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Rabbit Haemoglobin Synthesis in Frog Cells: the Translation of Reticulocyte 9 s RNA in Frog Oocytes

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Rabbit Haemoglobin Synthesis in Frog Cells: the Translation of Reticulocyte 9 s RNA in Frog Oocytes

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4 s + 5 s RNA, 9 s RNA, 18 s RNA, 28 s RNA and polyribosomes, all prepared from rabbit reticulocytes, were injected into living oocytes of the frog *Xenopus laevis*. Only after injection of 9 s RNA or polyribosomes was haemoglobin-like material formed. Haemoglobin was identified by various criteria: gel filtration, carboxymethyl cellulose chromatography, acrylamide gel electrophoresis and ion exchange chromatography of tryptic peptides. 9 s RNA promoted the formation of globin-like material, whether injected with haemin or without haemin.

It is concluded that when injected into a living cell, the 9 s RNA is fairly stable and has the properties of a haemoglobin messenger. The messenger requires no reticulocyte-specific factors for translation, and the translational machinery of the oocyte will accept the messenger RNA from a totally different cell type, from another species. At all stages of assembly and synthesis haemoglobin molecules are reasonably stable in oocytes.

1. Introduction

Several important questions in developmental biology could be answered if it were possible to combine messenger RNA from one kind of cell with the translational apparatus of another type of cell. Such an experiment could provide proof of the identity of a kind of messenger RNA, and could test the species and cell type specificity of translational systems. One way to accomplish this experiment would be to purify RNA from one cell and insert it into a different sort of cell. We describe here experiments involving the microinjection of rabbit reticulocyte 9 s RNA or polysomes into living frog oocytes.

2. Materials and Methods

(a) *Handling of Oocytes*

The *Xenopus laevis* females used to provide oocytes had been induced by hormone treatment to lay eggs between 2 and 4 weeks before the time when their oocytes were taken for micro-injection. The injection procedure and culture medium were as described by Gurdon (1968) and Gurdon, Lane, Woodland & Marbaix (1971). Dissected oocytes were maintained in culture medium for up to 6 hr before injection. The injection of the RNA preparations used appeared to have no adverse effect on the viability of oocytes, since

the injected cells were indistinguishable, for as long as 24 hr after injection, from uninjected or pricked oocytes. Oocytes were labelled by incubation in culture medium containing the appropriate labelled amino acid at between 0·1 and 1·0 mCi/ml. (specific activity of [^{3}H]histidine was 30 to 50 Ci/m-mole; and of [^{3}H]isoleucine was 400 mCi/m-mole). In most cases, labelling was begun immediately after micro-injection. For some experiments involving polyribosomes, the oocytes were preincubated in a solution of the radioactive amino acid for several hr. In other experiments, radioactive amino acids were injected separately or were injected mixed with the sample.

At the end of the labelling period, oocytes were given one wash with culture medium and were then stored at −50°C until homogenized.

(b) *Production of reticulocytes*

For the preparation of 9 s mRNA, rabbits were injected with a 2·5% w/v solution of phenylhydrazinium chloride in distilled water; 1 ml. of solution was injected each day for 4 days, and animals were killed on the sixth day. A more prolonged course of injections (up to 9 days) was given when polyribosomes were needed for micro-injection experiments. Reticulocytosis was confirmed by staining with brilliant cresyl blue.

(c) *Preparation and incubation of polyribosomes*

Polyribosome pellets were prepared according to the method of Luzzatto, Banks & Marks (1965), except that heparin 100 i.u./ml. was added to the lysis buffers (Rowley & Morris, 1967).

pH 7·4 buffer (0·5 M-Tris; 0·025 M-KCl, 0·005 M-$MgCl_2$ adjusted to pH 7·4 with HCl) was added to the 105,000 *g* pellet. After shaking at 4°C for 60 min, on a wrist-action shaker, the pellet was dispersed using a glass rod. The solution was shaken for a further 60 minutes. Suspensions at a concentration of approximately 40 mg ribosomes/ml. were used both for micro-injection experiments, and in cell-free systems.

Cell-free systems were prepared according to Luzzatto *et al.* (1965), except that the final incubate contained $4{\cdot}8 \times 10^{-5}$ M-asparagine and $3{\cdot}2 \times 10^{-5}$ M-glutamine. Moreover, haemin (prepared according to Zucker & Schulman, 1967) at a final concentration of $3{\cdot}2 \times 10^{-5}$ M was present, since it was required for the formation of whole haemoglobin molecules. [^{3}H]Histidine, at 100 to 3000 mCi/m-mole, was the only source of radioactivity. Following incubation at 20°C, 0·2 ml. samples were applied directly to Sephadex columns, having been mixed with an equal vol. of a solution of 0·5 M-KCl–0·05 M-Tris·HCl–haemoglobin 2 mg/ml. (pH 7·0). Sephadex columns were equilibrated and eluted with 0·5 M-KCl–0·05 M-Tris, pH 7·0.

(d) *Preparation of 9 s RNA from reticulocytes*

As described by Huez, Burny, Marbaix & Lebleu (1967) reticulocyte polyribosomes were treated with 33 mM-EDTA; the 14 s mRNA released was purified by sucrose gradient centrifugation. The sucrose gradient fractions which contained the mRNA were pooled and the solution was made 0·4 M in NaCl and 1% (w/v) in sodium dodecyl sulphate; 2 vol. of ethanol were added, then RNA and protein were allowed to precipitate by standing overnight at −20°C. The precipitate was collected by low speed centrifugation and was dissolved in a solution of 1% (w/v) sodium dodecyl sulphate in water (1 ml. of solution per 100 μg of RNA). 1-ml. portions of the RNA solution were layered on 36 ml. 10 to 20% sucrose gradients made in 10 mM-Tris·HCl (pH 7·4). After centrifugation (using a Spinco SW27 rotor) for 40 hr at 25,000 rev./min, fractions were collected by piercing the bottom of the tube. Fractions corresponding to the pure 9 s RNA were pooled and were made 0·4 M in NaCl. Two vol. of ethanol were added and the RNA was allowed to precipitate at −20°C overnight. The RNA was collected by low speed centrifugation and dissolved at a concentration of 250 μg RNA/ml. in 10 mM-Tris·HCl buffer (pH 7·4). Residual proteins were removed by 2 extractions with a mixture of chloroform–iso-amyl alcohol (24:1 v/v). The RNA solution was then dialysed for 15 hr against 2 changes of 1000 vol. of double-distilled water; the RNA solution was then lyophilized.

(e) *Preparation of 28 s, 18 s, 4+5 s RNA from reticulocytes*

Following the centrifugation of dissociated reticulocyte polyribosomes (as described previously), the appropriate regions of the sucrose gradient were pooled. The subsequent procedure was the same as that used for the preparation of 9 s RNA. In the case of 28 s RNA, the sucrose gradients used to purify the RNA were centrifuged for 12 hr at 25,000 rev./min, and consisted of 10 to 20% linear gradients made in Spinco SW27 tubes. In the case of 18 s RNA, either 1 or 2 successive sucrose gradient purifications were performed; 10 to 20% linear gradients were made in Spinco SW27 tubes, and were spun for 20 hr at 25,000 rev./min.

(f) *Injection mixture*

Samples were kept under oil and at 4°C, ready for micro-injection. Polyribosomes. suspended as described previously, were mixed with a haemin solution of 10 mg/ml, Tris·*in* KCl buffer, pH 7·8 (Zucker & Schulman, 1967), in the ratio 20:1 v/v. Using a micro-dialysis apparatus, RNA solutions were dialysed against 88 mM-NaCl, 1·0 mM-KCl, 15 mM-Tris·HCl (pH 7·6) for 20 min at 0°C. When desired, RNA solutions were mixed with the concentrated haemin solution, in the ratio 20:1 v/v.

(g) *Homogenization of oocytes*

Thawed samples of oocytes were homogenized in 0·0522 M-glycine–0·0522 M-Tris, containing 0·1% (w/v) histidine monohydrochloride, to which rabbit haemoglobin had been added to give a final concentration of from 2 to 5 mg/ml.; the final pH was 8·9. Homogenates were centrifuged at 3100 *g* for 15 min at room temperature (polyribosome experiments) or at 75,000 *g* for 30 min at 4°C (messenger RNA experiments), and the supernatant applied directly to a 140 cm × 1 cm G100 Sephadex column, equilibrated with 0·0522 M-glycine–0·0522 M-Tris (pH 8·9) buffer.

(h) *Acrylamide gel electrophoresis*

Samples were prepared for analysis according to Moss & Ingram (1968). Thus, a sample containing 2 to 5 μg of marker rabbit haemoglobin, dissolved in a solution containing 0·2% $K_3Fe(CN)_6$, 0·05% KCN and 0·01% $NaHCO_3$, was applied to each gel. The Tris·glycine pH 8·9 analytical gel electrophoresis system of these authors was used, but without a "spacer" gel. Gels were run for 90 min at a constant 5 mA per gel.

The distribution of radioactivity throughout the gel was measured by cutting the gel into 1-mm slices which were added to 0·5 ml. of a mixture of equal volumes of glacial acetic acid and 6% (w/v) hydrogen peroxide, and the slices were dissolved in this solution by heating overnight at 70°C; 0·3 ml. of 3% sodium dodecyl sulphate was added before the whole was dissolved in 15 ml. of the scintillation fluid described below.

Gels were scanned using a Gilford Spectrophotometer; some gels were stained using the benzidine–peroxide method before scanning.

(i) *Carboxymethyl cellulose chromatography*

Globin samples were prepared by the method of Schapira, Rossa, Maleknia & Padieu, except that the acid–acetone used contained 0·1% (v/v) β-mercaptoethanol and 0·1 (v/v) 12 N-HCl. A few drops of β-mercaptoethanol were added to samples, which were derived from Sephadex columns or homogenates, before the acid–acetone extraction step.

Globin was analysed by the method of Dintzis (1961), as outlined by Schapira *et al.* (1968), except that a 50 cm × 1 cm Whatman CM 52 column was used. When globin chains were rechromatographed slightly different buffers were used to those of Schapira *et al.*, in that gradients were from 0·239 N-formic acid–0·0196 N-pyridine to 2·39 N-formic–0·196 N-pyridine.

(j) *Tryptic digestion of haemoglobin samples*

^{14}C-labelled rabbit haemoglobin was prepared by suspending 2 ml. of rabbit reticulocytes in 5 ml. of an isotonic buffer (0·14 M-NaCl, 5 mM-Mg acetate, 15 mM-KCl, 5 mM-Tris·HCl (pH 7·4)) and incubating the suspension which contained 5 μCi [^{14}C]histidine/ml. for 1 hr at 37°C. Two vol. of distilled water were added to lyse the cells, and the lysate was left

for 2 min before centrifugation. The lysate was centrifuged at 15,000 *g* for 15 min at 4°C, and the resulting supernatant was adjusted to pH 5 with dilute acetic acid: the solution was given another low speed centrifugation and the final supernatant was dialysed, for 20 hr, against 2 changes of 1000 vol. of 0·1 M-NH_4HCO_3.

[^{3}H]histidine-containing material was collected from the haemoglobin region of Sephadex fractionations, and was dialysed for 20 hr against 2 changes of 1000 vol. of 0·1 M-NH_4HCO_3.

^{3}H-labelled haemoglobin from oocytes was mixed with ^{14}C-labelled carrier rabbit haemoglobin to give a ratio 10:1 ^{3}H:^{14}C counts. 1 mg of TPCK-trypsin (Worthington Biochemicals, 240 i.u./mg) was added to 8 ml. of the mixed haemoglobin solution; the enzymic digestion was allowed to proceed for 24 hr at 37°C. The solution then underwent 3 cycles of lyophilization and dissolution (in distilled water) before the peptides were finally dissolved in solution A (see below).

(k) *Cation exchange chromatography of peptides*

Peptides were separated on a 50 cm × 1 cm column of Technicon chromobeads (type P) maintained at 37°C; separation of peptides was achieved by eluting the column with a linear gradient produced by mixing 300 ml. of solution A (278 ml. acetic acid, 16·1 ml. pyridine, water 705·9 ml., pH 3·1) with 300 ml. of solution B (139 ml. acetic acid, 161 ml. pyridine, 700 ml. water, pH 5·0). The elution rate was 18 ml./hr and 3-ml. fractions were collected. 1-ml. samples were dissolved in 10 ml. of the scintillation fluid described below.

(l) *Scintillation counting*

Tritium-containing samples were dissolved in a scintillation fluid similar to that of Hall & Cocking (1965); it contained 625 ml. of Toluene, 375 ml. 2-ethoxyethanol, 4 g of 2,5,-diphenyloxazole, and 0·1 g of 1,4-bis-(5 phenyloxazolyl-2)-benzene/l. (Woodland & Gurdon, 1969). The radioactivity was assayed in a Nuclear Chicago 725 scintillation counter at an efficiency of about 15% for tritium. Double-labelled samples were dissolved in 10 ml. of a scintillation fluid prepared from a mixture of 1 l. of Toluene, 0·5 l. of Triton X100 and 4 g of Omnifluor (New England Nuclear) and were counted in a Packard Counter. Corrections were made for quenching.

3. Results

(a) *Haemoglobin synthesis by injected polyribosomes*

The incubation of rabbit reticulocyte polyribosomes in an *in vitro* system resulte d in the synthesis of radioactive molecules which eluted from Sephadex in the same fractions as marker rabbit haemoglobin (see Fig. 1(a)). This showed that the preparations of polysomes used were active. Polyribosome suspensions, containing haemin, were injected into oocytes which had been separately injected a few minutes beforehand with [^{3}H]histidine; the oocytes were frozen three hours after polysome injection, and subsequently analysed by Sephadex chromatography. A peak of radioactive material was found to elute with marker haemoglobin (see Fig. 1(b)). Injection of tritiated histidine, without subsequent injections of polysomes, results in no such peak (see Fig. 1(c)). A similar result is obtained if polyribosomes plus haemin are injected into oocytes which are labelled by incubation in a saline medium containing [^{3}H]histidine. These oocytes were incubated in label for about two hours before, and three hours after, injection. If polyribosomes are omitted from the injection mixture radioactive material similar to haemoglobin can no longer be detected. Table 1 shows that the presence of polyribosomes in the injection buffer is associated with the formation of radioactive material which coelutes with rabbit haemoglobulin.

The following controls were carried out to confirm that the peak of radioactivity coeluting with carrier haemoglobin results from the incorporation of [^{3}H]histidine

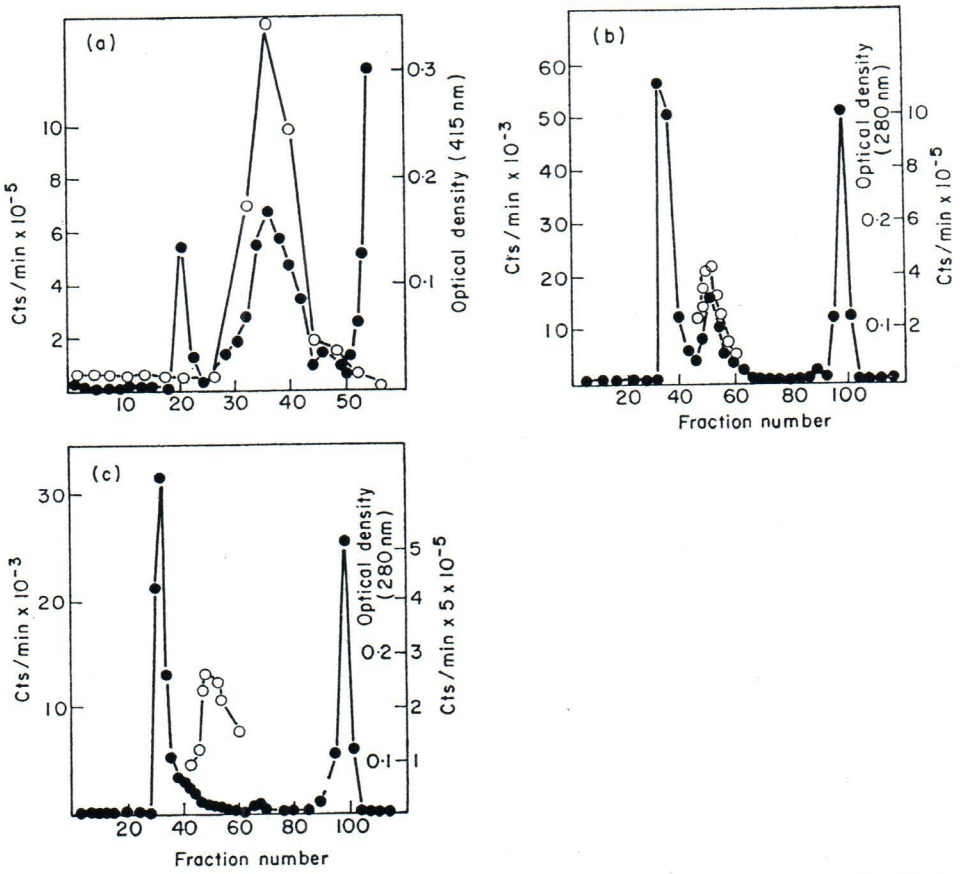

FIG. 1. (a) A cell-free system prepared and incubated as described by Luzzatto *et al.* (1965) and containing 100 μCi [^{3}H]histidine/ml., was passed down a G100 Sephadex column equilibrated with 0·5 M-KCl–0·05 M-Tris, pH 7·0. The elution profile obtained is shown.

(b) Approximately 30 oocytes, each injected with 2 μg of polyribosomes and (as a separate injection) with 0·5 μCi [^{3}H]histidine (40 Ci/m-mole), were homogenized and the supernatant analysed as described previously, using a 1 cm × 140 cm G100 Sephadex column equilibrated with 0·0522 M-Tris–0·0522 M-glycine (pH 8·9).

(c) Oocytes treated in the same way as in (b), except that the injection of polyribosomes is omitted. No labelled haemoglobin is observed.

Open circles (○ — ○ — ○) refer to optical density at 280 or 415 nm (as specified) and the closed circles (● — ● — ●) refer to radioactivity. The cts/min scale on the right side of (b) and (c) refers to the right-hand peak in (b) and (c).

into a haemoglobin-like molecule by a process similar to normal protein synthesis. If the oocytes are frozen immediately after injection, the peak of labelled material is not detected (Table 1). Thus, the labelled material is not the result of some rapid association phenomenon. A further test of haemoglobin synthesis is to use isoleucine—there are about six times as few isoleucine residues as there are histidine residues in rabbit haemoglobin (von Ehrenstein, 1966; Braunitzer, Best, Flamm & Schrank, 1966)—as the source of radioactivity. If [^{3}H]isoleucine is included in the culture medium, there is no difference between the elution profiles of oocytes injected with polyribo-

TABLE 1

Stimulation of haemoglobin synthesis by injected polysomes

Injected Material (a)	Duration of labelling: Before injection (min approx)	Duration of labelling: After injection (min approx)	Type of label used (b)	Cts/min per oocyte: Excluded region	Cts/min per oocyte: Total haemoglobin region	True haemoglobin peak (c)	True haemoglobin as percentage of excluded region
Reticulocyte polysomes	120 min	185 min	[^{3}H]histidine	113,600	18,600	8200	7·22
Control	120 min	240 min	[^{3}H]histidine	33,600	5240	—	—
Reticulocyte polysomes	320 min	0·5 min	[^{3}H]histidine	51,000	2340	74	0·15
Reticulocyte polysomes	140 min	225 min	[^{3}H]isoleucine	2243	263	—	—
Control	140 min	225 min	[^{3}H]isoleucine	2750	383	—	—

(a) Each sample was dissolved in pH 7·4 buffer containing 50 mM-Tris, 25 mM-KCl, 5 mM-$MgCl_2$ and haemin 0·5 mg/ml.
(b) 19 Histidine residues are present in the rabbit haemoglobin molecule. In contrast only 4 residues of isoleucine are present.
(c) Radioactivity in the true haemoglobin peak was estimated by normalizing the control and experimental curves with respect to the front peak and then calculating the difference in the haemoglobin region.

somes, the latter suspended in the haemin-containing buffer, and oocytes injected with the buffer alone. In neither experiment is there a peak of radioactive material that co-elutes with carrier haemoglobin (see Table 1).

We conclude that the injection of rabbit reticulocyte polyribosomes leads to synthesis of haemoglobin-like molecules, although this may only involve the completion of pre-existing nascent chains. Moreover, as can be seen from Table 2, translation of the reticulocyte message within the oocyte is an efficient process, not only when compared to the rate of translation in the polysome suspension before injection, but also when compared to the rate of translation when the suspension is added to a cell-free system. Thus, the intracellular environment of the oocyte is such as to permit rabbit reticulocyte polyribosomes to resume efficient translation; moreover, the products of translation are stable in this foreign milieu. It seems likely that globin chain synthesis may be reinitiated, rather than merely completed, when reticulocyte polyribosomes are injected into oocytes, for synthesis of haemoglobin continues for over four hours (Lane, 1971). However, proof of initiation can most convincingly be provided by demonstrating haemoglobin synthesis following the injection of 9 s messenger RNA, and experiments of this nature are discussed in the next section.

TABLE 2

Haemoglobin synthesis by reticulocyte polysomes in different translational environments

Nature of incubation (a)	Conditions of labelling	Haemoglobin synthesis (b) (cts/min per 50 mμl. of polysome suspension (c))
Polysomes injected into living oocytes (3 hr)	Injected oocytes incubated in [^{3}H]histidine at 1 mCi/ml. and 40 Ci/m-mole	8200
Polysomes incubated in complete cell-free system (1 hr)	[^{3}H]histidine added to incubate to give 0·1 mCi/ml., and 2·5 Ci/m-mole	2500 (d)
Polysomes in suspension with no additions (= control) (2 hr)	[^{3}H]histidine added to suspension to give 1 mCi/ml., and 40 Ci/m-mole	13

(a) Polysome preparation and cell-free system were as described in Materials and Methods.

(b) Determined by Sephadex chromatography, except in the case of the polysome suspension where trichloroacetic acid-precipitable counts were assumed to represent haemoglobin.

(c) No allowance has been made for the dilution of label by the presence of histidine in oocytes or in the preparations of polysomes. It is very likely that, if the contribution of unlabelled histidine could be taken into account this would increase the extent to which polysomes appear to be translated more efficiently than in a cell-free system.

(d) This value has been corrected for the lower specific activity and lower concentration of label compared to the other 2 incubates.

(b) *Haemoglobin synthesis by injected 9 s reticulocyte RNA*

(i) *Identification of whole haemoglobin*

Using the micro-injection and continuous labelling techniques described previously, the following experiments were carried out to test the effects of introducing 9 s rabbit reticulocyte RNA (see Materials and Methods) into living oocytes. The radioactive materials extracted from the injected oocytes have been fractionated and analysed by several different methods in order to characterize the products as haemoglobin-like.

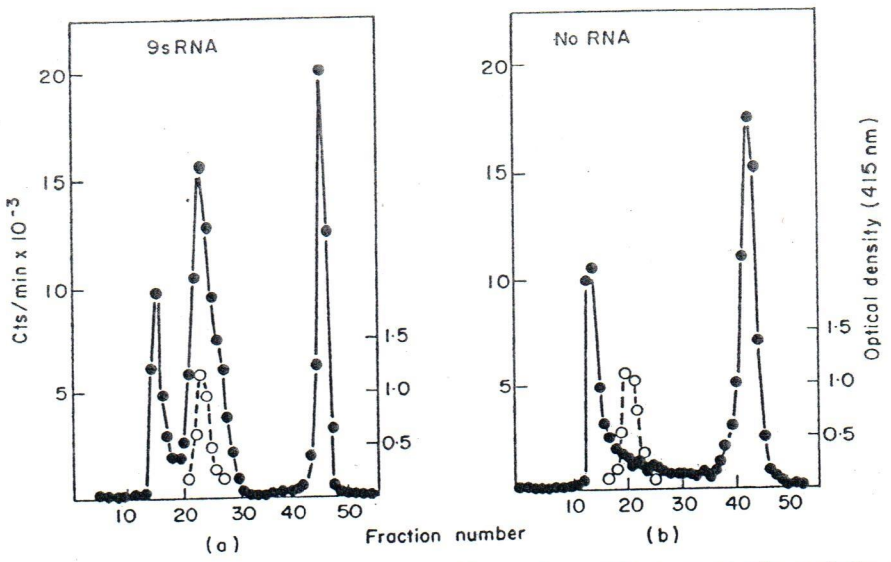

FIG. 2. Oocytes were injected, homogenized with marker rabbit haemoglobin, and the supernatant analysed on G100 Sephadex as described in Materials and Methods.

(a) Shows material from 30 oocytes which were injected with 9 s RNA (at 700 μg/ml. yielding an intracellular concentration of about 35 μg/ml.) dissolved in haemin-containing buffer, and cultured for 6 hr in [^{3}H]histidine at 1 mCi/ml.

(b) 30 oocytes were treated similarly except that the injectate contained no 9 s RNA. Cts/min refer to material from a single oocyte. The recovery of counts was 95 to 100%.

Open circles (-- ○ -- ○ --), refer to optical density at 415 nm and closed circles (— ● — ● —.) refer to cts/min.

Figure 2(a) and (b) show the Sephadex G100 elution profiles obtained from homogenates of oocytes injected with haemin and 9 s RNA and of controls injected with haemin but no RNA. The presence of 9 s RNA in the injectate is linked to the appearrance of a peak of radioactivity with an elution profile identical to that of added marker rabbit haemoglobin. If injected oocytes are homogenized with small quantities (less than 2 mg per batch of oocytes) of carrier rabbit haemoglobin, a pronounced shoulder is seen on the right of the haemoglobin peak. This shoulder is present both in terms of radioactivity and in terms of optical density at 415 nm and is probably the result of dissociation of the haemoglobin, a phenomenon known to be enhanced by Sephadex chromatography (Andrews, 1964). Thus, the labelled material and the carrier probably have similar dissociation constants.

Table 3 shows the results of three separate experiments, involving the injection of 9 s RNA into oocytes from different *Xenopus* females. Injected 9 s RNA consistently produces a four- to fivefold increase in the amount of radioactivity eluted from Sephadex G100 columns in the region of marker haemoglobin. In controls there is about one-half as much radioactivity in the haemoglobin peak as in the front peak. When allowance is made for the endogenous radioactivity in the Hb region of Sephadexes from 9 s RNA-injected samples, the radioactive material synthesized as a result of RNA injection amounts to about 60% of the total endogenous radioactive protein.

Material from the haemoglobin region of the Sephadex profile was mixed with the appropriate solutions and loaded onto a polyacrylamide gel. After electrophoresis

TABLE 3

Stimulation of amino-acid incorporation into protein by 9 s RNA injected into oocytes

Injected material (a)	Duration & method of labelling (b)	Cts/min per oocyte (Sephadex fractions) (c) Excluded region	Haemoglobin region	Low molecular weight region	Haemoglobin region ÷ excluded region	Relative increase in haemoglobin region
9 s RNA and haemin	6 hr injection	9005	6515	48,390	0·723	4·13
Haemin only (control)	6 hr injection	11,940	2063	37,740	0·175	
9 s RNA and haemin	7 hr injection	9850	18,510	8610	1·88	4·0
Haemin only (control)	7 hr injection	10,590	4960	19,220	0·47	
9 s RNA and haemin	10 hr incubation	165,300	333,380	357,000	2·02	5·61
Haemin only (control)	10 hr incubation	182,400	66,150	334,280	0·36	

(a) 9 s RNA was injected at about 700 μg/ml., to yield an intracellular concentration of about 35 μg/ml.

(b) Oocytes were labelled either by injection of [^{3}H]histidine mixed with RNA and haemin, or by incubating injected oocytes in [^{3}H]histidine-containing medium.

(c) 30 oocytes comprised each sample, which was analysed on Sephadex. The peaks are seen in Fig. 3.

using the method of Moss & Ingram (1968), a sharp peak of radioactive material was found to have moved with the marker haemoglobin (Fig. 3(a)). In controls lacking 9 s RNA, material taken from the optical density peak of haemoglobin (Fig. 2(b)) showed no preferential migration of radioactivity with carrier haemoglobin (Fig. 3(b)). We conclude that the molecules whose synthesis is caused by the injection of 9 s RNA are indistinguishable from haemoglobin in over-all charge distribution as well as in size. The type of electrophoretic analysis employed here has a high resolving capacity, as shown by the substantial separations of rabbit $\alpha_2\beta_2$, human β_4 and human $\alpha_2\beta_2$, from each other. Although frog and rabbit haemoglobins are not well resolved by this method, there is sufficient difference to enable a distinction to be drawn between co-electrophoresis with marker rabbit and coelectrophoresis with marker frog haemoglobin.

(ii) *Analysis of globin chains*

Material from the haemoglobin region of the Sephadex elution profile was treated as described by Schapira *et al.* (1968), with acetone–HCl containing β-mercaptoethanol, to yield a preparation of radioactive globin. If this protein preparation is analysed by chromatography on carboxymethyl-cellulose columns (after Dintzis, 1961), it is found that the presence of 9 s RNA in the injection mixture is linked to the appearance of radioactive material that co-chromatographs with carrier rabbit globin chains (Fig. 4(a)). If 9 s RNA is omitted from the injection mixture, no material which preferentially migrates with carrier globin chains can be detected.

In the eluate from these columns, there are two major peaks of optical density and radioactivity. By analogy with the results of Dintzis (1961), whose methods we have followed, the first peak is assumed to contain predominantly α chains, and the second peak predominantly β chains. If the peak tubes of the α region are pooled and the material rerun on a carboxymethyl cellulose column, there is one peak of optical density and one of radioactivity; the two peaks coincide with each other and with the region of the eluate expected to contain α chains (see Fig. 4(b)). Similarly, the rerunning of purified β chains results in a single peak of radioactivity which coincides with a single peak of optical density; and this peak occurs in the position expected of β chains (see Fig. 4(c)). The two major peaks are not always found in the proportions shown in Figure 4(a). However, this figure is typical in that the second peak is larger than the first. This difference may result from different recoveries of the two components, or to differential rates of synthesis or to differential rates of assembly into tetramers. The small peak of radioactivity seen on the right-hand side of Figure 4(a) probably consists of globin chains still associated with residual amounts of haem, for this region of the gradient is characterized by some absorbance at 415 nm.

(iii) *Analysis of peptides*

Haemoglobin uniformly labelled with [^{14}C]histidine was mixed with the haemoglobin-like material (labelled with [^{3}H]histidine and purified on G100 Sephadex) extracted from RNA-injected oocytes. The mixture was digested with trypsin, as described in the Materials and Methods section, thus yielding a solution of peptides. Analysis of this mixture on a cation-exchange resin shows that the presence of radioactive material, co-chromatographing with material derived from the carrier haemoglobin (in this case [^{14}C]histidine labelled), is linked to the presence of 9 s RNA in the injection mixture (see Fig. 5). There are some peptides derived from the carrier that

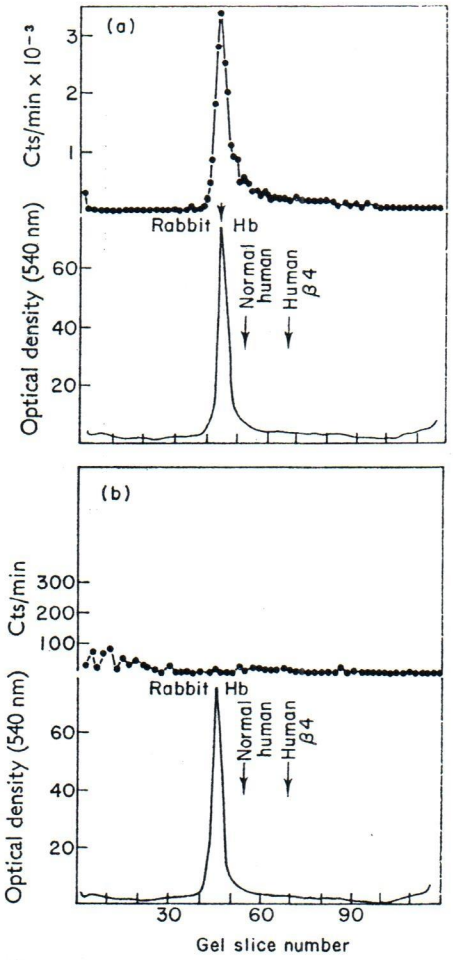

FIG. 3. (a), (b) Gels were prepared, stained and counted as described in Materials and Methods. The 2 arrows mark the positions of two marker haemoglobins, one a normal ($\alpha_2\beta_2$) human haemoglobin, the other an abnormal β_4 haemoglobin. 20 oocytes, each injected with 9 s RNA (at 700 μg/ml. yielding an intracellular concentration of about 35 μg/ml.) were incubated for 9 hr, homogenized, and the supernatant applied to a Sephadex column. A portion of the haemoglobin region from the Sephadex column was electrophoresed at 5 mA/gel for 90 min, giving the pattern shown in (a). (b) Shows the pattern given by material from oocytes injected with buffer, but no RNA. Recovery of counts was 60 to 70% in both cases. The continuous line (————) refers to optical density at 540 nm and the closed circles (— ● — ● —) to cts/min.

do not correspond to peptides present in the material synthesized in the oocytes. However, there are no peptides derived from the latter material that do not clearly correspond to peptides derived from the carrier haemoglobin. Moreover, there are seven major peaks showing co-chromatographic behaviour of ^{3}H and ^{14}C-labelled peptides. Theoretically, the tryptic digestion of rabbit haemoglobin should yield twelve different histidine-containing peptides, but in practice, fewer are sometimes obtained. The discrepancies noted may result from a deficiency of oocyte-derived α chains in the sample that was used for the tryptic digest. This, in turn, could result from deficient synthesis of α chains. Sephadex analysis would probably not distinguish

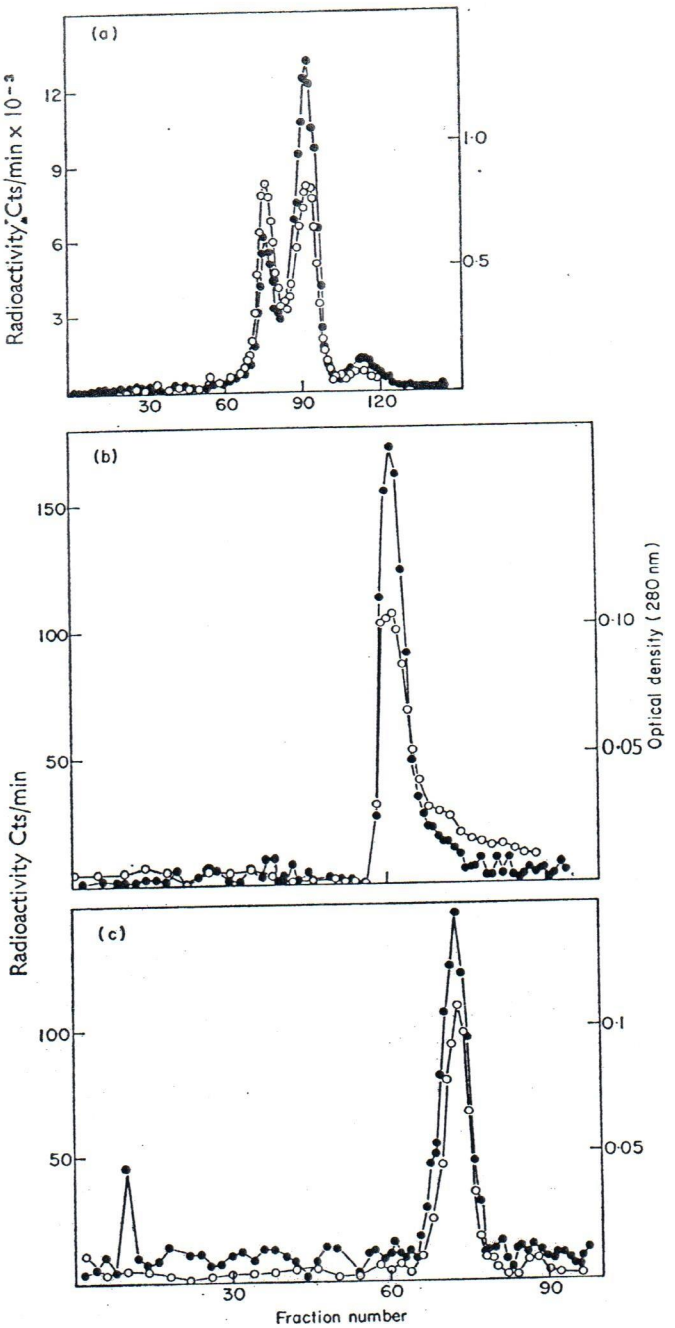

FIG. 4—*see legend on opposite page.*

between $\alpha_2\beta_2$ and β_4 molecules, and so the material obtained from Sephadex columns could be relatively rich in β chains. Moreover, if subunit exchange occurred between marker and oocyte-derived haemoglobins, the excess β chains could become incorporated into normal $\alpha_2\beta_2$ molecules. Even if α and β chains were made in equal amounts in the oocyte, unequal assembly could result in the tetramers being disproportionately rich in β chains. However, the relative abundance of α and β peptides in a sample depends on the losses that have occurred during processing, as well as on the initial quantities present.

As shown by Lane (1971), two basic ^{3}H-labelled peptides chromatograph and electrophorese on paper, separately from one another, but coincidentally with two marker ^{14}C-labelled peptides. For these two peptides at least the resemblance between marker and oocyte-derived peptides is very close.

The amino-acid sequences of *Xenopus laevis* haemoglobins are not known. However, the sequence of the β chain of *Rana esculenta* is known (Chauvet & Acher, 1970), and is very different to that of the rabbit β chain (Braunitzer *et al.*, 1966). Thus, it seems likely that there are substantial differences between the haemoglobins of frogs and those of rabbits. Since the cation exchange resin used is capable of resolving very similar types of peptides (Hirs, Moore & Stein, 1956*a*,*b*) it seems very likely that the material synthesized in the presence of 9 s RNA is similar to that of rabbit, as opposed to frog, haemoglobin.

(c) *9 s RNA as the sole requirement for stimulating globin synthesis in injected oocytes*

The results so far described have not excluded the possibility that the injection of RNA fractions other than 9 s can cause haemoglobin synthesis, nor that 9 s RNA serves as a messenger for globin only when injected with haemin. If it could be shown that 9 s is the only RNA that promotes globin synthesis, and that it can do so without haemin, this would strongly support the view that 9 s RNA has its effect according to accepted ideas regarding the function of messenger RNA.

Figure 6 shows the results of injecting different fractions of rabbit reticulocyte RNA, in each case mixed with haemin. Partially purified 18 s RNA causes a small amount of haemoglobin synthesis, but this synthesis is much reduced or even eliminated by further purification on sucrose gradients. This repurification step revealed the presence of small amounts of residual 9 s RNA. The Figures show that purified 28 s, 18 s and 4+5 s RNA fail to cause haemoglobin synthesis, and also that they have no effect on the over-all rate of incorporation of [^{3}H]histidine into protein.

FIG. 4. (a) Carboxymethyl cellulose columns were prepared and run as described in Materials and Methods. Oocytes, injected with 9 s RNA and haemin and incubated for 9 hr in [^{3}H]histidine (1 mCi/ml.), were homogenized and the supernatant applied to a Sephadex column. A portion of the haemoglobin region from the Sephadex column was used to prepare globin, as described in Materials and Methods. Further marker rabbit haemoglobin (in this experiment 20 mg) was added prior to this stage. The globin was chromatographed and the pattern obtained is shown in (a).

(b) & (c) Material (α chains) from the peak tubes of the first peak of a carboxymethyl cellulose run were pooled, and the chains rerun on carboxymethyl cellulose columns. The pattern obtained is shown in (b). Slightly different formic acid–pyridine gradients were used for the chromatography of separated globin chains (see Materials and Methods). (c) Shows the pattern obtained with purified chains from the second peak (β chains).

Open circles (— ○ — ○ —), refer to optical density at 280 nm and closed circles (— ● — ● —), to cts/min.

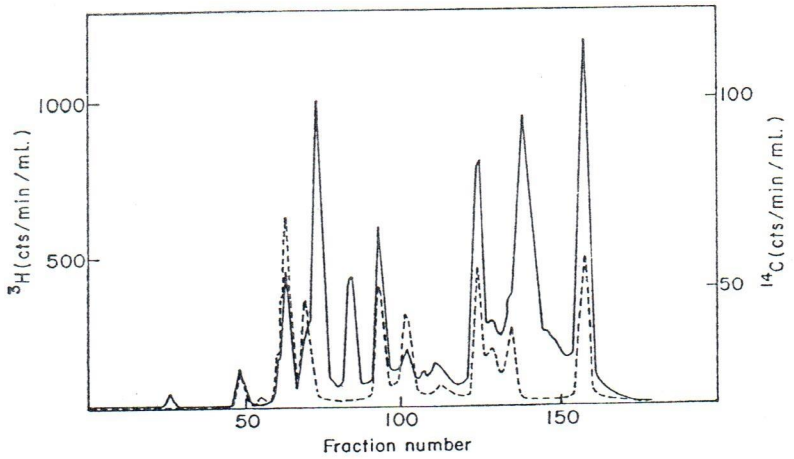

FIG. 5. ^{14}C-labelled haemoglobin from rabbit reticulocytes was mixed with 100,000 cts/min ^{3}H-labelled haemoglobin from oocytes (injected with 9 s RNA at 700 μg/ml. and incubated in [^{3}H]histidine for 5 hr), and the mixture digested with trypsin, as described in Materials and Methods. The peptide mixture was analysed on Technicon chromobeads (type P) cation exchange resin, eluted with a formic acid–pyridine gradient (see Materials and Methods), yielding the pattern shown. The continuous line (————) refers to ^{14}C cts/min/ml., and the broken line (– – – – –) to ^{3}H cts/min/ml.

The results described so far do not exclude the possibility that 9 s RNA from another tissue might also possess haemoglobin messenger activity. This has been tested by injecting a 9 s fraction of myeloma cell RNA, and haemin. This RNA failed to stimulate haemoglobin synthesis (Fig. 7), although it may induce injected oocytes to synthesize a myeloma-like protein (Gurdon *et al.*, 1971). Thus, the haemoglobin-forming activity is specifically associated with the 9 s fraction of RNA (Chantrenne, Burny & Marbaix, 1967) from reticulocytes.

It is possible that the presence of haemin is required for the translation of 9 s RNA in the oocyte. If haemin is omitted from the injection mixture containing 9 s reticulocyte RNA, translation of the 9 s RNA still occurs, for carboxymethyl-cellulose chromatography of the clarified oocyte homogenate reveals the presence of radioactive globin chains (Fig. 8(a)). Moreover, haemoglobin message translation in injected oocytes was not greatly enhanced by the presence of haemin in the injectate, since at the very least 40% as much globin was synthesized in oocytes lacking injected haemin as in those which received it. If both haemin and 9 s RNA are omitted from the injectate, no labelled globin chains can be detected (Fig. 8(b)). This is also true if haemin, but no 9 s RNA, is injected (Fig. 2(b)). Although these results show that added haemin is not needed for mRNA translation, we cannot exclude the possibility that haemin may play an important role in this process (as reviewed by London, Tavill, Vanderhoff, Hunt & Grayzel, 1967), because it, or a related compound, may be present in oocyte cytoplasm.

We have considered the possibility that globin chains might be present in the front peak of our Sephadex fractionations. To test this, carrier rabbit haemoglobin was added to front peak material, and the resulting mixture used to prepare globin, the latter being analysed for its content of radioactive α and β chains by carboxymethyl

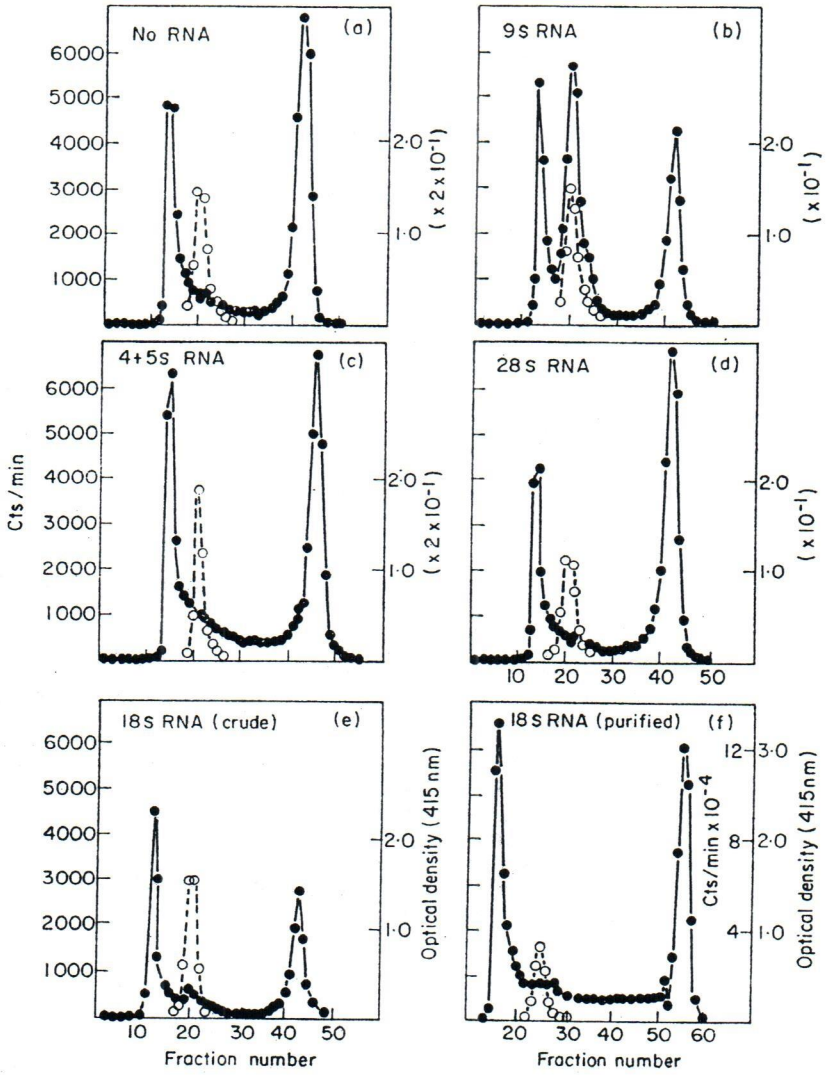

FIG. 6. Batches of 20 oocytes, injected with haemin and RNA as indicated in each Fig. and incubated in [^{3}H]histidine (1 mCi/ml.) for 7 hr, were homogenized with marker rabbit haemoglobin and the resulting supernatant was analysed on G100 Sephadex columns, as described in Materials and Methods. The concentration of RNA injected was about 1000 μg/ml. in each case, yielding an intracellular concentration of about 50 μg/ml. Cts/min refer to material from a single oocyte. Recovery of counts was 95 to 100% in all cases. The open circles (-- ○ -- ○ --), refer to optical density at 415 nm and the closed circles (— ● — ● —), to ^{3}H cts/min.

cellulose chromatography (see Materials and Methods). No radioactive chains could be found in front peak material derived from oocytes injected with 9 s RNA plus haemin, nor were any chains detectable in material from oocytes injected with haemin alone.

These results identify 9 s RNA from reticulocytes as the sole agent, among those we have tested, capable of causing haemoglobin synthesis. This is a reason for believing that it solely consists of, or at least contains, the mRNA for haemoglobin.

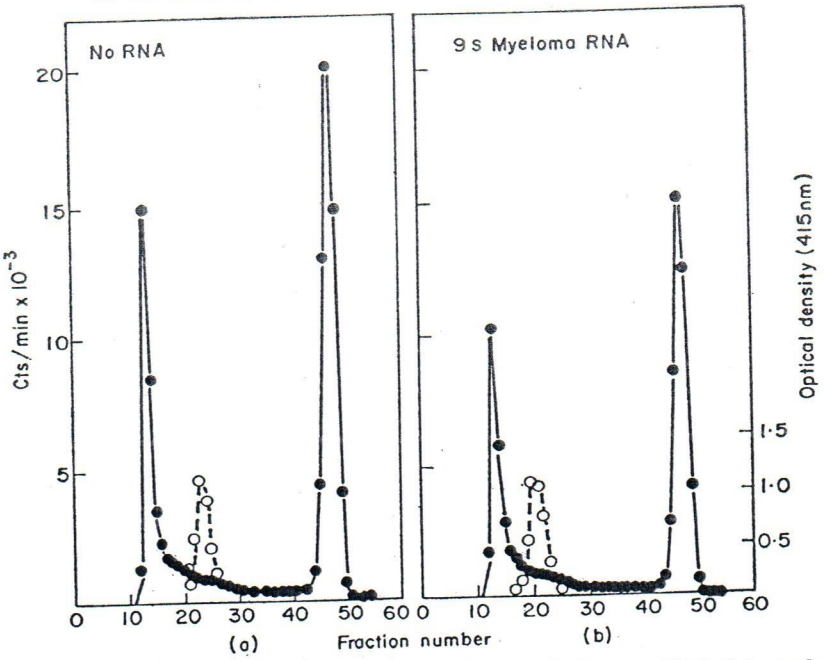

FIG. 7. Batches of 20 oocytes, injected with haemin and RNA as indicated in each Fig. and incubated in [^{3}H]histidine, were homogenized with rabbit haemoglobin and the resulting supernatant was analysed on G100 Sephadex columns, as described in Materials & Methods.

(a) Buffer plus haemin as injectate. Oocytes were incubated for 7 hr.

(b) Buffer plus haemin plus 9 s myeloma RNA (at 5 mg/ml. yielding an intracellular concentration of about 250 μg/ml.) as injectate. Oocytes were incubated for 10 hr.

Cts/min refer to material from a single oocyte. Recovery of counts was 95 to 100% in both cases. The open circles (– – ○ – – ○ – –) refer to optical density at 415 nm and the closed circles — ● — ● —), to ^{3}H cts/min.

Myeloma RNA was the kind gift of Drs Stavnezer and Huang and was prepared according to Stavnezer & Huang (1971).

4. Discussion

These results establish the surprising fact that purified mRNA, injected into the cytoplasm of a foreign living cell, is not only spared from rapid degradation but is also used with high efficiency for translation. The performance of injected polysomes is important because this adds confidence to the results obtained with pure 9 s RNA, and shows that the successful translation of the latter does not require its isolation from proteins and other components with which it is normally associated. Although we have now used three different preparations of 9 s RNA, and oocytes from about 30 different frogs, there is remarkably little variation in experimental results. The basic experiment, involving Sephadex analysis of oocytes injected with 9 s RNA, has been performed over 100 times, and in no case did the RNA fail to promote the formation of a haemoglobin-like molecule. Thus, the oocyte micro-injection system seems to yield results which are highly reproducible.

We have employed several different analytical methods to identify, as haemoglobin, the protein whose synthesis is caused by the injection of 9 s RNA. These include those generally applied to the identification of haemoglobin synthesized in cell-free systems

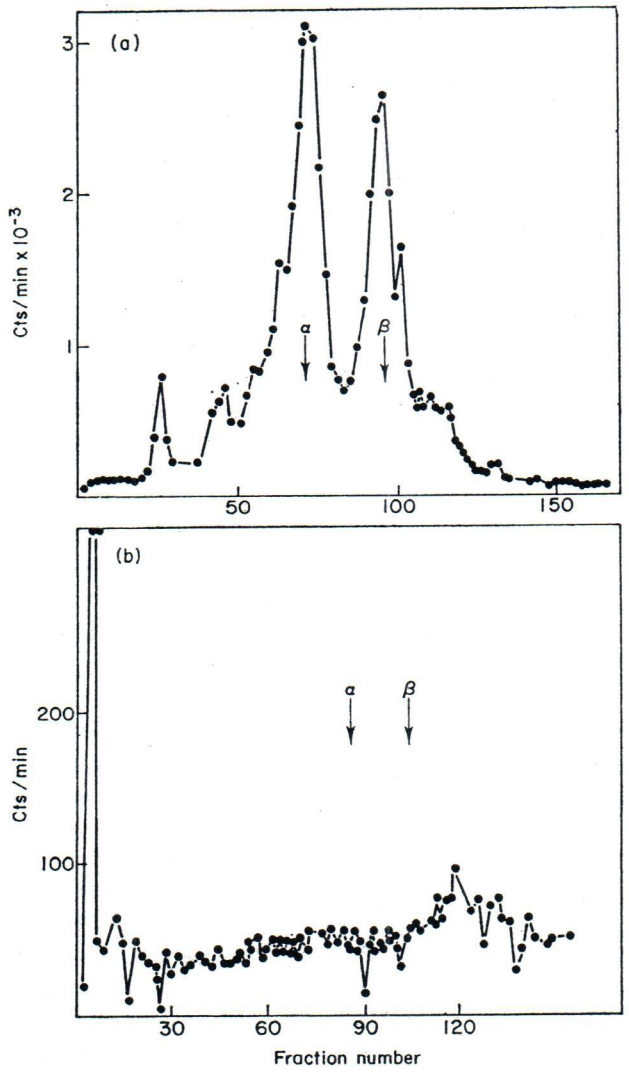

FIG. 8. Oocytes were injected with 9 s RNA (700 μg/ml.), incubated for 6 hr in 1 mCi [^{3}]histidine/ml., and homogenized with marker rabbit haemoglobin (5 mg), and the homogenate was dialysed against deionized water (1000 vol.) for 24 hr. Globin was prepared from the 3000 *g* supernatant of the dialysed homogenate (see Materials and Methods), and was chromatographed on carboxymethyl cellulose columns. The columns had previously been calibrated using a preparation of pure rabbit globin, as can be seen from the arrows in Fig. 8(a) and (b).

(a) Represents material from oocytes injected with 9 s RNA (700 μg/ml.) and injection medium, but no haemin.

(b) Represents material from oocytes injected with injection medium, but no haemin and no 9 s RNA.

30 oocytes were used in each experiment.

which are primed by 9 s RNA. We have identified the [^{3}H]histidine labelled proteins as haemoglobin on the grounds of size (Sephadex), over-all charge and size (acrylamide gels), their content of protein subunits which behave like α and β chains on carboxymethyl-cellulose, and by their content of haemoglobin-like peptides. The peptide analysis does not exclude the possibility of slight difference in amino acid content amongst the peptides found in both carrier and oocyte haemoglobin. Nonetheless, the precise coincidence between the marker and oocyte haemoglobin on gels, and the chromatographic identity of some peptides, both argue for detailed resemblances between the marker and oocyte haemoglobins and hence indicate that the synthesized molecules are rabbit, rather than frog, haemoglobin.

The results obtained not only confirm the deduction made by other workers (Laycock & Hunt, 1969; Lockard & Lingrel, 1969; Heywood, 1970) who used *in vitro* systems to provide evidence that the 9 s RNA fraction from reticulocytes contained haemoglobin messenger activity. Our results also extend their observations. Firstly, the assay system used was enormously sensitive; the presence of less than 10^{-15} moles of 9 s RNA could be detected, and the efficiency of translation was at least several hundred times greater than that of a cell-free system (Gurdon *et al.*, 1971). For this reason messenger activity is probably a property of the 9 s RNA itself. Second, our results apply to messenger RNA translation in a living cell. Moreover, as assayed in the living cell, 28 s RNA, purified 18 s RNA, and 4+5 s RNA from reticulocytes, and 9 s RNA from myeloma tissue, do not possess any detectable haemoglobin-forming.

The results reported here contribute to the question of the species and cell type specificity of the relationship between mRNA and other translational components. Since rabbit haemoglobin mRNA is successfully translated in frog oocytes, our results clearly indicate the following general principles: (1) that the components required to translate rabbit haemoglobin mRNA are present in cell types as different as oocytes and reticulocytes, and in species as unrelated as frogs and rabbits; and (2), that if haemoglobin mRNA specific components are required, then they are present and available in frog oocytes. It is possible that factors required specifically for haemoglobin mRNA translation do exist in frog oocytes. If it is assumed that such factors (e.g. the ribosome binding factors of Heywood, 1969, 1970) do exist in oocytes, then it is clear that their presence cannot be the only phenomenon determining the appearance of cell type specific proteins during cell differentiation.

The formation of haemoglobin-like molecules in the living oocyte shows not only that the 9 s RNA may be translated in the cytoplasm of the oocyte, but also proves that once formed, the globin chains may combine with each other, and form a tetramers, probably while still in the oocyte cytoplasm. Moreover, the proteins formed do not appear to be unstable, to any large extent, in this cytoplasmic environment. Thus, provided the correct information is supplied, an oocyte can produce, and permit the assembly of, a complex protein that is, under normal circumstances, completely uncharacteristic of this cell type.

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Note added in proof: subsequent peptide analyses of separated α and β globin chain shave shown that the material synthesized in message-injected oocytes is not distinguishable from that extracted from rabbit reticulocytes; moreover carboxymethyl cellulose chromatography of globin chains has shown that the haemoglobin synthesized in messenger-injected frog oocytes contains rabbit, and not frog, globin chains (Marbaix & Lane, manuscript in preparation).

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