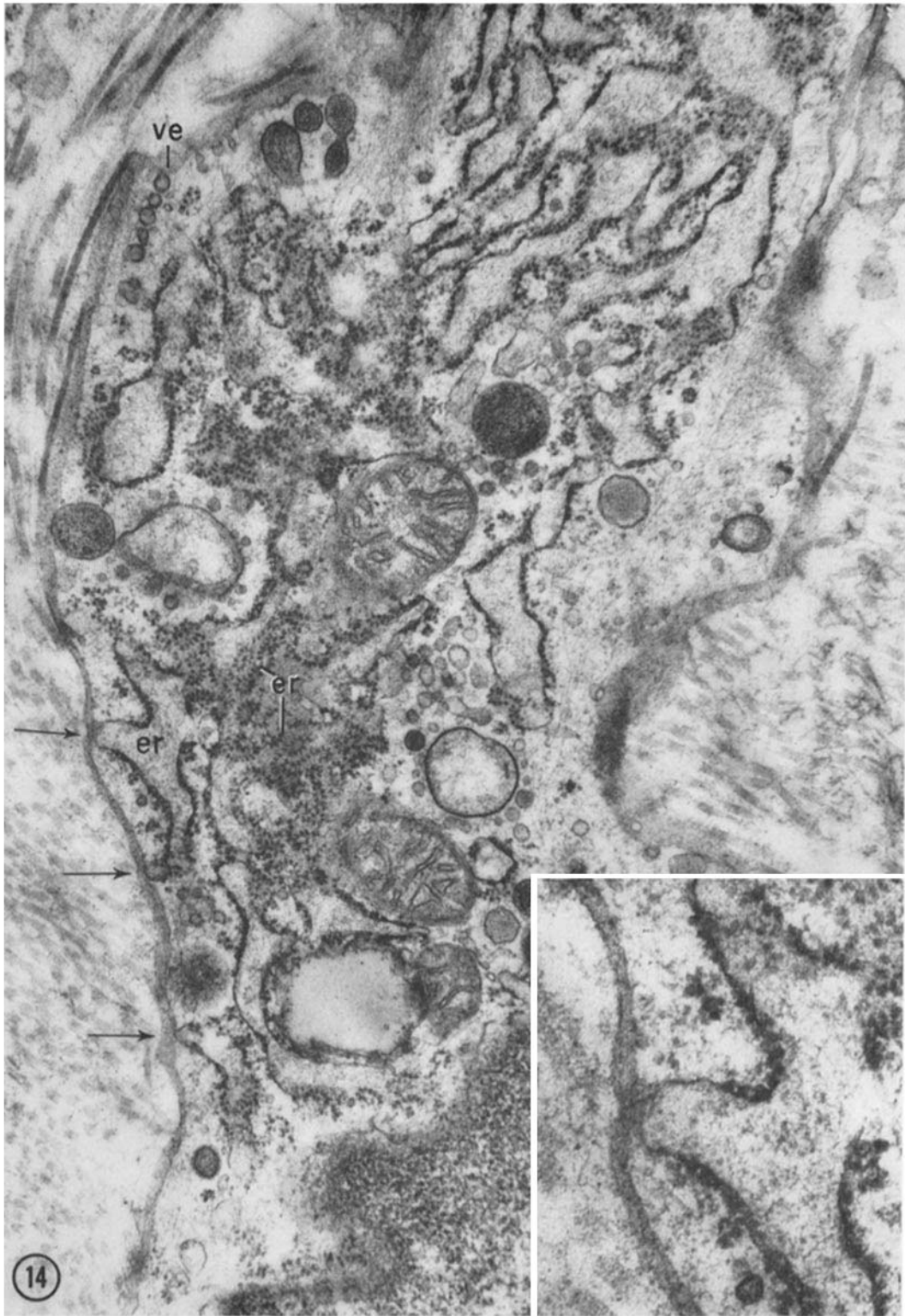


FIGURE 13 A portion of a 7-day wound, 4 hours after the administration of isotope. In this particular area collagen fibrils (*c*) are seen adjacent to a large fat cell (*F*). The collagen fibrils are labeled, whereas the fat contains no isotope. This micrograph demonstrates the relatively low background that is a feature of these electron microscope radioautographs. $\times 15,000$.

- as seen by electron microscope autoradiography, *Fed. Proc.*, 1963, **22**, 190.
9. WARSHAWSKY, H., LEBLOND, C. P., and DROZ, B., Synthesis and migration of proteins in the cells of the exocrine pancreas as revealed by specific activity determination from radioautographs, *J. Cell Biol.*, 1963, **16**, 1.
 10. ROSS, R., and BENDITT, E. P., Wound healing and collagen formation. I. Sequential changes in components of guinea pig skin wounds observed in the electron microscope, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 677.
 11. ROSS, R., and BENDITT, E. P., Wound healing and collagen formation. III. A quantitative radioautographic study of the utilization of proline- H^3 in wounds from normal and scorbutic guinea pigs, *J. Cell Biol.*, 1962, **15**, 99.
 12. LUFT, J. H., Improvements in epoxy embedding methods, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 409.
 13. CARO, L. G., and VAN TUBERGEN, R. P., High resolution autoradiography. I. Methods, *J. Cell Biol.*, 1962, **15**, 173.
 14. MILLONIG, G., A modified procedure for lead staining of thin sections, *J. Biophysic. and Biochem. Cytol.*, 1961, **11**, 736.
 15. CHALKLEY, H. W., CORNFIELD, J., and PARK, H. A., A method for estimating volume-surface ratios, *Science*, 1949, **110**, 295.
 16. LOUD, A. V., A method for quantitative estimation of cytoplasmic structures, *J. Cell Biol.*, 1962, **15**, 481.
 17. CARO, L. G., High resolution autoradiography. II. The problem of resolution, *J. Cell Biol.*, 1962, **15**, 189.
 18. PELC, S. R., Theory of electron autoradiography, *J. Roy. Micr. Soc.*, 1963, **81**, 131.
 19. GRANBOULAN, P., Resolving power and sensitivity of a new emulsion in electron microscope autoradiography, *J. Roy. Micr. Soc.*, 1963, **81**, 165.
 20. BACHMANN, L., and SALPETER, M. M., Autoradiography with the electron microscope. A quantitative evaluation in "Symposium on quantitative electron microscopy." (G. Bahr, editor), in press.
 21. ROSS, R., and BENDITT, E. P., Wound healing and collagen formation. IV. Distortion of ribosomal patterns of fibroblasts in scurvy, *J. Cell Biol.*, 1964, **22**, 365.
 22. GODMAN, G. C., and LANE N., On the site of sulfation in the chondrocyte, *J. Cell Biol.*, 1964, **21**, 353.
 23. PETERSON, M., and LEBLOND, C. P., Synthesis of complex carbohydrates in the Golgi region, as shown by radioautography after injection of labeled glucose, *J. Cell Biol.*, 1964, **21**, 143.
 24. FEWER, D., THREADGOLD, J., and SHELDON, H., Studies on cartilage V. Electron microscopic observations on the autoradiographic localization of S^{35} in cells and matrix, *J. Ultrastruct. Research*, 1964, **11**, 166.
 25. SALPETER, M. M., and BACHMANN, L., Autoradiography with the electron microscope. I. A procedure for improving resolution, sensitivity, and contrast, *J. Cell Biol.*, 1964, **22**, 469.
 26. YOUNG, B. A., and KOPRIWA, B. M., The use of the gevaert NUC-307 nuclear emulsion for radioautography at the electron microscope level, *J. Histochem. and Cytochem.*, 1964, **12**, 438.
 27. GODMAN, G. C., and PORTER, K. R., Chondrogenesis studied with the electron microscope, *J. Cell Biol.*, 1960, **8**, 719.
 28. KARRER, H. E., Electron microscopic study of developing chick embryo aorta, *J. Ultrastruct. Research*, 1960, **4**, 420.
 29. YARDLEY, J. H., HEATON, M. W., GAINES, L. M., JR., and SHULMAN, L. E., Collagen formation by fibroblasts, *Bull. Johns Hopkins Hosp.*, 1961, **106**, 381.
 30. PORTER, K. R., Cell fine structure and biosynthesis of intercellular macromolecules, *Biophysic. J.*, 1964, **4**, 167.

FIGURE 14 Electron micrograph of a typical area from a 7-day wound in which a portion of a fibroblast is seen. In this particular fibroblast the cisternae of the endoplasmic reticulum have been cut tangentially so that characteristic aggregates of ribosomes lining the cisternal membrane can be seen. In addition, at the margin of the cell several areas (arrows) are seen where the membranes of the cisternae of the rough endoplasmic reticulum (*er*) seem to approximate or merge with the plasma membrane of the cell. In another area a string of small vesicles (*ve*) can be seen to run from the plasma membrane in the direction of the ergastoplasm. $\times 23,000$. The inset is a higher magnification of one of these areas of proximity of the endoplasmic reticulum to the plasma membrane, demonstrating what could be interpreted as a communication between the endoplasmic reticulum and the extracellular regions. Fig. 14, $\times 23,000$; inset, $\times 60,000$.



31. CHAPMAN, J. A., Morphological and chemical studies of collagen formation. I. The fine structure of guinea pig granulomata, *J. Biophysic. and Biochem. Cytol.*, 1961, **9**, 639.
32. GOLDBERG, B., and GREEN, H., An analysis of collagen secretion by established mouse fibroblast lines, *J. Cell Biol.*, 1964, **22**, 227.
33. STEARNS, M. L., Studies on the development of connective tissue in transparent chambers in the rabbit's ear. I, *Am. J. Anat.*, 1940, **66**, 133.
34. STEARNS, M. L., Studies on the development of connective tissue in transparent chambers in the rabbit's ear. II, *Am. J. Anat.*, 1940, **67**, 55.
35. GLÜCKSMANN, A., HOWARD, A., and PELC, S. R., The uptake of radioactive sulfate by cells, fibres and ground substance of mature and developing connective tissue in the adult mouse, *J. Anat. and Physiol.*, London, 1956, **90**, 478.
36. GLÜCKSMANN, A., Cell turnover in the dermis, in *Advances in Biology of Skin: Wound Healing*, (W. Montagna and R. E. Billingham, editors), Pergamon Press, London, 1964, 76.
37. GROSSFELD, H., MEYER, KARL, and GODMAN, GABRIEL C., Acid mucopolysaccharides produced in tissue culture, *Anat. Rec.*, 1956, **124**, 489.
38. SOLOMON, A. K., The kinetics of biological processes: Special problems connected with the use of tracers, *Advances Biol. and Med. Physics*, 1953, **3**, 65.
39. PARTRIDGE, S. M., and ELSDEN, D. F., The chemistry of connective tissues. VII. Dissociation of the chondroitin sulfate-protein complex of cartilage with alkali, *Biochem. J.*, 1961, **79**, 26.
40. RUSSELL, J. A., The use of isotopic tracers in estimating rates of metabolic reactions, *Perspectives Biol. and Med.*, 1958, **1**, 138.
41. KOULISH, S., and KLEINFELD, R. G., The role of the nucleolus. I. Triated cytidine activity in liver parenchymal cells of thioacetamide-treated rats, *J. Cell Biol.*, 1964, **23**, 39.
42. AMANO, M., and LEBLOND, C. P., Comparison of the specific activity time curves of ribonucleic acid in chromatin, nucleolus and cytoplasm, *Exp. Cell Research*, 1960, **20**, 250.
43. ZILVERSMIT, D. B., ENTENMAN, C., and FISHLER, M. C., On the calculation of turnover time and turnover rate from experiments involving the use of labeling agents, *J. Gen. Physiol.*, 1943, **26**, 325.
44. NADLER, N. J., Calculations of the turnover times of proteins in each region of the acinar cells of the pancreas, *J. Cell Biol.*, 1963, **16**, 24.
45. GROSS, J. I., MATTHEWS, M. B., and DORFMAN, A., Sodium chondroitin sulfate-protein complexes of cartilage, *J. Biol. Chem.*, 1960, **235**, 2889.
46. GOULD, B. S., Collagen formation and fibrogenesis with special reference to the role of ascorbic acid, *Internat. Rev. Cytol.*, 1963, **15**, 301.