Microtechnique for Determining the Specific Activity of Radioactive Intracellular Leucine and Applications to \textit{in Vivo} Studies of Protein Synthesis* \\

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SUMMARY

A microtechnique has been developed for the determination of specific activities of radioactive leucine in the nanogram range, with an accuracy of at least 7\%. The technique depends on the coupling of [3H]leucine to [14C]2,4-dinitrofluorobenzene. By use of inulin in the incubation medium, a correction can be made for extracellular leucine contaminating the tissue, and thus the specific activity of the intracellular leucine pool can be estimated.

The technique has been applied to determining rates of protein synthesis in a protein-secreting gland, the galea of the silkmoth, \textit{Antheraea polyphemus}, after progressive exposure to actinomycin D. Actinomycin D is shown to "superinduce" incorporation of [3H]leucine in the differentiation-specific product of the galea, the zymogen of cocoonase. However, superinduction is illusory; when changes in specific activity of the intracellular pool are taken into account, no actinomycin effect on zymogen synthesis is apparent.

In recent years protein synthesis has been used extensively as a key index of cellular response to developmental stimuli, hormones, and other effectors. Most frequently, rates of protein synthesis have been inferred from rates of radioactive amino acid incorporation into acid-insoluble material. At best, however, such estimates are semiquantitative. For converting incorporation data into absolute rates of protein synthesis, it is essential to know the specific activity of the amino acid pool utilized in protein synthesis. For determining relative rates of protein synthesis, the specific activity of the precursor must be estimated, unless it is known to be constant for a given set of samples. Failure to take specific activities into consideration may lead to serious errors. Thus, actinomycin D is now known to inhibit histone synthesis during early sea urchin development (1); yet this phenomenon was long unsuspected, because the inhibitor increases the intracellular specific activity of leucine by decreasing the leucine pool size (2), so that actinomycin-treated embryos incorporate at least as much label in total protein as do the controls (2, 3).

In principle, the simplest method of estimating specific activity involves measuring both the quantity of an amino acid in a tissue aliquot and the radioactivity associated with that amino acid. In practice, direct quantitation of amino acids (e.g. by use of an amino acid analyzer) may require elaborate equipment, may depend on careful attention to losses, and may be impractical if the amount of tissue is small. This paper reports a simple microtechnique based on the reaction of amino acids with 1-fluoro-2,4-dinitrobenzene. The specific activity of [3H]leucine is determined by reaction with [14C]FDNB and measuring the ratio of [3H] to [14C] in the resulting dinitrophenyl-leucine, after purification by two-dimensional chromatography. The technique requires no elaborate equipment other than a scintillation counter, it is sensitive with nanogram amounts or less, provided enough radioactivity is present, it is unaffected by losses during analysis, and it should be easily modifiable for use with other tissues and other amino acids.

The radioactive amino acid in a tissue homogenate will be derived partly from the intracellular pool and partly from extracellular amino acid, such as might be trapped between cells. Since only the intracellular specific activity is of interest, a correction must be made if extracellular amino acid is a significant fraction of the total. Inulin is a classical tool for quantifying extracellular spaces (4); in our experience, use of [14C]-inulin contributes to the reliability of the [3H]leucine specific activity estimates.

As an example of possible applications, the microtechnique was used to determine rates of protein synthesis after actinomycin D treatment of the galea (5), a differentiated gland of the giant silkmoth, \textit{Antheraea polyphemus}. We find that the technique leads to a significant improvement in estimates of mRNA stability, by revealing that actinomycin increases the intracellular specific activity of [3H]leucine in this tissue.

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1 The abbreviations used are: FDNB, 1-fluoro-2,4-dinitrobenzene; DNP-, dinitrophenyl-.
METHODS

Tissue Incubation—The method was perfected with the use of wing tissue from developing silkmoths, A. polyphemus, on Day 5 of the pupa-to-adult transformation (6). The pupal wing cuticle was removed with nickel-plated, short-bladed scissors (Vigor), and the underlying tissue was excised and transferred to a black watch glass with solid square base (Turbox) containing about 2 ml of commercial (plasma-free) Grace's (7) medium (Grand Island Biological Company, Grand Island, New York) fortified with 200 µg of streptomycin, 60 µg of penicillin G, and 20 µg of phenylthiourea. The tissue was minced to approximately 2 mm, transferred to a 12-ml test tube, pelleted at room temperature (<1720 × g), and washed four times by resuspending in 5 ml of Grace's medium and pelleting. The washed tissue was transferred to a clean watch glass and cultured with 0.60 ml of fortified Grace's medium for the indicated prior incubation period, usually 2 hours. The medium was then removed with a Pasteur pipette, and the tissue was rinsed four times with 1.5 ml each of modified Grace's lacking leucine. Modified Grace's medium (0.50 ml) was then added, containing the indicated concentration of nonradioactive l-leucine plus L-[4,5-3H]leucine (Schwarz BioResearch, 15 Ci per mmole or 38.5 Ci per mmole) and [carboxyl-14C] inulin (International Chemical and Nuclear Corporation, Irvine, California; see text for specific activities). [3H]Inulin (Schwarz BioResearch, 380 µCi per mg) was used in some experiments. After labeling for 1 hour at 25° (with shaking at 70 rpm), the tissue was transferred to a chilled conical test tube and pelleted as before. All subsequent steps were performed at 2°. Within 10 min, the tissue was washed 10 times by resuspending in fresh 7 ml of 90% Weevers' solution (8) and pelleting. Finally, the tissue was transferred to a ground glass microhomogenizer (Bolab, Inc., Reading, Massachusetts) and stored at -80° until used.

After vigorous homogenization in 0.20 ml of 0.01 M Tris-HCl (pH 7.6 at 23°) or distilled water, the tissue homogenate was clarified by centrifugation first at about 1,200 × g for 3 min and then at 13,000 × g for 5 min. The final supernatant was stored in a microanalysis tube (Wettermann and Company, Basel, Switzerland) at -20°.

Protein concentrations were determined by a slight modification of the method of Lowry et al. (9), with bovine serum albumin (Nutritional Biochemicals) as a standard. Isoelectric incorporation into protein was estimated by precipitation with cold 7% trichloroacetic acid in the presence of 0.01 M leucine and 50 µg of bovine serum albumin. The suspension was heated at 90° for 15 min, cooled to 4° and Millipore filtered, and the precipitate was counted by liquid scintillation in a toluene-based mixture containing 10% Bio-Solv Solubilizer-3 (Beckman Instruments).

Preparation of DNP Derivatives—The aliquots of tissue supernatant used contained at least 1500 cpm of soluble [3H]leucine and 2 to 50 µg of protein. The aliquots were placed in microhomogenizer tubes and desiccated to dryness under slight vacuum at room temperature. Fifteen microliters of 0.2 M sodium phosphate (pH 9.3 at 23°) and 10 µl of a mixture of nonradioactive FDNR (Eastman) plus [U-14C]FDNR (Amersham-Searle, 31.5 µCi per mmole) in 76% ethanol were added, and the tube was stoppered. The mixture was shaken vigorously and reacted at 40° in the dark in a water bath; all subsequent steps were performed in the dark or under subdued light. After 16 to 24 hours, the mixture was desiccated to dryness, redissolved in about 25 µl of 0.2 M sodium carbonate (pH 10.5 at 23°), and extracted twice with 90 µl of anhydrous diethyl ether (Malinckrodt Chemical Works). These ether layers were discarded. The aqueous layer was acidified with 80 µl of 0.4 M citric acid (pH 1.8 at 23°) and extracted twice with 90 µl of ether. The new ether layers were pooled in a clean microanalysis tube, 3 µg each of carrier DNP-leucine, DNP-isoleucine, DNP-valine, and DNP-phenylalanine (Schwarz BioResearch) were added, the solvent was evaporated, and the DNP derivatives were redissolved in about 25 µl of anhydrous ether and stored overnight at -20°.

Chromatography—Chromatograms (on silica gel layers, 20 × 20 cm, 0.1 mm thick, with ethylene terephthalate backing, Eastman sheet 6060) were developed in groups of four to eight with a Multiple Thin Plate TLC Chromatank (Shandon, Sewickley, Pennsylvania) by ascending two-dimensional chromatography. In the first dimension a slight modification of the "toluene" system described by Biserte and Osteux (10) was used. The thin layer was exposed to vapors of the aqueous phase supplemented with 1.5 volumes of 0.8 M NaOH for at least 3 days. After development, chromatograms were air dried for approximately 45 min, rotated 90°, and developed in the second dimension in the "benzene" system of Brenner and Niederwieser (11). Infrequently, development in the benzene system was repeated to improve separation. The developing times for the two dimensions were about 3 and 2 hours, respectively. All solvents were purified as described by Stahl (12), except that benzene (Eastman, spectro grade) was used without further purification in later experiments. Upon chromatography, the spot containing DNP-leucine plus DNP-isoleucine was cut out, placed in a scintillation vial, scraped off with a spatula, and counted as described above.

For the separation of DNP-leucine from DNP-isoleucine, a somewhat different procedure was followed. Previously coated thin layer chromatography silica gel plates (Brinkmann Instruments, Inc., Westbury, New York; layer thickness, 0.25 mm) were developed as above with the tolune system in the first dimension. The second dimension was carried out with a B-N chamber (Brinkmann Instruments) for continuous horizontal chromatography based on solvent evaporation from the leading edge of the plate. The temperature of the chamber was kept at 15° with a Lauda-Brinkmann circulator (Brinkmann Instruments). The solvent system was chloroform, methanol, and glacial acetic acid (85:5:1). Chloroform (Merek and Company) and methanol (Fisher) were ACS grade.

Correction of Contaminating Extracellular Leucine—For estimating extracellular space, tissues were incubated in the presence of [14C]Inulin as well as [3H]leucine; after incubation they were washed, homogenized, and counted as described previously. On the assumption that extracellular spaces contained [14C]Inulin and [3H]leucine in the same ratio as the incubation medium, the amount of [3H]leucine reasonably ascribed to extracellular contamination could be calculated as shown in Equation 1.

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\text{Extracellular [3H]leucine (fraction of the total)} = \frac{[\text{14C}]\text{Inulin cpm per aliquot homogenate}}{[\text{3H}]\text{leucine cpm per aliquot homogenate}} \times \frac{[\text{3H}]\text{leucine cpm per aliquot incubation medium}}{[\text{14C}]\text{Inulin cpm per aliquot incubation medium}}
\]
Homogenate. The first assumption was confirmed directly by chromatography—endogenous leucine plus isoleucine in the DNP-leucine plus DNP-isoleucine spot is identified in free leucine, and (b) the efficiency of recovering leucine and isoleucine was calculated by determining the volume of the inulin-defined extracellular space and multiplying by the known concentrations of leucine and isoleucine in the incubation medium. The specific activity of extracellular leucine plus isoleucine was determined by reacting the volume of the inulin-defined extracellular space with an excess of nonradioactive FDNB (0.6 pmole), extracted, and chromatographed as usual. The entire chromatogram was assayed for radioactivity. No significant radioactivity was encountered anywhere but in the DNP-leucine plus DNP-isoleucine spot and its immediate vicinity. Areas equal in size to the spot and immediately adjacent to it in all directions contained no more than 8% of the spot's radioactivity, and frequently considerably less. Thus, in the vicinity of the spot, no major amount of trichloroacetic acid-soluble extract of labeled wing homogenate were fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, which permits identification of the characteristic product of this gland, coconase zymogen (14). The rates of incorporation of [3H]leucine into zymogen and nonzymogen proteins were calculated from the radioactivity associated with the corresponding bands. Results were normalized for the amount of zymogen or nonzymogen protein in each sample, estimated from the A<sub>478</sub> difference between control and actinomycin D samples was always less than 40% and usually less than 20%, no major error was introduced by nonlinearity of dye binding.

**RESULTS**

**Chromatographic Separation of DNP-Leucine plus DNP-Isoleucine from All Other Leucine and FDNB Derivatives**—In our experience, repeatable separation of DNP-leucine from DNP-isoleucine by thin layer chromatography requires some kind of continuous chromatography; therefore, the two derivatives were treated as a single spot in all experiments except those of Table II, in which continuous, horizontal chromatography was used. Under our chromatographic conditions, DNP-valine and DNP-phenylalanine were the two common DNP-amino acids which migrated closest to the DNP-leucine plus DNP-isoleucine spot (12). Both of these possible contaminants, as well as the breakdown products, dinitrophenol and dinitroaniline, were invariably well separated from the spot.

The adequacy of separation of the DNP-leucine plus DNP-isoleucine spot from all other compounds derived from the reaction of a tissue homogenate with FDNB was documented as follows. In one type of experiment 0.5 µCi of [3H]leucine (15 Ci per mmole) was added to an aliquot of nonradioactive homogenate (containing about 30 µg of protein and about 38 ng of endogenous leucine plus isoleucine). The mixture was reacted with an excess of nonradioactive FDNB (0.6 µmole), extracted, and chromatographed as usual. The entire chromatogram was assayed for radioactivity. No significant radioactivity was encountered anywhere but in the DNP-leucine plus DNP-isoleucine spot and its immediate vicinity. Areas equal in size to the spot and immediately adjacent to it in all directions contained no more than 8% of the spot's radioactivity, and frequently considerably less. Thus, in the vicinity of the spot, no major amount of radioactivity was found, except that expected from tailing and slightly variable cutting procedures. The efficiency of recovery of the added [3H]leucine was 40 to 50% in 10 recent trials.

In a second type of experiment, an aliquot of nonradioactive homogenate was reacted with excess [14C]FDNB and processed as usual. As expected, radioactivity was recovered in many areas of the chromatogram. For example, in one experiment an aliquot of homogenate (containing about 30 µg of protein and about 38 ng of endogenous leucine plus isoleucine) was reacted with 0.6 µmole of [14C]FDNB (0.4 µCi per µmole). The DNP-phenylalanine, DNP-valine, dinitroaniline, and dinitrophenol spots con-
tained 0.34, 0.60, 31, and 390 times as many counts per min as the DNP-leucine plus DNP-isoleucine spot. Still, the proportion of radioactivity in the immediate vicinity of the DNP-leucine plus DNP-isoleucine spot was as low as in the preceding experiment, indicating that the derivatives of interest were well separated from all other contaminants.

**Accuracy of Specific Activity Determinations**—A variable amount of [3H]leucine (2500 to 6550 cpm) was added to aliquots of wing homogenate (each corresponding to about 30 μg of protein). The radioactive leucine did not add significantly to total leucine, since it represented less than 1% of the total. All samples were reacted with identical aliquots of [14C]FDNB. The results (Fig. 1) fit well a straight line which extrapolates to zero. The maximum deviation was 7%, with the counting statistics error alone being 8%. Clearly, the specific activity of soluble leucine in a tissue sample was determined with a fair degree of accuracy.

**Correction for Extracellular Leucine**—The level of extracellular leucine inevitably contaminating a tissue sample must be known, so that a correction might be made if necessary. This level was estimated by using [3H]inulin in combination with [3H]leucine. In one wing experiment (Table I) extracellular [3H]leucine was estimated as approximately 38% of the total. Estimates were reproducible in any one experiment, and their averages normally varied between 20 and 40% for different experiments.

This correction depends on whether inulin is in fact excluded from cells and on whether the same extracellular space is equally available to both inulin and leucine. The following evidence supports these assumptions.

When minced wing tissue was incubated for various periods of time in the presence of [3H]inulin and [3H]leucine, the inulin recovered in the washed tissue reached a maximum at 20 min and remained constant thereafter (Fig. 2A). The time for attainment of equilibrium is evidently needed for penetration between the two closely apposed epithelial layers which make up the wing (16); this interpretation is supported by the similar kinetics of leucine uptake. In any case, slow leakage of inulin into the cells, after 20 min, does not occur. In a more complete series of in vivo experiments, Cherbas and Cherbas (17) showed that inulin injected into A. polyphemus attains its final physiological volume as soon as dispersion throughout the blood is complete (2 hours post-injection). In their experiments, no uptake into cells (i.e., increase in physiological volume) was detected even 24 hours after injection. The estimate of Cherbas and Cherbas of extracellular space in A. polyphemus, with inulin, agreed closely with their direct measurement of blood volume, again indicating that inulin is in fact excluded from insect cells.

Physiological "extracellular space" is an operational parameter, which is affected not only by true physical extracellular space but also by adsorption to tissue surfaces and by influx and efflux from extracellular compartments. In addition, the physical extracellular space itself may vary with different substances, since all spaces may not be equally accessible to molecules of widely varying size or charge. An argument in favor of equating inulin extracellular space with leucine extracellular space was the observation that inulin and leucine eluted with equal efficiency from labeled tissue during washing; for approximately the first five wash cycles, the ratio of [3H]leucine to [3H]inulin in the washing solution was substantially equal to that in the incubation medium. Upon further washing the ratio increased slightly, presumably because of the gradual extraction of intracellular [3H]leucine. This secondary increase was not due to the presence of a fraction of tightly bound inulin, more resistant to extraction than extracellular leucine. Binding is easily revealed by saturation kinetics, whereas the inulin content of washed wing is strictly propor-

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**Fig. 1. Accuracy of specific activity determinations.** Identical 10-μl aliquots of [14C]FDNB (2.5 μCi, 9.4 μCi per mole) were reacted with a series of 10-μl wing homogenate aliquots to which increasing amounts of [3H]leucine were added (85.3 Ci per mole), representing a negligible fraction of the total leucine pool. The ratio of H to C in DNP-leucine plus DNP-isoleucine was determined as described under "Methods." As expected, the ratio is linearly related to the amount of [3H]leucine added.

**TABLE I**

Calculation of extracellular leucine contamination

In two different experiments, a total of 12 wing tissue samples were incubated in [3H]leucine (approximately 410 μCi per mole, 230 μCi per ml) and [3H]inulin (approximately 3 μCi per mg, 4.3 μCi per ml) for 1 hour. The tissues were washed by centrifugation with ice-cold 80% Weever's medium and processed as described under "Methods." Values in Column C represent the difference between total and cold trichloroacetic acid-precipitable radioactivity.

| Sample | Radioactivity in incubation medium | Radioactivity in tissue | E, Volume of extracellular space (E = D/B) | F, Fraction of tissue leucine which is extracellular (F = (A×D)/B) |
|--------|-----------------------------------|------------------------|------------------------------------------|-------------------------------------------------|
|        | [3H]leucine cpm/μl               | [3H]leucine cpm/μl     | [3H] leucine cpm/μg protein              | [3H] leucine cpm/μg protein                      |
|        |                                   |                        |                                         | µg protein                                       |
| 1      |                                   | 2010                   | 24.5                                    | 5.17                                            | 0.391                                            |
| 2      |                                   | 2340                   | 30.3                                    | 6.39                                            | 0.415                                            |
| 3      |                                   | 1440                   | 15.4                                    | 3.25                                            | 0.363                                            |
| 4      |                                   | 1500                   | 18.4                                    | 3.88                                            | 0.371                                            |
| 5      |                                   | 1610                   | 17.8                                    | 3.76                                            | 0.355                                            |
| 6      |                                   | 1510                   | 16.9                                    | 3.57                                            | 0.306                                            |
| 7      |                                   | 1970                   | 24.5                                    | 6.11                                            | 0.444                                            |
| 8      |                                   | 2110                   | 26.3                                    | 6.56                                            | 0.445                                            |
| 9      |                                   | 1430                   | 14.5                                    | 3.61                                            | 0.366                                            |
| 10     |                                   | 1520                   | 15.3                                    | 3.81                                            | 0.359                                            |
| 11     |                                   | 1810                   | 18.7                                    | 4.66                                            | 0.368                                            |
| 12     |                                   | 1770                   | 16.9                                    | 4.21                                            | 0.340                                            |
Fig. 3. Tissue-associated inulin as a function of inulin concentration in the medium. Samples of wing tissue were incubated in the presence of [3H]inulin (Schwarz BioResearch, 380 μCi per mg) for 1 hour. The tissue was washed by centrifugation, homogenized, and counted by the usual procedures.

In cellular specific radioactivity of intracellular leucine. Samples of wing tissue were incubated in the presence of [3H]leucine (571 mCi per mmole, 322 μCi per ml) and [14C]inulin (2.5 μCi per mg, 3.33 μCi per ml) for increasing periods of time. After washing the tissue and homogenizing as described under "Methods," aliquots of the homogenates were counted. Soluble leucine radioactivity was calculated by subtracting trichloroacetic acid-precipitable from total [H] counts per min. Results were normalized for the amount of tissue in each sample by determining protein content with bovine serum albumin as a standard. O-O, soluble [3H]leucine; O---O, [14C]inulin. B, kinetics of leucine incorporation into trichloroacetic acid-precipitable material (TCA PPT). Details as in A.

Kinetics of Leucine Labeling—Steady state labeling of the soluble leucine pool is attained at about 25 min (Fig. 2A). As in the case of inulin, we interpret the long lag time as being due to the difficulty of permeation between the epithelial layers. A corollary is that leucine uptake takes place primarily through the cells' basal surfaces, which are apposed in the epithelial bilayer and which border the blood in vivo.

Fig. 2B displays the incorporation of [3H]leucine into trichloroacetic acid-precipitable material as a function of time. As expected from Fig. 2A, a constant rate of incorporation is attained after roughly 25 min.

mRNA Stability in the Galeae—As an example of applications of the microtechnique, we investigated the effects of actinomycin D on protein synthesis in the silkmoth galeae. The galeae of each animal are a pair of small glands (0.5 mg each, wet weight), which contain secretory cells highly specialized for the production of a specific protein, the zymogen of the protease, cocoonase (18-20). Depending on the stage of development, zymogen production amounts up to 70% of total protein synthesis in the glands (21). Previous studies (22) based simply on isotope incorporation, had indicated that actinomycin D specifically depresses the synthesis of nonzymogen proteins, while leaving zymogen production relatively unaffected. In the present experiments, the effect of actinomycin was evaluated both in terms of isotope incorporation alone and in terms of actual rates of protein synthesis, as determined from incorporation values plus estimates of intracellular specific activities.

Paired galeae were incubated in the presence and absence of actinomycin D for variable periods of time; thereafter, the glands were exposed to [3H]leucine for 1 hour, washed, and frozen. After homogenization, aliquots were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis to permit protein fractionation. The radioactivity in the zymogen or nonzymogen region of each gel (21) was determined and normalized against the corresponding amount of protein. Fig. 4A presents the results, in terms of leucine incorporation alone. Relative to controls, actinomycin-treated glands incorporated [3H]leucine into nonzymogen proteins at rates consistent with an average mRNA half-life of 7.6 hours. By contrast, incorporation into zymogen was actually stimulated by actinomycin. Such "superinduction" has been reported in many other systems (23).

Intracellular specific activities were also determined from aliquots of the same glands (Fig. 4B). Quite consistently, relative
The effects of actinomycin D (ACT. D) on zymogen and nonzymogen protein synthesis in the silkmoth galeae. Galeae (day 11 to 15) were incubated in the presence or absence of actinomycin D (60 μg per ml) and were then labeled with [3H]leucine (4.8 mCi per mole, 350 μCi per ml) and [14C]inulin (2.9 μCi per mg, 12 μCi per ml) for 1 hour. Time on the abscissa corresponds to the prior incubation time plus a fraction of the labeling period (0.49 hour, or approximately half of the labeling period but with a correction for the exponential nature of decay). After labeling, the tissues were washed and homogenized, and aliquots were used for sodium dodecyl sulfate-polyacrylamide electrophoresis of the proteins and for determination of intracellular specific activity as described in the text. A, incorporation into zymogen (O—O) and nonzymogen (O—O) protein in actinomycin D-treated tissue, as a fraction of the corresponding control value. B, change in specific activity of intracellular leucine as a function of time in actinomycin D. Specific activity in each actinomycin D-treated tissue is reported relative to the corresponding control value. C, rate of actual zymogen (○—○) and nonzymogen (○—○) protein synthesis in actinomycin D-treated tissue, as a fraction of the corresponding value in the control. Incorporation data from A were divided by intracellular specific activity measurements from B. The best-fit zymogen line has a slope of -0.007 (half-life = 99 hours).

**TABLE II**

Comparison of specific activities based on the [3H]FDNB derivatives of leucine or leucine plus isoleucine

Galeae (incubated in the presence or absence of actinomycin D for 1, 5, and 7 hours) were pooled and labeled for 1 hour in [3H]leucine. Specific activities of the total soluble [3H]leucine were determined as described in the text with the [3H]FDNB derivatives of either leucine alone or leucine plus isoleucine as measures of pool size.

|       | A. [H] cpm DNP-leucine/ [H] cpm (leucine + isoleucine) | B. [H] cpm DNP-leucine/ [H] cpm DNP-leucine | B/A |
|-------|---------------------------------------------------------|---------------------------------------------|-----|
| 1.   | + Actinomycin                                           | 1.16                                       | 2.58 | 2.44 |
| 2.   | - Actinomycin (control)                                 | 0.671                                      | 1.39 | 2.07 |
| 3.   | + Actinomycin/ - actinomycin                           | 1.73                                       | 1.93 | 2.07 |

Times higher than that based on leucine plus isoleucine. These values indicate that DNP-leucine is 43 and 48%, respectively, of the DNP-leucine plus isoleucine total in the spot; these values are in good agreement (11% difference), considering that the experimental error is 7%. When correction was made for extracelluar contamination, the agreement between the two methods proved equally good. The estimates of the stimulation by actinomycin (based on leucine alone or on leucine plus isoleucine) differed by only 10%. In the presence and absence of actinomycin, the intracellular leucine was 51 and 56%, respectively, of the combined intracellular leucine plus isoleucine.
DISCUSSION

Specific Activity of Free Intracellular Leucine—In principle, the absolute rate of protein synthesis can be calculated by dividing the radioactivity incorporated into protein per unit time by the specific activity of the isotopically labeled intracellular amino acid pool. This involves several testable assumptions: (a) breakdown of newly synthesized proteins is not significant during the labeling period; (b) the specific activity of the intracellular pool can be determined, with due corrections for extracellular contamination; (c) the average specific activity of the amino acid pool over the time of exposure to isotope is not significantly different from the specific activity at the end of exposure; and (d) the specific activity of the labeled amino acid in newly synthesized protein is identical to that in the total intracellular free amino acid pool (i.e. that the total free pool serves as a precursor for protein synthesis).

In the present case, the first assumption is valid, since the average protein half-life in the galea is about 21 hours. The second assumption is shown in this paper to be correct within experimental error (see Figs. 2 and 3 and text). The third assumption is only approximately correct for the wing, since the lag in uptake (Fig. 2A) causes underestimation of synthesis by 10 to 20%. In the case of the galea, no correction for uptake delay was necessary, since rates of synthesis in the actinomycin-treated glands were expressed relative to the controls (rather than in absolute terms), and since the open structure of the galea, unlike the bilayered nature of the wing, permits rapid pool equilibration (18). As discussed later, validity of the fourth assumption, namely that the intracellular amino acid pool is not compartmentalized, is suggested (although not proved rigorously) by Fig. 2.

The importance of determining specific activities has been emphasized recently by Fry and Gross (24, 25). They and others report that the rate of incorporation of [14C]leucine into fertilized sea urchin eggs is about constant up to the first cleavage metaphase. At this time there is a transient decrease in rate of incorporation. By analogy with the inhibition of protein synthesis during metaphase in mammalian cell cultures (26), this transient decrease has been interpreted as a decrease in protein synthesis (27). However, Fry and Gross report that there is a corresponding decrease in leucine specific activity at cleavage metaphase, so that apparently protein synthesis remains constant (24). In another study, these authors report that actinomycin D treatment of cleaving sea urchin embryos leads to an increase in intracellular leucine specific activity (2); unless correction is made for this effect, the inhibition of protein synthesis by actinomycin is not apparent (3). Kemp and Sutton (28), using tobacco callus tissue, find that the rate of incorporation of [3H]leucine per cell is a function of tissue size, whereas the actual rate of protein synthesis is size independent. Stewart and Papacostantinou (29), in a series of experiments similar to those described here for the galea, find that actinomycin has a stimulatory effect on the incorporation of a radioactive amino acid mixture by adult bovine lens epithelial and fiber cells. The stimulation is not observed in experiments with young calf lenses. It would be worth testing whether the anomalous stimulation is due to an increase in specific activity (as in the galea), especially since in our experience actinomycin seems to affect differently the specific activities of different tissues, or possibly even of the same tissue at different developmental stages.

Specific activities of amino acids have usually been determined with the aid of an amino acid analyzer (25). Recently, an alternative technique has been reported, involving coupling an unknown amount of nonradioactive leucine (optimally 10 to 20 nmols) and a known amount of radioactive leucine to tRNA^Leu (30). The microtechnique described in the present report permits determinations with only 0.05 nmol of leucine. Such sensitivity was essential for our purposes, since an entire galea contains only about 0.5 nmol of free leucine.

After 1 hour of incubation, the intracellular specific activity of leucine was considerably lower than that of the incubation medium (20% of the medium in the case of the galea). The corresponding values found by Gan and Jeffay (31) were 50% in rat liver and 70% in rat muscle. Leucine is reported to be an essential amino acid for insects (32, 33), perhaps suggesting that most of the pool dilution comes from protein catabolism (at least under our experimental conditions, which involved incubation in a low leucine medium).

The use of inulin as an index of extracellular space seems justified. As discussed under "Results," inulin was shown to be excluded from an intracellular space in the wing and in elite from the extracellular space in the same manner as leucine, without binding preferentially to the tissue surfaces.

The fourth assumption (that the total free pool the specific activity of which we measure is in fact the precursor pool for protein synthesis) is crucial. If there are soluble amino acid compartments which exchange slowly with each other and the medium, protein synthesis rates cannot be determined accurately by this method. The bulk of the evidence in the literature argues against the existence of such compartments. For example, Lottfield and Harris (34) induced de novo ferritin synthesis in rat liver while simultaneously exposing the tissue to radioactive amino acids. They then isolated the newly synthesized ferritin, hydrolyzed it, and determined the specific activities of its amino acids through chromatography. The values obtained for valine, isoleucine, and leucine were nearly identical with the specific activities of the respective total free amino acid pools. Fry and Gross (25) expanded the intracellular cold leucine pool in sea urchin embryos. When labeled with [14C]leucine, these embryos showed significantly lower incorporation into protein than did control embryos exposed to the same labeling medium; these results suggest that exogenous label does not bypass the intracellular pool. Finally, Henshaw et al. (35) compared in rat liver the specific activities of the free lysine pool and of the lysine attached to tRNA and found them to differ by less than 10%.

However, there is evidence for compartmentalization of some intracellular metabolites. Thus, the mono- and diphosphate esters of uridine are compartmentalized in logarithmically growing Escherichia coli (36) and glucose 6-phosphate and phosphoglyceric acid in rat diaphragm (37). In both cases, a particular substance in total extracts was found to have a specific activity higher than that of one or more of its metabolic preursors. There are also several studies in the literature which suggest compartmentalization of amino acid pools (for references, see Reference 38). In general, such studies claim that exogenous amino acid is utilized preferentially, since they observe that incorporation of label into trichloroacetic acid-precipitable material in-

1 F. C. Kafatos and P. B. Moore, submitted for publication.
2 F. C. Kafatos and P. B. Moore, submitted for publication.
3 J. C. Regier and F. C. Kafatos, unpublished observations.
creases linearly, whereas the specific activity increases exponentially. These findings are contradicted by the previously mentioned studies as well as by our own data. When the change of intracellular specific activity with time is calculated from Fig. 2A and compared with the time course of incorporation shown on Fig. 2B, the results are more in accord with the idea of a single soluble pool than with the compartmentalized pool model.

Actinomycin D Effects—Actinomycin D (39) is a well known inhibitor of RNA synthesis (40-42). Much evidence has accumulated that the inhibition of protein synthesis observed upon actinomycin D treatment results from the decay of mRNA. For example, Levinthal, Keynan, and Higa (43) report that, upon treatment of *Bacillus subtilis*, the rapidly turning over mRNA fraction and the protein synthetic capacity decay exponentially with comparable decay times (2 and 3 to 4 min, respectively). In an independent study, the half-life was similarly estimated as 3 min (44) without the use of actinomycin. Thus, at least in bacteria, actinomycin D seems a valid tool for determining mRNA stability.

Wilson and Hoagland (45) used actinomycin to determine the stability of rat liver polysomes. They found that after exposure to actinomycin the number of ribosomes in the polysome region was proportional to the protein synthetic rate. This fact, implying that actinomycin D does not affect the translation rate per active ribosome, supports the view that mRNA decay is the cause of declining protein synthesis (although there are other possible explanations). These authors also report that actinomycin increases the intracellular specific activity of [14C]valine, just as for leucine in sea urchin embryos (2) and in insect tissues (this report).

Many studies describe generalized actinomycin D effects on intermediary metabolism. For example, Lasala et al. (46) report for human leukemia leukocytes that, in addition to lowering the rate of RNA synthesis, actinomycin inhibits respiration and anaerobic glycolysis and lowers ATP concentration. Since these changes were observed under conditions of RNA synthesis inhibition, they may well be secondary effects.

There have been reports (47) that actinomycin accelerates breakdown of some RNA, independent of suppression of RNA synthesis. One such report was based on the observation that in actinomycin-treated *B. subtilis* a fraction of the chloramphenicol RNA is rapidly degraded, whereas in 6-azauracil-treated *B. subtilis*, in which RNA synthesis is also inhibited, chloramphenicol RNA is not degraded. The interpretation has been challenged by Chantrenne (48) and Leive (49) among others. Chantrenne pulsed *Bacillus cereus* with [14C]azaguanine and then chased with unlabeled guanosine in the presence and absence of actinomycin. He found similar kinetics of RNA decay. Leive induced *E. coli* for β-galactosidase synthesis, divided the culture, removed inducer, and added actinomycin to one aliquot. She observed similar rates of decay of the ability to make β-galactosidase in both the control and the actinomycin-treated bacteria.

A more serious criticism of the type of experiment shown in Fig. 4, A and B, is that actinomycin-induced decay of protein synthesis is not necessarily proportional to the decay of mRNA. Thus, Fan, Higa, and Levinthal (50) found that, although the initial rate of decline of mRNA content and protein synthetic ability were similar in *E. coli*, after some time the rate of protein synthesis remained higher than expected. Kennel (51) observed a similar phenomenon in *B. subtilis*; noting that the decline in protein synthesis was closely paralleled by a decline in 23 S ribosomal RNA, he suggested that ribosomes and not mRNA are rate-limiting. Tomkins et al. (23) also interpret enzyme superinduction as a reflection of control mechanisms operating at the level of mRNA accessibility, rather than abundance.

In any case, until it becomes feasible to measure accurately and directly specific eucaryotic mRNA species, it seems reasonable to interpret, tentatively, actinomycin effects in terms of mRNA decay.

If incorporation alone is considered, nonzymogen mRNA in the gase appears to decay with a half-life of about 7.6 hours; zymogen synthesis appears to rise upon exposure to actinomycin (70% stimulation in 5 hours). When specific activities are taken into account, so that actual protein synthesis instead of incorporation is evaluated, a rather different picture emerges: nonzymogen mRNA decays with a half-life of only about 3.6 hours, and zymogen synthesis is unaffected by actinomycin for the duration of the experiment. These results are in agreement with previous autoradiographic studies (22), performed under somewhat different conditions. The corrected value for average nonzymogen mRNA half-life is also in good agreement with the value (2.50 ± 0.24 hours) obtained by Kafatos and Moore in an extensive study based on the assumption that zymogen synthesis is unaffected by actinomycin.

In sum, the differential effect of actinomycin on differentiation-specific and nonspecific synthetic functions of the galea seems well established. Since the comparison involves the same cells at the same time, and particularly since actinomycin D does not perturb zymogen synthesis (rather than superinducing it), we conclude that the differential effect probably reflects differential rates of specific and nonspecific mRNA breakdown.

Note Added in Proof—Dr. Argiris Efstratiadis has recently observed in this laboratory that the use of Protosol (New England Nuclear) markedly increases the counting efficiency of radioactive DNP-leucine spot. After 2 hours 15 ml of the regular toluene-based liquid scintillation mixture containing 0.3% glacial acetic acid are added.

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