CYTOPLASMIC PARTICLES CARRYING RAPIDLY LABELED RNA IN DEVELOPING INSECT EPIDERMIS

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The fate of messenger RNA, from chromosome to polysome, is of obvious interest. In many cell types, the cytoplasmic particles which contain the most rapidly labeled RNA are significantly smaller than ribosomes. Following a short exposure to radioactive uridine, preparations of cytoplasm can be obtained which, when fractionated on sucrose density gradients, show considerable labeling in the region of 40S to 50S, where the smaller of the two ribosomal subunits also occurs free.[1-6] At least part of this labeled RNA may be ribosomal, but messenger and possibly other RNA species are also represented. In HeLa cells, this newly synthesized RNA hybridizes with DNA more efficiently than the bulk ribosomal RNA;[2] in cells infected with vaccinia virus, vaccinia mRNA is associated with 40S particles.[3] It seems that, upon its entry into the cytoplasm, newly synthesized mRNA may be found in association with particles similar in size to the small ribosomal subunit. However, in very early embryos such particles occur in discrete size classes, none of which coincides with ribosomal subunits.[4]

These observations have been interpreted in two different ways. According to one view, newly synthesized mRNA attaches itself to a small ribosomal subunit in the nucleus, and traverses the cytoplasm in the form of such a complex.[2, 3, 5] Alternatively, messenger-containing particles ("informosomes") distinct from ribosomal subunits have been postulated.[4, 5] Two kinds of evidence for the first view have been offered: the unique sedimentation value of pulse-labeled particles in HeLa, which nearly coincide with the small subunit, and the well-known capacity of that subunit to bind mRNA. Binding of mRNA to the small subunit may, in fact, represent an obligatory step in the initiation of protein synthesis.[7] In contrast, the informosome concept receives support from the polydisperse nature of the particles in sea urchin and fish embryos and from their relatively low buoyant density. When the labeled particles are fixed with formaldehyde and then banded in a CsCl gradient, they are seen to be lighter and hence probably richer in protein than the small subunit itself;[6] an mRNA-charged subunit might be expected to be richer in RNA and hence *denser* than the subunit alone.

As Spirin has pointed out,[8] the two theories need not be mutually exclusive. In developmentally stable cells such as HeLa, a limited repertoire of messengers may be transported into the cytoplasm on small subunits for immediate translation. In developmentally active systems such as embryos, where the pattern of protein synthesis undergoes programmed and radical changes, informosomes may present augmented opportunities for controlling the translation of numerous new messages. The present study is part of an attempt to follow the fate of mRNA in the epidermal cells of giant silkworms undergoing metamorphosis.

At the beginning of adult development, the wing of a silkworm pupa is clearly differentiated: it is irreversibly epithelial in nature and has already secreted a pupal cuticle. Yet it is also embarked on a program of rapid and profound change, involving cell proliferation, elaboration of a distinctive adult cuticle, production of molting fluid, and morphogenesis of intricate cuticular scales. Elsewhere in the insect, epidermal cells may specialize to produce lipids, specific proteins, or enzymes.[9] Thus, the epidermis of developing silk moths is an outstanding example of a differentiated yet transforming tissue. In addition, it forms spatially distinct, specialized organs; it is available in relatively large quantities and at a variety of predictable developmental stages; it responds to the developmental hormones, ecdysone and juvenile hormone. In sum, it is a favorable system for studying the molecular aspects of development,[9] including the transport of mRNA within metazoan cells.

Materials and Methods.—Epidermal tissues were dissected from developing silk-moth (*Antheraea polyphemus*) wings from animals in the first 6 days of adult development and galeae (paired epidermal exocrine glands) from animals at day 9–11. The tissue was rinsed with Grace's medium[10] fortified with streptomycin and with phenylthiourea (PTU) to inhibit melanization; it was then transferred into fresh medium containing uridine-H^3 at a concentration of 200 μc/ml (*ca.* 4 c/mmole) and placed on a rotary shaker at 25°. At the end of the desired period of incubation, the tissue was chilled and washed repeatedly with ice-cold Weevers' saline,[11] which was diluted with 0.2 vol H_2O so that it would reach isotonic concentration. The tissue was subsequently minced and transferred to 20% sucrose in HEK–1 mM Mg^{++} (0.01 M HEPES–KOH buffer,[12] pH 7.4; 0.01 M KCl; and 1 mM $MgSO_4$). Three min later, the sucrose concentration was lowered to 8% by the addition of 1.5 vol HEK–1 mM Mg^{++} without sucrose. The tissue was finally exposed to an osmotic shock by rinsing and resuspending in *ca.* 5 vol of fresh, sucrose-free HEK–1 mM Mg^{++}. The following RNase inhibitors were added: 1 mg/ml washed Macaloid (Baroid Division, National Lead Co.), 5 μg/ml polyvinyl sulfate, and 3 mg/ml commercial RNA (*E. coli* sRNA, General Biochemical Co., or highly polymerized yeast RNA, grade A, Calbiochem). After 3–5 min, the cells were gently homogenized with several strokes of a Dounce homogenizer (typically, five to ten with the loose-fitting A pestle and two with the B pestle). A nonionic detergent (Tween-40 or Brij) was then added to a concentration of 0.5%, and the homogenate exposed to another two strokes of the A pestle. Homogenization was monitored with a phase microscope and was terminated when the majority of nuclei were free of cytoplasm but retaining their granularity and physical integrity. Special care was taken to preserve the nuclei intact, so as to avoid contamination of the cytoplasm. Debris, nuclei, mitochondria, etc., were removed by sedimentation (first at 1,000 g for 2 min and then at 10,000 g for 5 min). The supernatant was clarified with 0.5% sodium deoxycholate (DOC) and layered on linear sucrose density gradients (usually 20–35%, in HEK–1 mM Mg^{++}). After centrifugation in a Spinco model-L or International B-60 ultracentrifuge, fractions were collected, either with an automatic ISCO density gradient fractionator-ultraviolet analyzer or manually. Aliquots were precipitated with cold 7% TCA, collected on millipore filters, and counted in a liquid scintillation spectrometer. In occasional experiments, slightly different conditions were used, as indicated.

Preparatory to centrifugation in CsCl, particles obtained from sucrose gradients were aldehyde-fixed for 12–24 hr at 4°. The fixative was either 5% formaldehyde,[13, 14] or 5% formaldehyde plus 1.2% acrolein, in HEK–1 mM Mg^{++}. In some experiments, the unfractionated cytoplasmic preparation, immediately after homogenization, was lightly prefixed for 3 hr with 3% formaldehyde plus 0.8% acrolein; following centrifugation in sucrose gradients, aliquots were again postfixed in the usual manner. Fixed particles were dialyzed and incorporated in the lighter of the two solutions used to form gradients of CsCl (Harshaw Chemical, optical grade). Mg^{++} was added either in a uniform con-

centration of 2 or 20 mM, or in a gradient of 20–40 mM, paralleling the CsCl gradient. Upon centrifugation for *ca.* 15 hr, in either the Spinco SW-39L or the International SB-405 rotor, fractions were collected in 0.01% gelatin;[15] after gravimetric or refracto-metric determination of the density at 25°C, they were precipitated with TCA and counted.

Results.—Sucrose density gradients: The UV-absorption profile of cytoplasmic preparations includes a major peak of single ribosomes (monoribosomes) and two minor peaks attributable to ribosomal subunits (Fig. 1). The monoribosome peak is often skewed toward the heavy side because of ribosome dimers and degraded polysomes. Assuming a value of 80*S* for the sedimentation coefficient of mono-ribosomes,[16] the large and small subunits correspond to about 65*S* and 42*S*, respectively.

With very short pulses of uridine, acid-precipitable radioactivity is largely confined to the top of the gradient, at 20*S* or less. In the galea, a pulse of about one hour is sufficient for a radioactive peak to appear in the region of the small subunit, above a background of polydisperse label (Fig. 1*A*). Shortly there-after, distinct peaks are also observed in the regions of the large subunit and the monoribosomes (Fig. 1*A*). The monoribosome peak gradually increases, both in absolute magnitude and relative to the other two peaks; by five hours, radio-activity is nearly coincident with absorbance (Fig. 1*B*). Similar results are ob-tained without DOC treatment and with any Mg^{++} concentration within the range of 1–3 mM. Higher Mg^{++} levels cause extensive and variable precipita-tion at the low concentration of monovalent cations employed.

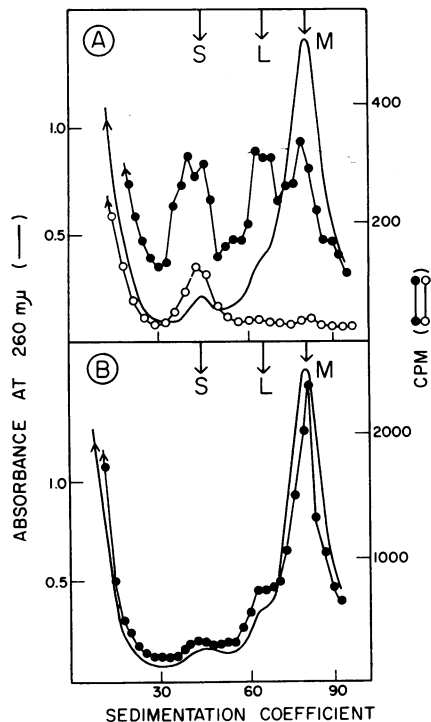In the wing, cytoplasmic labeling is more delayed (Fig. 2). At 1.25 hours, a

FIG. 1.—Sedimentation analysis of cyto-plasmic particles from galea. Cytoplasm was prepared as described under *Materials and Methods* and was centrifuged in 15–30% sucrose gradients containing 0.01 *M* pH 7.4 Tris–HCl buffer, 0.01 *M* KCl, and 1 mM Mg^{++} (except for —O—; see below).

Centrifugation was for 4 hr, at 35,000 rpm in the SW-39L rotor. Sedimentation is repre-sented from left to right in this and in all subsequent figures. Sedimentation coefficients were assigned by using the monoribosomes at 80S as a standard. The approximate posi-tions of the bulk monoribosomes (*M*) and the large (*L*) and small (*S*) ribosomal subunits are indicated.

(*A*) —O—: Radioactivity of a preparation labeled for 0.8 hr (homogenized and centri-fuged in 3 mM Mg^{++}); ——: absorbance, and —●—: radioactivity of a preparation labeled for 1.25 hr.

(*B*) ——: Absorbance, and —●—: radio-activity of a preparation labeled for 5 hr.

low level of polydisperse radioactivity can be detected, decreasing from a minor peak at 20S to a low plateau at about 50S.   At 2.5 hours, definite peaks coincide with the small and large subunits and with the ribosomes, the one at 42S being the more pronounced.   However, the majority of the label is polydisperse in distribution; the specific activity (cpm per A_{254}) is maximal in the "troughs" between the peaks, as well as in the polysome region.   Even at 5 hours, most of the label in subribosomal particles is polydisperse, although considerable ribosomal label has accumulated.

CsCl gradients: The polydisperse distribution of radioactivity suggests that, at least in the wing cytoplasm, some of the most rapidly labeled particles are not newly formed subunits.   This is confirmed by equilibrium density centrifugation of particles derived from the wing.

When particles from the 30S to 60S region of sucrose gradients are fixed and centrifuged in CsCl, they form a single major UV-absorbing band, which corresponds to the bulk, or mature small subunit.   The average buoyant density, ρ, of this band is 1.50 ± 0.02 gm/cm^3.   Radioactive material is distributed in a strikingly different manner (Fig. 3).   No distinct peak is coincident with the absorption band itself.   Instead, a radioactive peak 0.04 density units lighter than the bulk particles occurs regularly; presumably it corresponds to newly syn-
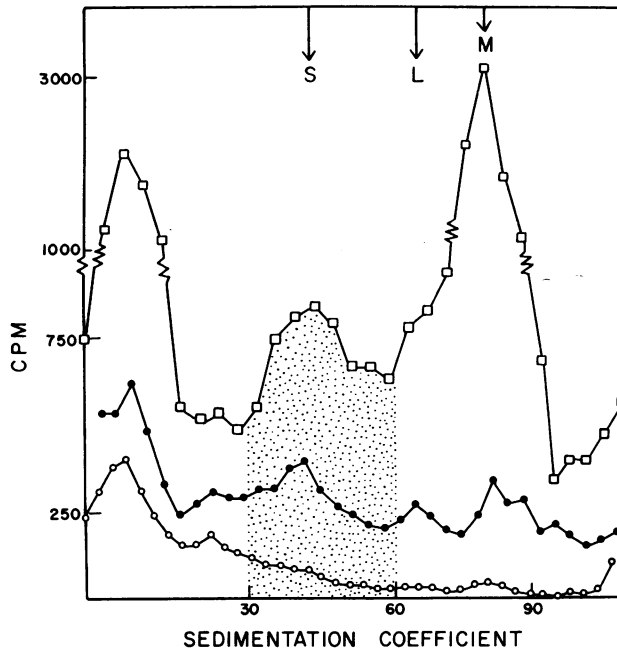

FIG. 2.—Sedimentation analysis of cytoplasmic particles from wing (only radioactivity is displayed).   Each preparation was derived from the wings of one day-4 animal, labeled for 1.25 hr (—O—), 2.5 hr (—●—), or 5 hr (—□—), respectively.   Sedimentation in 20–35% sucrose gradients, containing HEK–1 mM Mg^{++}, at 37,000 rpm for 7 hr (SB-283).   Other details are as in Fig. 1.   The stipling indicates the range of particles commonly selected for further analysis on CsCl gradients.

thesized subunit. A similar density difference between new and "mature" subunits has been reported in L cells.[14]

More significantly, the vast majority of radioactivity is polydisperse in density and coincides with only traces of UV-absorbing material (Figs. 3 and 4). This polydisperse label is most evident in the region of $\rho = 1.32$ to $\rho = 1.42$, although it also occurs outside this range; it is thus 0.08–0.18 unit lighter than the bulk subunit. By contrast, four-hour-labeled particles in the region of 80S are nearly coincident in density with the bulk monoribosomes.

Occurrence and stability of light particles: Particles banding at $\rho = 1.32$ to $\rho = 1.42$ are observed whether formaldehyde alone or formaldehyde plus acrolein are used for fixation (Fig. 3), and irrespective of whether or not the unfractionated cytoplasm was prefixed. They survive dialysis against 0.1 M KCl at pH 8.3 prior to fixation, although like the subunit they shift to slightly higher density; these conditions of pH and ionic strength tend to dissociate extraneous protein from ribosomes,[16] and would thus be expected to minimize artifacts caused by preferential protein binding. They are observed in CsCl gradients containing as little as 2 mM Mg^{++}.

To some extent the light particles are distinguishable from the bulk subunit even in sucrose gradients: they correspond to the most rapidly labeled, polydisperse material. Thus, they are more abundant, relative to newly synthesized small subunit, in preparations labeled for short periods (Fig. 4); the newly synthesized subunit is more abundant in sucrose gradient fractions taken from the middle of the UV-absorbing 42S band (Fig. 4); and the light particles are more abundant in fractions from either side of the 42S absorbance peak (Fig. 3A).

In vitro formation of light particles: Are these particles native cell components,

FIG. 3.—Equilibrium density centrifugation analysis of 30–60S particles in CsCl gradients. Particles were obtained from wing cytoplasm labeled for 4.5 hr and fractionated on sucrose gradients without fixation. The 30–37S and 47–60S fractions were derived from two animals, the 38–46S from only one; thus, the pooled preparation is enriched in particles which are polydisperse in sucrose gradients and do not coincide with the small subunit.

The particles were divided into two equal aliquots, fixed for 14 hr in formaldehyde (A) or formaldehyde plus acrolein (B), dialyzed against HEK–1 mM Mg^{++}, and banded in CsCl containing 20 mM Mg^{++}. Centrifugation was in the SW–39L rotor for 11 hr at 39,000 rpm. The position of the UV-absorbing bulk subunit (S) and the approximate density range of light particles are indicated.

———: Bulk particles (predominantly mature small subunits), detected through their absorbance; —O—: newly synthesized particles, detected through their radioactivity; —●—: density of the buoyant solution.
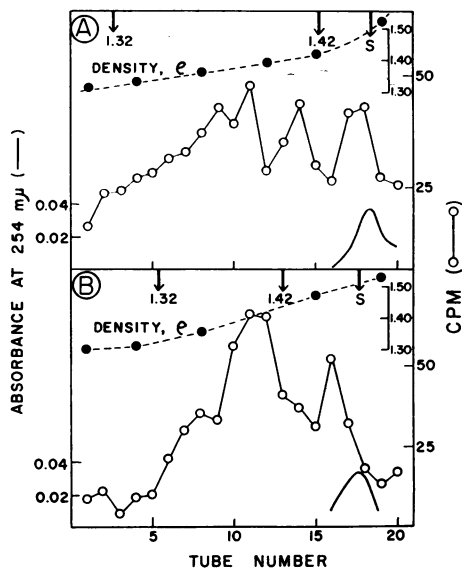
or are they artifacts of homogenization and subsequent processing—formed, for example, by association of free, newly synthesized RNA with cytoplasmic proteins? In a preliminary attempt to answer this question, labeled nuclear RNA was extracted with a modified cold phenol–1% sodium dodecyl sulfate method[17] from wings incubated for four hours with uridine, and was added to unlabeled wings at the time of homogenization. Aliquots of the cytoplasmic homogenate were fractionated as usual on sucrose gradients with or without a preliminary fixation. The 35S to 55S fractions of each gradient were pooled, fixed in formaldehyde or formaldehyde plus acrolein, and centrifuged in CsCl. Purified RNA alone, not mixed with cytoplasm, sedimented to the bottom of the tube, since it was denser than a saturated solution of CsCl. In the presence of cytoplasm, most of the labeled RNA became lighter and remained in solution. More than half of it banded at densities at least as high as the UV-absorbing bulk subunit; presumably, this RNA had combined with free subunits[7] or with very small amounts of protein. However, some labeled particles of low density were also formed; the radioactivity profile varied with the method of fixation, indicating that some fixation procedures can, indeed, introduce artifacts. Light particles were most abundant in preparations not exposed to preliminary fixation, but postfixed with both formaldehyde and acrolein, after fractionation on the sucrose gradient (Fig. 5). Even in this case, however, the artificial light aggregates were not quite as light as the native particles: they had an average density of about 1.41 gm/cm³.

Discussion.—In the cells of developing insect epidermis, newly synthesized small subunits are the first ribosomal components to reach the cytoplasm. This is confirmed by sedimentation analysis of rapidly labeled, phenol-extracted RNA: in both wing and galea, labeled 17S ribosomal RNA appears in the cytoplasm prior to the 27S species.[17] The new subunits are distinguished from
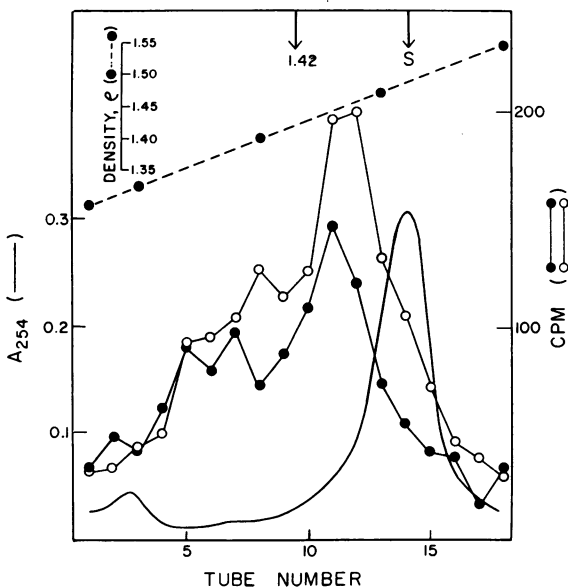

FIG. 4.—Relative abundance of light particles as a function of incubation time. Cytoplasmic homogenates from wings labeled for 2 or 6 hr were analyzed on separate sucrose gradients, without preliminary fixation. Particles from the 37–47S region (which includes most of the bulk small subunit) were fixed with 5% formaldehyde for 16 hr, dialyzed, and centrifuged in CsCl gradients (SB-405 rotor, 50,000 rpm, 20 hr, 20–40 mM Mg++).
—●—: Radioactivity of the 2-hr particles (3.5 animals).
—○—: Radioactivity, and ——: absorbance of the 6-hr particles (2 animals).
— —●— —: Density of the buoyant solution.

Fig. 5.—Formation of artificial light aggregates by incubation of labeled purified RNA with unlabeled wing cytoplasm. RNA and wing cytoplasm were mixed as described in the text and fractionated on a sucrose gradient, without preliminary fixation. The 35–55S fractions were fixed for 17 hr with formaldehyde (—O—) or formaldehyde and acrolein (—●—). They were then dialyzed and centrifuged in CsCl containing 20 mM Mg^{++} (SW-39L).
———: UV-absorbing material from the formaldehyde-fixed preparation, with the peak at $\rho = 1.51$ corresponding to the small subunit (S).
—●—: Density of the buoyant solution.
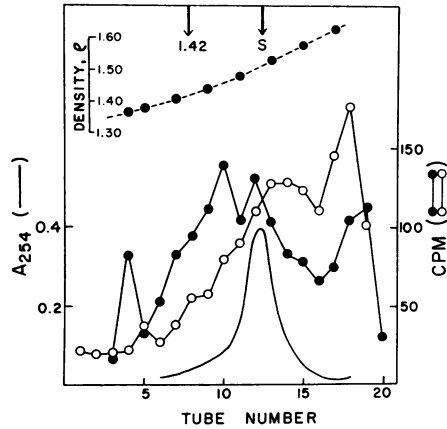
the mature small subunits by their lower buoyant density, which may indicate an additional protein component (about 10% of the total protein). These observations are in complete agreement with the results of Perry and Kelley,[14] which were obtained with mammalian cells.

Taken at face value, the present study also suggests that the cytoplasm of developing wing epidermis contains additional, distinct particles which serve as carriers for rapidly labeled RNA. These particles are polydisperse in sucrose gradients, having sedimentation coefficients of approximately 20–70S. Because of their very low buoyant density, they can be distinguished in CsCl gradients from both mature and newly synthesized ribosomal subunits. Their density indicates that, if they consist exclusively of protein and RNA, they may have a protein content of 75 per cent on the average, and up to 85 per cent in some cases.[14] Clearly, they are not merely mRNA-charged small subunits: mature and newly synthesized subunits contain 55 and 60 per cent protein, respectively. It is tempting to identify the light particles with the informosomes described in developing embryos. The properties of their RNA, apart from a high turnover rate, remain to be elucidated.

Light particles have been observed after four different fixation procedures, in two media of different pH and ionic strength, and in 2–40 mM Mg^{++}. Since they occur in preparations fixed immediately after homogenization, they are probably not the result of preferential RNase degradation[18] of newly synthesized subunits. There is some evidence of their existence even in sucrose gradients, prior to fixation. They are also observed in gradients of Cs_2SO_4 and in cesium polycarboxylates.[19] In sum, they cannot be easily dismissed as artifacts.

However, their existence in the intact cell is by no means established as yet. They could be formed either during homogenization or during fixation. Spontaneous *in vitro* association of mRNA with cytoplasmic proteins[20] would be interesting in itself; fixation artifacts would merely be misleading. Figure 5 indicates that some fixation procedures can, indeed, introduce artifacts; this emphasizes the potential pitfalls in our present methods, even though the artificial and native particles were not identical in that particular experiment. Clearly, methods should be developed for equilibrium density centrifugation of

unfixed particles, both to circumvent the uncertainties introduced by fixation and to allow additional studies, after fractionation according to buoyancy. Such improved techniques are the subject of a forthcoming paper.

Summary.—After a pulse of uridine-H³, the first cytoplasmic particles to be labeled are smaller than the 80S ribosomes. Some of the radioactivity is attributable to newly synthesized ribosomal subunits, which enter the cytoplasm independently, the small subunit prior to the large. However, the polydisperse nature of the radioactivity, especially after short pulses, indicates the presence of additional components in the wing cytoplasm. When the 30–60S fractions from sucrose gradients are aldehyde-fixed and banded in CsCl, radioactivity is observed in two major classes of particles: newly synthesized small subunits (which band at a density 0.04 gm/cm³ lighter than the mature subunits) and polydisperse particles, banding predominantly at $\rho = 1.32$ to $\rho = 1.42$. Thus, a major fraction of newly synthesized RNA occurs in the cytoplasm in particles which may contain 75 per cent or more protein. These light particles are stable under a variety of conditions and are observed with several different fixation procedures. If they proved to be native cell constituents, they would support the informosome model of mRNA transport.

Abbreviations used: mRNA, messenger RNA; PTU, phenylthiourea; HEPES, N-2, hydroxyethylpiperazine-N′-2-ethanesulfonic acid; HEK, 0.01 M HEPES–KOH, pH 7.4, plus 0.01 M KCl; DOC, sodium deoxycholate.

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