These observations combined with the physiological results of Brown and Brown (*2*) suggest that light absorption by the pigment contained in the granules causes release of Ca from the granules themselves. The 50-Å globular particles in the illuminated pigmented granules may be rearranged into membrane-like lamellae as a result of alteration of their ionic environment—in particular, a reduction in the intragranular Ca concentration. However, there is a possibility that some conformational change responsible for the transformation from globule to lamella precedes the release of Ca. These observations bear on several other aspects of cell biology, for example, mechanisms of membrane assembly (*12*), similar morphologic changes seen in lysosomes under certain conditions (*13*), and the possibility that a similar mechanism may exist in the vertebrate pigment epithelium (*14, 15*).

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2. H. M. Brown and A. M. Brown, *Science* **178**, 755 (1972); A. M. Brown and H. M. Brown, *J. Gen. Physiol.* **62**, 239 (1973).
3. R. W. Meech and F. Strumwasser, *Fed. Proc.* **29**, 834 (1970); R. W. Meech, *J. Physiol. (Lond.)* **237**, 259 (1974).
4. N. Chalazonitis and A. Arvanitaki, *Arch. Sci. Physiol.* **10**, 291 (1956).
5. In previous morphologic studies of *Aplysia* neurons these granules have been called grains, lipid bodies, lipochondria, pigment-containing bodies, and pigmented granules [Arvanitaki and Chalazonitis (*1*); Rosenbluth (*6*); Coggeshall (*7*)]. I will call the whole structure a granule. Granules are of the order of 1 μm in diameter. The contents of granules can also be described as granular, but to avoid confusion I will call the substructure of the granule contents globular. The globular particles which make up the contents of dark-adapted granules are approximately 50 Å in diameter.
6. J. Rosenbluth, *Z. Zellforsch. Mikrosk. Anat.* **60**, 213 (1963).
7. R. E. Coggeshall, *J. Neurophysiol.* **30**, 1263 (1967).
8. The composition of the ASW was (in millimoles per liter) 500 NaCl, 10 KCl, 10 $CaCl_2$, 50 $MgCl_2$, and 10 tris-Cl, *p*H 7.8.
9. The composition of the fixative was 3 percent glutaraldehyde, 0.1*M* cacodylate buffer, *p*H 7.6, and 0.8*M* sucrose. Cells were rinsed in buffer plus sucrose, postfixed in 1 percent OsO_4 in 0.1*M* cacodylate buffer, *p*H 7.6, dehydrated in ethanol, and embedded in Epon. Some cells were fixed in 3 percent glutaraldehyde, 0.1*M* phosphate buffer, *p*H 7.6, and 0.8*M* sucrose saturated with $CaCl_2$.
10. Rosenbluth (*6*), describing the pigment granules of *Aplysia* neurons after fixation in OsO_4 in veronal acetate, ASW, or saturated $CaCl_2$, noted that they sometimes contained crystalline material or precipitates.
11. When Ca is replaced by Sr in the ASW, these neurons generate bursting pacemaker potentials [J. Barker and H. Gainer, *Brain Res.* **65**, 516 (1974)]. Since Ca acts as a carrier of current and measurable Ca influx occurs during the action potential in these cells [D. Geduldig and D. Junge, *J. Physiol. (Lond.)* **199**, 347 (1968); J. Stinnakre and L. Tauc, *Nat. New Biol.* **242**, 113 (1973)], and Sr can carry current in the Ca spike mechanism, at least in the barnacle muscle [S. Hagiwara and K. Naka, *J. Gen. Physiol.* **48**, 141 (1964)], cells that burst in Sr-ASW should become loaded with Sr.
12. Images that suggest the association of 50-Å globular units into various arrays including membrane-like lamellae are reminiscent of images seen in electron microscope studies of lipid-water systems and soaps [for example, see W. Stoeckenius, *J. Cell Biol.* **12**, 221 (1962); J. A. Lucy and A. M. Glauert, *J. Mol. Biol.* **8**, 727 (1964)]. Although it is not possible at this time to interpret the structural changes in *Aplysia* granules in molecular terms, they are interesting as an example of structural changes in a cell similar to phase changes described in model lipid systems.
13. As Rosenbluth (*6*) and Coggeshall (*7*) have pointed out, the pigmented granules resemble lysosomal dense bodies. Treatments that cause lysosomal dense bodies to be transformed into lamellar forms have been reviewed by H. Koenig [in *Lysosomes in Biology and Pathology*, J. T. Dingle and H. B. Fell, Eds. (American Elsevier, New York, 1969), vol. 2, pp. 150–154]. The mechanism of these transformations should be considered in terms of possible photosensitization or possible correlation of Ca release with the structural transformation.
14. The bases for this suggestion are (i) published electron micrographs of phagosome-derived granules in globular and lamellar forms strikingly similar to those I have described [M. Spitznas and M. J. Hogan, *Arch. Ophthalmol.* **84**, 810 (1970); H. Kolb and P. Gouras, *Invest. Ophthalmol.* **13**, 487 (1974)] and (ii) physiological studies showing that cells of the pigment epithelium hyperpolarize in response to light (*15*). Images of phagosomes in these configurations have been interpreted as stages in digestion of rod disk membranes. Part of the mechanism of membrane breakdown might include packaging of membrane subunits with Ca, the reverse of the process of membrane formation that occurs in *Aplysia* granules. The images of phagosome-related bodies in pigment epithelium could also be interpreted, however, as stages in light-induced conversion of uniformly globular forms to membrane-like lamellae. Thus, the structure of the phagosome contents after packing into globular form could undergo a light-induced change back to lamellar form, as in the *Aplysia* granules.
15. R. H. Steinberg, R. Schmidt, K. T. Brown, *Nature (Lond.)* **227**, 728 (1970); R. Schmidt and R. H. Steinberg, *J. Physiol. (Lond.)* **217**, 71 (1971). The hyperpolarization of the pigment epithelium in response to light is thought to be rod-dependent principally because the action spectrum of the light response corresponds to the absorption spectrum of rhodopsin, but the available evidence is not unequivocal. The hyperpolarizing light response in the vertebrate pigment epithelium results in current flow sufficient to be recorded in the electroretinogram as the c-wave and may be an important component of retinal physiology.
16. I thank G. Neises for technical assistance and H. M. Brown and A. M. Brown for discussing their unpublished work with me.

5 December 1974

Phototransduction in Aplysia Neurons: Calcium Release from Pigmented Granules Is Essential

Abstract. *Increased free intracellular calcium mediates increased membrane potassium conductance in illuminated* Aplysia *giant neurons. The calcium source was examined by microprobe analysis of cytoplasmic pigmented granules. Illumination markedly depleted granules of calcium and altered them structurally. Release of calcium from granules is essential for phototransduction.*

Henkart (*1*) has presented structural evidence suggesting that when an *Aplysia* giant neuron is illuminated Ca may be released from lipochondria or pigmented granules present in the cytoplasm. To investigate Ca release more directly, the Ca content of pigmented granules from nonilluminated neurons and from neurons illuminated with white light has been compared by using electron microprobe analysis. In addition, the concentration of free intracellular Ca required to mediate the light-evoked surface membrane hyperpolarization (*2*) known to occur in this neuron has been determined by pressure injection of Ca ethylene glycol-bis-(aminoethylether)-*N,N'*-tetraacetic acid (EGTA) buffers into the neuron before illumination. We found that the Ca content of illuminated ("light") granules is considerably less than that of nonilluminated ("dark") granules; this result is taken as direct evidence that illumination releases Ca from the granules. The light-evoked rise in free intracellular Ca then increased plasma membrane potassium conductance. Since the potassium equilibrium potential is more negative than the transmembrane potential, the neuron is hyperpolarized (*2*).

Table 1. Elemental peak integrals (mean ± standard deviation) from 36 nonilluminated granules (three neurons) and 12 illuminated granules (two neurons). Data are normalized to the chlorine peak integral (*5*).

Granules	Counts per elemental peak integral			
	Ca	P	Na	S
Nonilluminated	251 ± 100	1413 ± 606	377 ± 110	190 ± 89
Illuminated	63 ± 20	340 ± 231	186 ± 66	89 ± 18

In the experiments involving electron microscopy and electron microprobe analysis, the abdominal and pleural ganglia of *Aplysia californica* were removed under faint red light, which has no effect on the light response of the giant neurons. Then the giant neuron of each ganglion was exposed by dissection. Each ganglion of a pair was placed in artificial seawater (*3*) for 5, 10, 15, 30, or 60 minutes. During this time one ganglion was exposed to 10^4 erg cm^{-2} sec^{-1} of light transmitted by a fiber optics bundle connected to a tungsten halide source. The ganglia were then fixed in a 5 percent glutaraldehyde solution [0.1*M* *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) buffer, 20°C, *p*H 7.6, brought to about 1 osm with sucrose] for 2 to 6 hours. To ensure measurable levels of Ca, 10 m*M* $CaCl_2$ was added to the fixative in some instances. Individual giant neurons were then removed from the ganglia with forceps and washed three times, for 30 minutes per wash, in the same buffer solution. The neurons were postfixed for 30 minutes in a 1 percent OsO_4 solution; this was followed by three water rinses. The neurons were dehydrated in 75, 95, and 100 percent ethyl alcohol solutions and embedded in a modified Luft epoxy resin mixture (*4*). Sections 700 to 800 Å thick were cut with diamond knives on an Ivan Sorvall MT-2 ultramicrotome and mounted on Formvar-coated 200-mesh copper grids. Detailed ultrastructural studies were made with a Philips 300 transmission electron microscope.

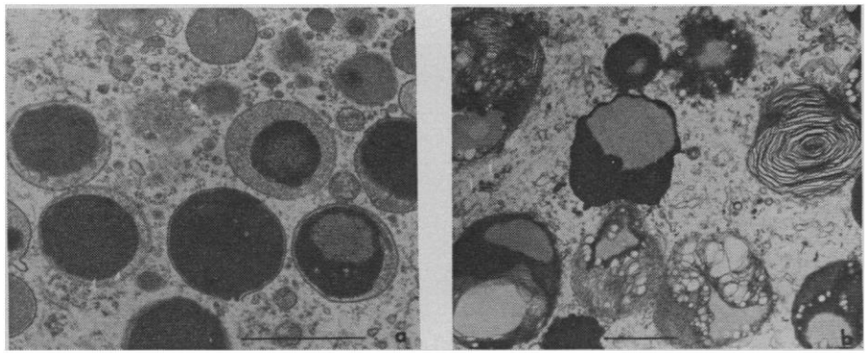

Fig. 1. Pigmented granules from *Aplysia* giant neurons. Representative granules from nonilluminated and illuminated neurons were subsequently analyzed with the electron microprobe; their spectra are shown in Fig. 2. (a) Granules from nonilluminated neurons prepared with 10 m*M* $CaCl_2$ in the fixative (see text). The single limiting membrane is evident. Electron-opaque material varies from granule to granule with material of lesser density appearing as ground substance. Membrane configurations were observed rarely in granules found in nonilluminated neurons. (b) Granules observed in a neuron illuminated for 10 minutes and fixed under the same conditions as in (a). The granules in these cells consisted of membranes in complex formations and arrays of vesicles within or in close proximity to the original single limiting membrane. Cytoplasmic clearing was commonly observed. Scale bars, 1 μm.

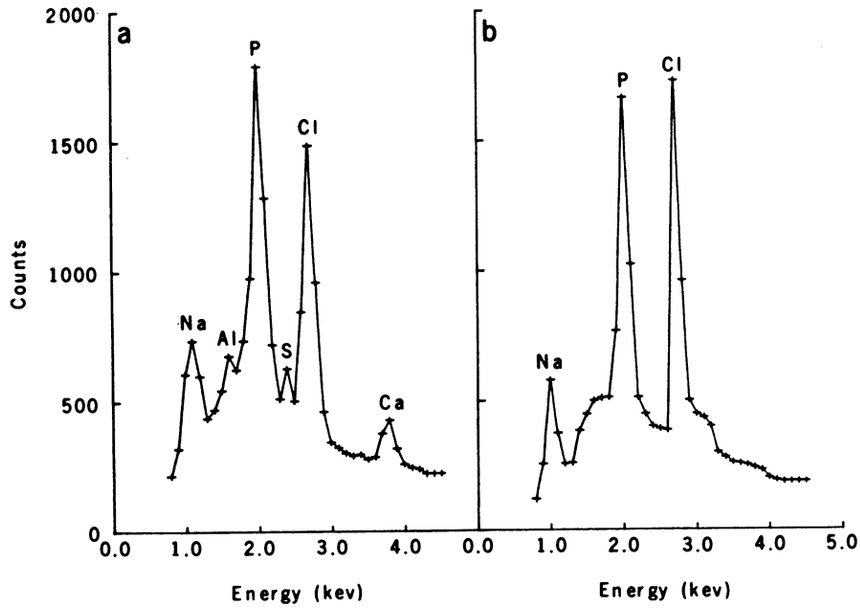

Fig. 2. Multichannel x-ray spectra obtained by electron microprobe. The energies of the emitted x-rays (abscissa) are functions of the atomic numbers of the elements being ionized by the incident electron beam. The elemental content is given by the area under each peak. Counting time was 200 seconds at an accelerating voltage of 20 kv. (a) Spectra from six nonilluminated pigmented granules from the cytoplasm of an *Aplysia* giant neuron. The Al peak is due to emissions from the coating on the SEM scintillator. (b) Spectra from six pigmented granules equivalent in volume to those used in (a) and illuminated with white light for 10 minutes. The major difference between the spectra in (a) and (b) is the total absence of the Ca and S peaks in (b). The Al peak is eliminated in (b) because the detector is now shielded from the scintillator.

Pigmented granules have a variety of appearances, but certain forms predominate in dark- as opposed to light-treated neurons. Hence dark granules commonly consisted of a moderately opaque background material bounded by a single membrane. In most instances various amounts of an electron-opaque osmiophilic aggregate were also present. These aggregates often contained smaller, clear areas less than 100 nm in diameter, some of which were stippled (Fig. 1a). In light-treated neurons the granules were commonly converted into complex arrangements of membranes or vesicles, or both (Fig. 1b). These changes were only readily apparent after a minimum of 10 minutes of illumination at the light intensity used. Taking into account differences in methods of fixation and illumination, these observations are similar to those reported by Henkart (*1*).

For electron microprobe analysis we used an energy-dispersive x-ray detector (Edax and Kevex). Grids prepared from the same samples used for ultrastructural studies were mounted in graphite specimen holders and examined on a Cambridge (S4-10) scanning electron microscope (SEM) in the transmission mode. The x-ray detector was positioned 5.5 mm from the sample

holders. All of the granules were observed at × 20,000 at an accelerating voltage of 20 kv. The images were positioned on the screen, and the scanning area was adjusted to include five to six dense granules or an equivalent area of adjacent cytoplasm. No other organelles were visualized in the scanning area. Equivalent periods of analysis were used for dark and light granules and ranged from 200 to 500 seconds, depending on count rates. In one experiment each of five dark granules was scanned for 200 to 500 seconds; the individual granule counts were summed and compared to similarly obtained sums for light granules.

Figure 2 shows two representative x-ray spectra, from which background has been subtracted, obtained from dark and light granules of two neurons fixed in the presence of added Ca. The characteristic peaks of the elements were definitely altered by illumination. There was less bremsstrahlung underlying the elemental peaks of the light granule spectra because all the elements were reduced in these spectra compared to dark granule spectra. In Fig. 2, for example, the spectrum obtained from light granules showed smaller peaks for Na and P, and Ca and S peaks were absent. The results were similar in the experiment in which individual dark and light granules were scanned.

Table 1 contains the mean integrated peak values for Ca, S, P, and Na for 48 pigmented granules from two illuminated and two nonilluminated neurons. Since the pairs of samples to be compared were of the same thickness and the areas scanned were similar, the volumes analyzed were almost equivalent. The chlorine in the resin used in the embedding process is a satisfactory standard for calibrating the elemental content of the sample (*5*). Table 1 shows that in light granules Ca and P were reduced to one-fourth and S and Na to one-half of the amounts present in dark granules.

One pair of giant neurons fixed in the absence of added Ca gave similar results for light and dark granules. However, in several other neurons fixed similarly, Ca was not detected in dark granules. The addition of 10 m*M* $CaCl_2$ to the fixative was sufficient to raise the Ca content of the dark granules to detectable levels. Thus, the spectra for illuminated and nonilluminated neurons probably reflect only the effects of illumination on elements that were bound or sequestered in the granules and were not removed by the fixation process, and the true elemental spectra of the granules may differ from the spectra shown in Fig. 2.

The Ca peak was greatly reduced or not evident in neurons illuminated for 10 minutes, whereas the more striking morphological alterations usually required at least that amount of illumination. It is possible that mobilization of specific elements from the pigmented granules precedes the ultrastructural changes.

Having demonstrated a light-induced release of Ca from the pigmented granules and knowing that both illumination and an increase in free intracellular Ca enhance membrane K conductance (*6*), we attempted to estimate the concentration of free Ca necessary for the light response to occur. The method for pressure injection of various solutions into *Aplysia* giant neurons has been described (*6*). Pressure injection of a Ca EGTA buffer with a free Ca concentration of $10^{-7}M$ or less reduced resting membrane conductance. The light response was abolished; but after 20 minutes it was restored (Fig. 3). However, a second or third injection abolished the response irreversibly. Control pressure injections of 0.2*M* KCl buffered to *p*H 7.2 had no effect on the light response. Injection of Ca EGTA buffers having a free Ca concentration of $10^{-6}M$ usually increased resting membrane conductance, but the light response, although reduced, persisted. This may be due to inadequate distribution of the injected solution such that localized light-evoked increases of Ca can still occur.

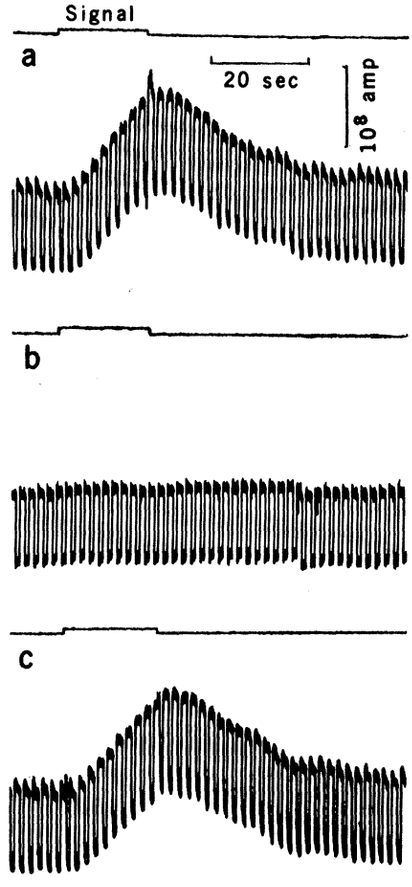

Fig. 3. Effect on the light response of intracellular injection of Ca EGTA buffer containing $10^{-8}M$ Ca into an *Aplysia* giant neuron. Neuron was voltage-clamped to −50 mv with 6-mv command hyperpolarizing pulses superimposed. Records show associated inward (downward) current pulses. (a) Control response to illumination at signal; note typical outward current and conductance increase. (b) Record 30 seconds after about 10^{-11} liter of the Ca EGTA solution was injected. A slight inward current and reduction in nonilluminated membrane conductance occur. The light response is abolished. (c) Twenty minutes later. The light response is restored.

These results indicate that (i) light produces a decrease in the Ca content of pigmented granules, and (ii) the light-evoked conductance change in the plasma membrane is abolished when free intracellular Ca is maintained below about $10^{-7}M$ (*6*); they are strong evidence for the essential role of Ca in the phototransduction process in the *Aplysia* giant neuron. We explain the light response of the *Aplysia* giant neuron as follows. The lipochondria granules in the neuronal cytoplasm contain a light-absorbing pigment or pigments (*7*) and Ca, which may be bound to the pigments or sequestered in the granules. Absorption of photons by the pigment releases Ca, and when the free intracellular Ca content doubles (*6*) an increase in plasma or surface membrane K conductance occurs. The amplitude and duration of the response are limited by the cellular mechanisms that regulate free Ca concentrations. Hence Ca release appears to be essential for this particular phototransduction process. While Ca has been implicated in vertebrate phototransduction, its role has not been clearly established, so the general applicability of these findings awaits further experimental evidence from other photoreceptors (*8*). However, there are similarities between the pigmented granules and rod outer segment disks since both are the major cellular loci for photopigment and both

are separate from the surface membrane. Hence phototransduction in both the *Aplysia* neuron and the vertebrate rod requires the release of a transmitter to couple photon absorption by pigment to current flow across the plasma membrane. It is also noteworthy that the light-induced morphological alterations of these large granules, which ultrastructurally resemble lysosomes, may be preceded by a depletion of calcium and other elements.

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3. The giant cells of the pleural and abdominal ganglia (referred to as R_2 in this ganglion) are homologous structurally and have equivalent responses to illumination.
4. J. H. Luft, *J. Biophys. Biochem. Cytol.* **9**, 409 (1961).
5. Intracellular chloride of cells fixed in the HEPES glutaraldehyde buffer was measured by using a chloride liquid ion exchanger microelectrode, as described by J. M. Russell and A. M. Brown [*J. Gen. Physiol.* **60**, 499 (1972)]. After fixation there was no measurable intracellular chloride, and since the chloride is free in solution in R_2 (see Russell and Brown) the only source of chlorine in the spectra is the epoxy resin mixture in which the specimen was embedded.
6. Calcium EGTA buffers were prepared by the method of R. W. Meech [*J. Physiol. (Lond.)* **237**, 259 (1974)]. The intracellular *p*H in R_2, which we measured with an H^+ glass microelectrode, ranges from 7.2 to 7.3. At *p*H 7.26 the apparent association constant for Ca EGTA is $10^{7.25}$ [J. Bjerrum, G. Schwarzenbach, L. G. Sillén, in *Stability Constants* (The Chemical Society, London, ed. 2, 1964), part 1, pp. 76 and 90; H. Portzehl, P. C. Caldwell, J. C. Rüegg, *Biochim. Biophys. Acta* **79**, 581 (1964)]. This calculation is only approximate for intracellular Ca since it ignores the effects of any intracellular Mg, which would increase the level of free Ca. A plot of the change in membrane conductance against the free Ca contained in a variety of Ca EGTA buffers injected into R_2 indicates a control level of about $1.1 \times 10^{-7} M$, which is slightly less than the values of $3 \times 10^{-7} M$ reported for squid [P. F. Baker, A. L. Hodgkin, E. B. Ridgway, *J. Physiol. (Lond.)* **218**, 709 (1971)] and $9 \times 10^{-7} M$ reported for *Helix* by Meech (see above). Illumination increases membrane conductance by 15 percent (*2*), and for such an increase the free intracellular Ca should have doubled.
7. A. Arvanitaki and N. Chalazonitis have reported [in *Nervous Inhibition*, E. Florey, Ed. (Pergamon, New York, 1961), pp. 194–231] and we have confirmed that the cytoplasmic pigmented granules demonstrate an absorbance peak at about 490 mm. The electrophysiological action spectrum also peaks at this wavelength (*2*).
8. W. A. Hagins, *Annu. Rev. Biophys. Bioeng.* **1**, 131 (1972).

19 November 1974

Partial Amino Acid Sequence of the Precursor of Immunoglobulin Light Chain Programmed by Messenger RNA in vitro

Abstract. *The five proteins programmed in a cell-free system by a mouse kappa light chain messenger RNA were labeled with [^3H]leucine and subjected to amino acid sequence analyses. In all five proteins, 20 amino acid residues precede the amino terminus of the mature protein, indicating that there is one major point for the initiation of messenger RNA translation. The abundance (30 percent) of leucine residues in the extra piece (leucine at positions 6, 7, 8, 11, 12, and 13) indicates that this moiety is hydrophobic. Furthermore, it seems that the precursor may have an additional extra piece at the carboxyl terminus.*

The possibility that the messenger RNA (mRNA) coding for the MOPC-321 immunoglobulin light (L) chain directs the synthesis of an L-chain precursor was initially suggested from the size of the cell-free products and from two-dimensional peptide mapping of their tryptic digests (*1*). In a cell-free system this mRNA programmed the synthesis of five proteins with molecular weights of about 28,700, 25,300, 19,700, 18,200, and 17,200. Despite the fact that none of these proteins are of the same size as the authentic L chain (24,020 molecular weight) (*2*), the peptide patterns they yield are composed almost entirely of L-chain peptides. Sequence analysis of one of the cell-free products showed that it contains an extra piece composed of 20 amino acid residues coupled to the NH_2-terminus of the mature protein (*1*). We report here radioactive amino acid sequence analyses of all of the five cell-free products that were labeled with [^{3}H]leucine and suggest how they are interrelated.

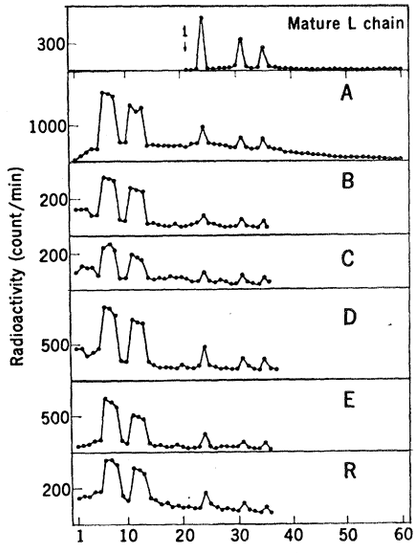

Fig. 1. Radioactivity determined from each cycle of Sequenator analyses of the mature M-321 L-chain marker (^{14}C, upper panel) and of cell-free products (^{3}H, samples A to R, lower panels) programmed by MOPC-321 L-chain messenger RNA. Molecular weights of the samples analyzed are given in Table 1. Numbers on the abscissa correspond to the cell-free products; the first Sequenator cycle for the mature L chain is indicated by the arrow.

The preparation of polysomes from MOPC-321 mouse myeloma, the isolation of L-chain mRNA from polysomes specifically precipitated with antibodies to L chain, and the translation of mRNA in the Krebs 2 ascites cell-free system have been described (*1*, *3*). More than 95 percent of the protein programmed by this mRNA was M-321 L chain (*1*). In our study the cell-free products labeled with [^{3}H]leucine (29.8 c/mmole) were resolved by electrophoresis on sodium dodecyl sulfate–10 percent polyacrylamide gels with 0.42*M* tris · HCl (*p*H 9.18) as the running buffer (*4*). After the electrophoresis (3 hours; 2 ma per tube), 110 slices per gel were prepared. The material was eluted by shaking each slice in 0.5 ml of 8 m*M* tris · HCl (*p*H 9.18) for 24 hours at room temperature. Portions (25 μl) were counted; the radioactive pattern obtained showed five peaks that corresponded to the five protein bands previously characterized in the cell-free reaction mixture (*1*). The peaks were collected separately. They are designated here A, B, C, D, and E (Table 1). To gain information on the totality of cell-free products, the small amount of material that was eluted between each of the peaks A to E was also collected and pooled to yield sample R. The radioactivity eluted in samples A to E and sample R was 72 percent of the amount applied to the gel. Nonradioactive M-321 L-chain protein (6 mg) was added to each sample. Sample A was precipitated with 10 percent trichloroacetic acid, washed once with 5 percent trichloroacetic acid and three times with a mixture of 95 percent acetone and 5 percent water (by