BIOSYNTHESIS OF HEMOGLOBIN: ATTACHMENT OF HEME TO GLOBIN

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ABSTRACT

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by Katherine Liang

The biosynthetic pathways of heme and globin are known. The details of the regulation and coordination of heme and globin synthesis remain to be investigated.

The purpose of this study was to investigate the stage of hemoglobin biosynthesis at which the attachment of heme to globin chains occurs in rabbit reticulocytes.

C^{14} α-aminolevulinic acid (α-ALA), a known precursor of heme, was used to introduce a radioactive label into heme in lysates of rabbit reticulocytes. The C^{14} label associated with the ribosome fraction was analyzed on sucrose density gradients. The release of C^{14} labelled material from ribosomes in the presence of protein synthesis was studied. The effect of puromycin on the release of C^{14} labelled material was also investigated. Finally C^{14} α-ALA labelled ribosomes were dissociated and analyzed on ECTEOLA cellulose columns for the presence of C^{14} heme-peptidyl sRNA.

Results from sucrose density gradient analyses showed a small amount of radioactivity from C^{14} α-ALA attached to the polysomal fractions. Studies on the release of C^{14} labelled material on ribosomes and the effect of puromycin indicate that this C^{14} labelled material (heme or heme intermediates) is not attached to the globin chains on polysomes.

Finally, the absence of heme-peptidyl-sRNA from dissociated C^{14} δ-ALA ribosomes indicate that heme is attached to globin chains after the latter are released from polysomes.

To Peter

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INTRODUCTION

Studies on the physical and chemical structures of the vertebrate hemoglobins, their mode of synthesis and functioning, and on their genetic control have occupied many years of research. A full understanding of the mechanism and regulation of their biosynthesis has been the goal of these studies.

The biosynthetic pathway of heme, the prosthetic group of hemoglobin, is well defined; the complete amino acid sequence of the four polypeptide globin chains and their tertiary and quarternary structures are also known. However, the details of the regulation and coordination of heme synthesis and globin synthesis remain to be investigated.

This study was to investigate the stage of hemoglobin biosynthesis at which the attachment of heme to globin chains occurs in rabbit reticulocytes.

The ultimate goal of this investigation and other investigations of a similar nature in relating the syntheses of heme and globin are two fold: (a) To determine, in general, how prosthetic groups of proteins are combined with their apoproteins during synthesis and (b) to obtain an insight into the control of protein synthesis at the molecular level.

HISTORICAL

Borsook and Kruh (1) first described the close coordination of heme and globin syntheses in the intact rabbit reticulocytes. They also demonstrated (2) that although added iron might be essential for optimal synthesis of hemoglobin, it did not stimulate synthesis in the absence of certain essential amino acids. Similar parallelism in the rates of heme and globin syntheses was also reported by Morell and his coworkers (3) in rabbit bone marrow and by Mizet (4) in dog reticulocytes.

Hammel and Bessman (5) noted an increase in protein synthesis when hemin and other porphyrins were added to avian erythrocyte nuclei. The degree of stimulation was dependent on the concentration of hemin. They postulated that porphyrins stimulated amino acid incorporation into globin.

Gribble and Schwartz (6) have reported that protoporphyrin enhanced the release from ribosomes of newly formed globin chains in their cell-free system. They suggested that such a release might involve the coiling of the globin chains around the protoporphyrin and/or heme groups.

Bruns and London (7) showed that hemin, at a concentration of 10^{-4} M, inhibited the utilization of glycine into hemin by rabbit reticulocytes, and also increased the incorporation of C^{14} valine into newly formed hemoglobin.

Rabinovitz and Waxman (8) studied the influence of iron on the state of aggregation and activity of the ribosomes in the intact reticulocytes. Their data indicate that iron or hemin could bring about reaggregation of polysomes which have become disaggregated upon incubation of reticulocytes

with an amino acid mixture and glucose. They also reported (9) that cells incubated with iron and transferrin had an increased polysome content. Their results were inconsistent with the tape theory of protein synthesis, which included the concept that terminal events, such as the association of α and β polypeptide chains or the insertion of heme have no role in the peptide forming process.

Most recently Grayzel and his coworkers (10) reported that in reticulocytes of iron-deficient rabbits there occurred an increase (in the presence of added hemin) in size and proportion of polysomes, in specific activity of the polypeptide chains attached to the polysomes and in the specific activity of soluble hemoglobin. They postulated a mechanism in which heme might attach to nascent chains of globin on polysomes and thereby promote conformational changes in the polypeptide chains.

Towards the end of the present studies, Felicetti and Baglioni in Italy (11) reported their findings that an added Fe^{59} label does not seem to be present in heme in a heme-nascent globin form while the globin molecules were being synthesized on the polysome. They also postulated that α and β globin subunits are probably intermediates in the assembly of hemoglobin.

EXPERIMENTAL

I. Analytical Procedures

A. Preparation of Protoporphyrin: The Ferrous Sulfate Method

A modification of the procedure by Falk (12) was used. One hundred ml. of glacial acetic acid was added to a solution of 10 mg. of crystalline hemin in 1 ml. of pyridine. The reaction was performed at room temperature and was carried out in an atmosphere of nitrogen. A freshly prepared solution of 0.4 g. of ferrous sulfate in 0.4 ml. of concentrated hydrochloric acid was added to the reaction mixture. The passage of nitrogen over the solution was continued for 5 minutes. The mixture was then transferred to a separatory funnel containing a solution of 70 ml. of 4.5M sodium acetate (NaAc) and 500 ml. of fresh ether. The mixture was shaken vigorously. Protoporphyrin was transferred to the ether phase. The aqueous phase was reextracted with 100 ml. of fresh ether. Thirty ml. of 3N hydrochloric acid were added to the combined ether extracts and the mixture shaken vigorously. The aqueous layer was removed and the pH of the solution adjusted to 4. Protoporphyrin was precipitated from the aqueous solution at this pH. The brown precipitates were collected by centrifugation and dried *in vacuo* in the absence of light.

B. Extraction of Hemin and Porphyrins from Incubation Mixtures

The incubation mixtures were stirred overnight with 50 ml. of ethyl acetate:acetic acid solution (3:1 v/v) and filtered.

The filtrate was washed with water, to remove residual δ-aminolevulinic acid (δ-ALA), and transferred to a separatory funnel. The ethyl acetate phase was then concentrated to dryness. The residue was taken up in 10 ml. of formic acid, 0.5 ml. aliquot of which was plated with 5 mg. of carrier hemin onto a tared aluminum plachet, dried *in vacuo*, and radioactivity determined in a low background Nuclear Chicago Automatic Geiger counter. Results have been corrected for self absorption of the β particles by the sample.

C. Analysis of Extractable Products by Paper Chromatography

The residue from the ethyl acetate phase during extraction was taken up in 0.5 ml. of 2,6 lutidine. Two µl of the solution was spotted on a 30 x 40 cm. Whatman No. 1 paper chromatogram. The solvent system used was a mixture of 2,6 lutidine:H_2O (1:1 v/v) (13). The chamber was saturated with 25% ammonia. The chromatogram was developed descendingly for 18 to 20 hours, dried and cut in 2 inch strips and radioactivity determined in a Packard Model 7201 radiochromatogram scanner.

II. Biological Materials

A. Preparation of Rabbit Reticulocytes

Male New Zealand white rabbits weighing, six to eight pounds, were made reticulocytic by four daily subcutaneous injections of 0.175 ml./pound body weight of 2.5% neutralized phenylhydrazine. On the sixth day after the initial injection the animals received a solution containing 2000 i.u. of heparin and

100 mg. of Nembutol by intravenous injection. Blood was collected immediately by heart puncture. The red blood cells were separated from the plasma by centrifugation for 20 minutes at 2000 x g. in a Servall centrifuge. The plasma was decanted and its volume recorded. The cells were resuspended in NKM solution (a solution containing 0.13M NaCl, 0.005M KCl and 0.0075M $MgCl_2$), using a volume equal to the plasma. The suspension was filtered through glass wool. The filtrate, containing the cells, was centrifuged (20 min. x 2000 g.). The cells, which sedimented, were resuspended in NKM solution and the suspension was centrifuged once more. The supernatant was removed. The packed cells were lysed by adding two volumes of a 0.0025M $MgCl_2$ solution and stirring gently for 10 minutes. After centrifugation at 15,000 g. for 10 minutes, the supernatant was decanted as the lysate.

B. Prelabelling of Ribosomes

Two preincubation procedures were used, one in the presence of active protein synthesis and the other in its absence.

1. The incubation medium contained, per ml. of lysate, 0.1 µC C^{14} α-aminolevulinic acid (α-ALA) (specific activity 24.5 mC/mM) and buffered with 5 µmoles Tris-HCl buffer pH 7.5. Reaction mixtures were incubated for various times at 37°C and the reaction was stopped by the addition of two volumes of a cold solution containing 0.25M sucrose, 0.0175M $KHCO_3$ and 0.002M $MgCl_2$ (medium B). Ribosomes were isolated from the reticulocytes by centrifugation at 78,000 g. for 90 minutes in a

Beckman L-2 ultracentrifuge. The aqueous phase was decanted as "supernatant." Ribosomal pellets thus obtained were designated once sedimented (1X) C^{14}-ALA ribosomes. The pellets were resuspended in a small volume of 0.25M sucrose by gently homogenizing with a glass homogenizer and a Teflon pestle. The ribosomal suspension thus obtained was either directly layered on a sucrose density gradient and analyzed or diluted with medium B (to a concentration of 0.5 mg. ribosomes/ml. medium B) and centrifuged at 78,000 g. for 90 minutes to yield 2X C^{14}-ALA ribosomes. The ribosomal suspension was centrifuged at 15,000 g. for 10 minutes to remove any insoluble material. The ribosome concentration was determined by its absorption at 260 mμ. (1 mg. ribosomes/ml. = 11.3 O. D.)

2. Reticulocyte lysates were incubated in the presence of active protein synthesis by a modification of the method of Lamfrom and Knopf (14). The incubation mixture contained, per ml. of lysate, 0.1 μC. C^{14}-ALA (specific activity 24.5), 4 μmoles $MgCl_2$, 50 μmoles KCl, 0.05 μmoles each of an equimolar mixture of 20 amino acids, 2 μmoles Tris-HCl buffer pH 7.5, 0.2 μmoles ATP, 0.05 μmoles GTP, 1 μmole phosphoenolpyruvic acid and 10 μg. pyruvate kinase. Other details of the preincubation procedure were identical to those in procedure 1.

III. Sucrose Density Gradient Analysis

A. Analysis of Polysomes

Linear sucrose gradients containing 15 to 30% sucrose, 1.5×10^{-4} M $MgCl_2$, 10^{-2} M KCl, 10^{-2} M Tris pH 7.5 were prepared at 4°C. The ribosomal suspension was gently layered onto the gradient contained in a 30 ml. ultracentrifuge tube. The tubes were then placed in an S.W. 25.1 rotor and centrifuged for $3\frac{1}{2}$ hours at 75,500 g. at 4°C. Contents of each tube were then analyzed for materials absorbing at 260 mμ by pumping through a Gilford spectrophotometer equipped with a flow cell (0.5 cm. path length). Results were plotted automatically by a Sargeant Model SR recorder. Flow rate through the cell was maintained at 5 ml. per minute using a Buchler polystaltic pump. Effluent from the flow cell was collected in 1.0 ml. portions with a Packard Model 231 fraction collector. To each fraction was added 1 mg. of bovine serum albumin and the precipitate which formed in 10% TCA solutions was collected on nitrocellulose filters. The filters were washed twice with 5 ml. of 10% TCA. The dried filters were placed in a counting vial, 15 ml. of toluene, PPO, POPOP counting fluid was added to each vial and radioactivity was determined by a Packard Model 3003 liquid scintillation spectrometer.

B. Analyses of sRNA

A modification of the procedure of Traut and Monro (15) was used. The method was identical to the one just described except for the following: Linear sucrose density gradients

containing 5 to 20% sucrose, 0.1M $LiCl_2$, 0.5% sodium dodecyl sulfate (SDS), and 5 x 10^{-3}M Tris-HCl buffer pH 7.0 were prepared at room temperature. Materials to be analyzed were adjusted to 0.5% SDS and layered onto a gradient of 56 ml. Centrifugation was carried out for 48 hours at 20°C in a S.W. 25.2 rotor at 75,500 g.

IV. Polyacrylamide Gel Electrophoresis of Supernatant Hemoglobin

A. Preparation of Methemoglobin from Supernatant

The concentration of hemoglobin in the supernatant was determined by the method of Drabkin and Austin (16). Potassium ferricyanide ($K_3Fe(CN)_6$) was added to a sample containing 1.5-3.2 mg. hemoglobin/ml. to a final concentration of 6 x 10^{-4}M $K_3Fe(CN)_6$. The reaction mixture was stirred and allowed to stand for 2 minutes to permit complete formation of methemoglobin from hemoglobin. Potassium cyanide (KCN) was then added to a final concentration of 8 x 10^{-4}M and allowed to stand for 2 minutes to convert methemoglobin to the methemoglobin-CN complex. The solution was measured for absorbance at 540 mμ. (16 mg. hemoglobin/ml. = 11.5 O.D.) To prepare methemoglobin from supernatant, the concentration of which was determined as described above, one equivalent of $K_3Fe(CN)_6$ was added to the solution dropwise and allowed to stand for 30 minutes at room temperature. The solution was then dialyzed against distilled water overnight.

B. Polyacrylamide Gel Electrophoresis of Methemoglobin

Procedures for preparing the sample gels were obtained from Canal Industrial Corp., Bethesda, Md. A 7% gel solution and 9 x 1 cm. glass columns were used. The following stock solutions were necessary to prepare the separating and stacking gel solutions:

(A)	1N HCl	48 ml.	
	Tris	36.3 g.	
	Tetramethylethylenediamine	0.23 ml.	
	H_2O to make	100 ml.	(pH 8.8-9.0)
(B)	1N HCl	48 ml.	
	Tris	5.98 g.	
	Tetramethylethylenediamine	0.46 ml.	
	H_2O to make	100 ml.	(pH 6.6-6.8)
(C)	Acrylamide	28 g.	
	Methylene bisacrylamide	0.735 g.	
	H_2O to make	100 ml.	
(D)	Acrylamide	10 g.	
	Methylene bisacrylamide	2.5 g.	
	H_2O to make	100 ml.	
(E)	Riboflavin	4.0 mg.	
	H_2O to make	100 ml.	
(F)	Sucrose	40 g.	
	H_2O to make	100 ml.	
(G)	Ammonium Persulfate	0.14 g.	
	H_2O to make	100 ml.	

The following working solutions were prepared from the stock solutions:

(A) Separating gel solution:

1 part A
2 parts C
1 part H_2O

The above was mixed immediately before use with an equal volume of a freshly prepared solution of ammonium persulfate (G). The mixture was used to fill a glass column (9 x 1 cm.) to about 3 cm. and allowed to stand for 40 minutes for polymerization.

(B) Stacking gel solution:

1 part B
2 parts D
1 part E
4 parts F

The solution was layered on top of the separating gel solution and exposed to fluorescent light to facilitate polymerication. The methemoglobin sample was then mixed with 1 ml. of the stacking gel solution and layered over the stacking gel. After polymerization, the sample tube was placed between the two chambers of the electrophoresis apparatus. The chambers contained a Tris-glycine buffer (.3% Tris, 1.44% gly). The current was maintained at 5 milliamperes per tube for the 60 minute running time. The sample tubes were then removed from the apparatus and the gel columns extruded from the glass cylinder. The major methemoglobin band was cut from the column and eluted with a small amount of Tris-glycine buffer. The eluent was then filtered through glass wool. The filtrate was read for absorbance at 409 mμ. 15 mg. of carrier bovine serum albumin was then added to the filtrate and the solution precipitated with 5% TCA. The solution was centrifuged and the pellets dissolved in 0.25 ml. 1N NaOH and transferred to scintillation counting vials. 15 ml. of thixotropic counting fluid was added to each vial and the radioactivity determined in a liquid scintillation spectrometer.

V. Release of Protein Bound Radioactivity from C^{14} δ-ALA Ribosomes

A. In a Complete Cell-Free System of Protein Synthesis

In the complete cell-free system, the incubation mixtures contained, per ml. of assay, 0.25 µmoles GTP, 1 µmole ATP, 5 µmoles phosphoenolpyruvate, 40 µg. pyruvate kinase, 50 µmoles Tris-Cl buffer pH 7.5, 20 µmoles glutathione, 0.05 µmoles of an equimolar amino acid mixture (10^{-3}M), 50 µmoles KCl, 4 µmoles $MgCl_2$, 6 mg. of a 40-70% ammonium sulfate precipitated enzyme from supernatant, and 2 mg. of C^{14} δ-ALA ribosomes. The reaction was carried out at 37°C for the time periods indicated. Each assay was then chilled and transferred to a 4 ml. spinco tube, the tube filled with a 0.25M sucrose, 0.01M $MgCl_2$ solution and the mixture was centrifuged at 100,000 g. for 60 minutes. Supernatants were decanted into respective tubes and 15 mg. of carrier bovine serum albumin was then added to each assay. The precipitate which formed in 5% TCA was centrifuged and reprecipitated with 5% TCA three times. Each pellet was dissolved in 0.25 ml. of 1N NaOH and transferred to a scintillation counting vial. 15 ml. of thixotropic counting fluid was added to each vial and the radioactivity determined by a liquid scintillation spectrometer.

B. Effect of Deoxycholate Treatment of Ribosomes

Six mg. of C^{14} δ-ALA ribosomes were suspended in a 0.5% sodium deoxycholate solution at 0°C for 10 minutes then diluted to 30 ml. with medium B. Ribosomes were reisolated by centrifugation at 75,000 g. for 90 minutes. The pellet was homogenized

in a small volume of 0.25M sucrose and used in a release assay as described above.

C. Effect of Incubation with Puromycin

One μmole of puromycin, pH 7.0, was added to the release assay as described in A.

VI. Column Chromatography of C^{14} α-ALA Labelled Ribosomes

A modification of the procedure by Ganoza and Nakamoto (24) was used. An ECTEOLA cellulose column (3 x 1 cm.) was packed under a pressure of 0.5 lb. per square inch. The column was equilibrated with a solution of 0.1M NaCl, 0.1M NH_4COOH pH 4.7 and 0.5% Brij-35 at room temperature. Before loading, the C^{14} α-ALA ribosomes were diluted to 1 ml. with the buffered detergent, 2 mg. of carrier sRNA added and incubated at 0°C for 10 minutes. The reaction mixture was loaded onto the column and eluted with 80 ml. of a linear gradient containing 0.1M NaCl to 2.0M NaCl and 0.1M NH_4COOH pH 4.7, 0.5% Brij-35. Flow rates were maintained at 1-2 ml. per minute. Eluants were collected in 3.0 ml. portions. Each fraction was determined for absorbance at 260 mμ. To each fraction was added 1 mg. bovine serum albumin and the precipitate which formed in 10% TCA solution was collected on nitrocellulose filters. Fifteen ml. of toluene, PPO, POPOP counting fluid was added and each vial containing a dried filter, and radioactivity determined by a liquid scintillation spectrometer.

RESULTS

It has been established by Shemin, Neuberger and Scott (15, 16) that δ-aminolevulinic acid (δ-ALA) originating from glycine and succinyl CoA is a specific precursor of heme and porphyrins.

In the present studies, rabbit reticulocytes were incubated with C^{14} δ-ALA *in vitro*. Incubation mixtures were extracted with ethyl acetate-acetic acid mixtures and the extracts, containing hemin, porphyrin and possibly other heme intermediates were analyzed for radioactivity. The results, in Table I, show that C^{14} δ-ALA was incorporated better into extractable porphyrins by lysates than by whole cells of rabbit reticulocytes. This result is probably due to poor penetration of the δ-ALA into the intact cells.

Since δ-ALA is not extractable by the procedure employed, the total radioactivity present in the extract of each incubation mixture is a measure of the incorporation of C^{14} δ-ALA into heme or heme intermediates.

Figure 1 shows the incorporation of C^{14} δ-ALA into lysate was linear for the first 10 minutes and reached a plateau around 40 minutes.

The extracts from various incubation mixtures were analyzed for product composition by paper chromatography. The radioactivities of the various components in the chromatogram were measured. As shown in Table II, incorporation of δ-ALA into hemin was detected after five minutes of incubation and continued after 40 minutes of incubation.

TABLE I

INCORPORATION OF δ-AMINOLEVULINIC ACID
INTO EXTRACTABLE PRODUCTS

	δ-ALA Added	Total Radioactivity in Extracts (DPM)
Whole Cells*	2 μC.	39,110
	0.1 μC.	3,225
Lysate	2 μC.	683,879
	0.1 μC.	77,710

*Equal amounts of whole cells and lysate were incubated for 10 minutes

FIGURE 1

INCORPORATION OF C^{14}-α-ALA
INTO EXTRACTABLE PRODUCTS VS. TIME

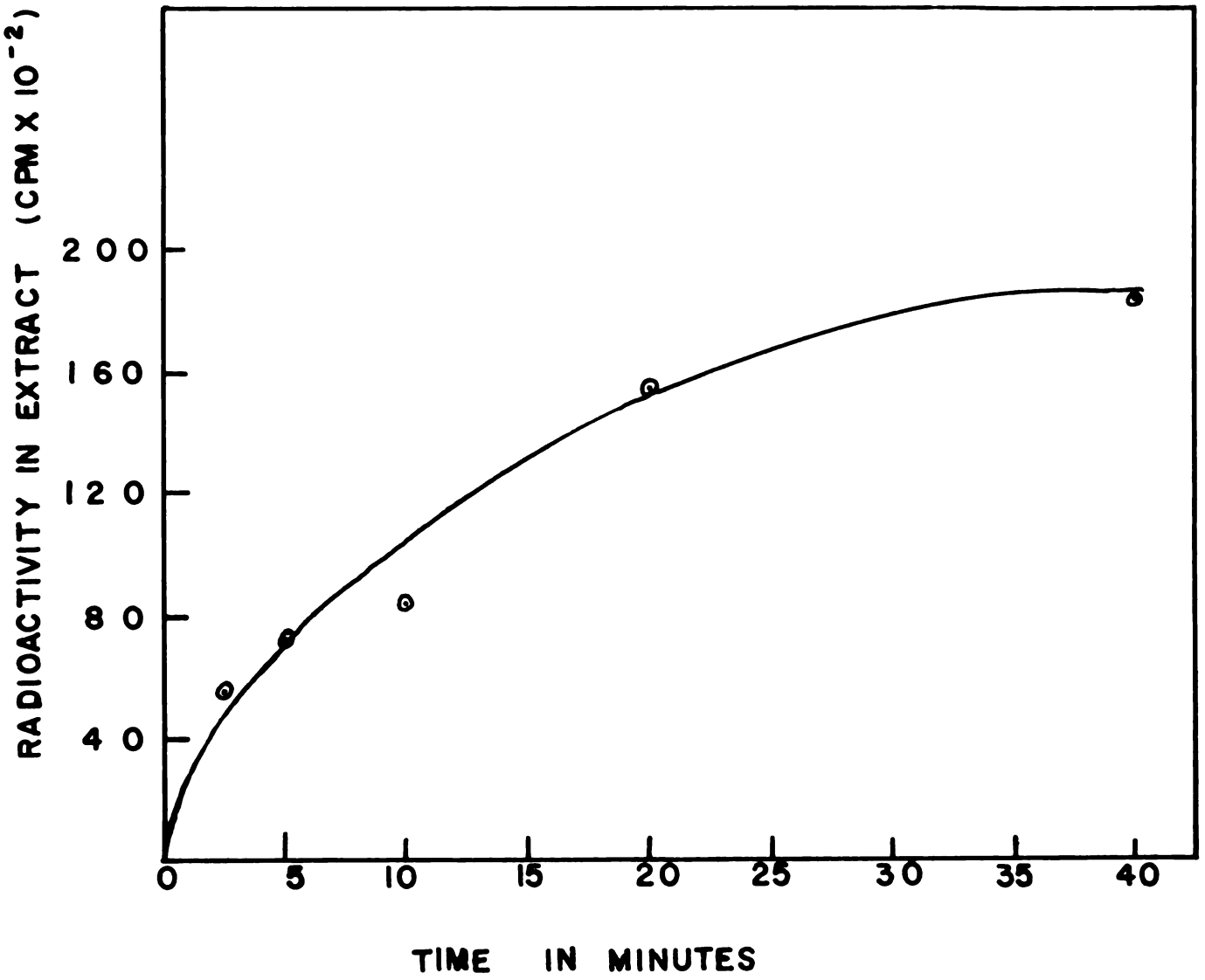

RADIOACTIVITY IN EXTRACT (CPM X 10⁻²)
200
160
120
80
40
0
5
10
15
20
25
30
35
40
TIME IN MINUTES

TABLE II

PAPER CHROMATOGRAPHIC ANALYSIS OF HEMIN EXTRACTS

Compounds Chromatographed	R_f of Major Radioactive Components in extracts	R_f	R_H*
Extract, 5 min. incubation	0.90		0.99
Extract, 40 min. incubation	0.91		1.00
Standard Hemin	0.91		1.00
Standard Protoporphyrin	0.93		1.02

* $R_H = \frac{R_f \text{ of Compound}}{R_f \text{ of Hemin on the Same Chromatogram}}$

Figures 2 and 3 show analyses of sucrose density gradients of ribosomes from rabbit reticulocyte lysates incubated with δ-ALA for 5 minutes and 40 minutes respectively. The patterns of ribosomal and polysomal distribution are almost identical. There is slightly more radioactivity associated with the polysomes from reticulocytes incubated for 40 minutes. In both cases, most of the radioactivity was associated with the soluble hemoglobin fractions.

In order to determine the radioactivity associated with the hemoglobin of the supernatant fractions from reticulocytes, methemoglobin was prepared from the hemoglobin present in these supernatants and analyzed on polyacrylamide gel electrophoretic columns. The results, in Table III, showed that there is an increase of incorporation of C^{14} δ-ALA into the soluble hemoglobin with increased time of incubation.

To study further the radioactivity associated with the polysomal fractions, ribosomes from reticulocytes preincubated with C^{14} δ-ALA were washed with medium B and resedimented several times. Figure 4 shows the decrease in radioactivity associated with the ribosomes after five successive washings.

If the radioactivity associated with the polysomes were attached to the peptidyl globin chains, it should be released into the supernatant with the completed globin chains when the ribosomes were incubated with the necessary components for protein synthesis in a cell-free system (17).

Table IV shows the absence of such a release from two different preparations of ribosomes and at two different stages of washing (2X and 3X). C^{14} valine labelled ribosomes, which were used as controls for these

FIGURE 2

SUCROSE DENSITY GRADIENT ANALYSIS OF RIBOSOMES FROM A RABBIT RETICULOCYTE LYSATE INCUBATED WITH C^{14} δ-AMINOLEVULINIC ACID (δ-ALA) FOR 5 MINUTES

(Solid line denotes optical density at 260 mμ
Dotted line denotes radioactivity in counts/min.)

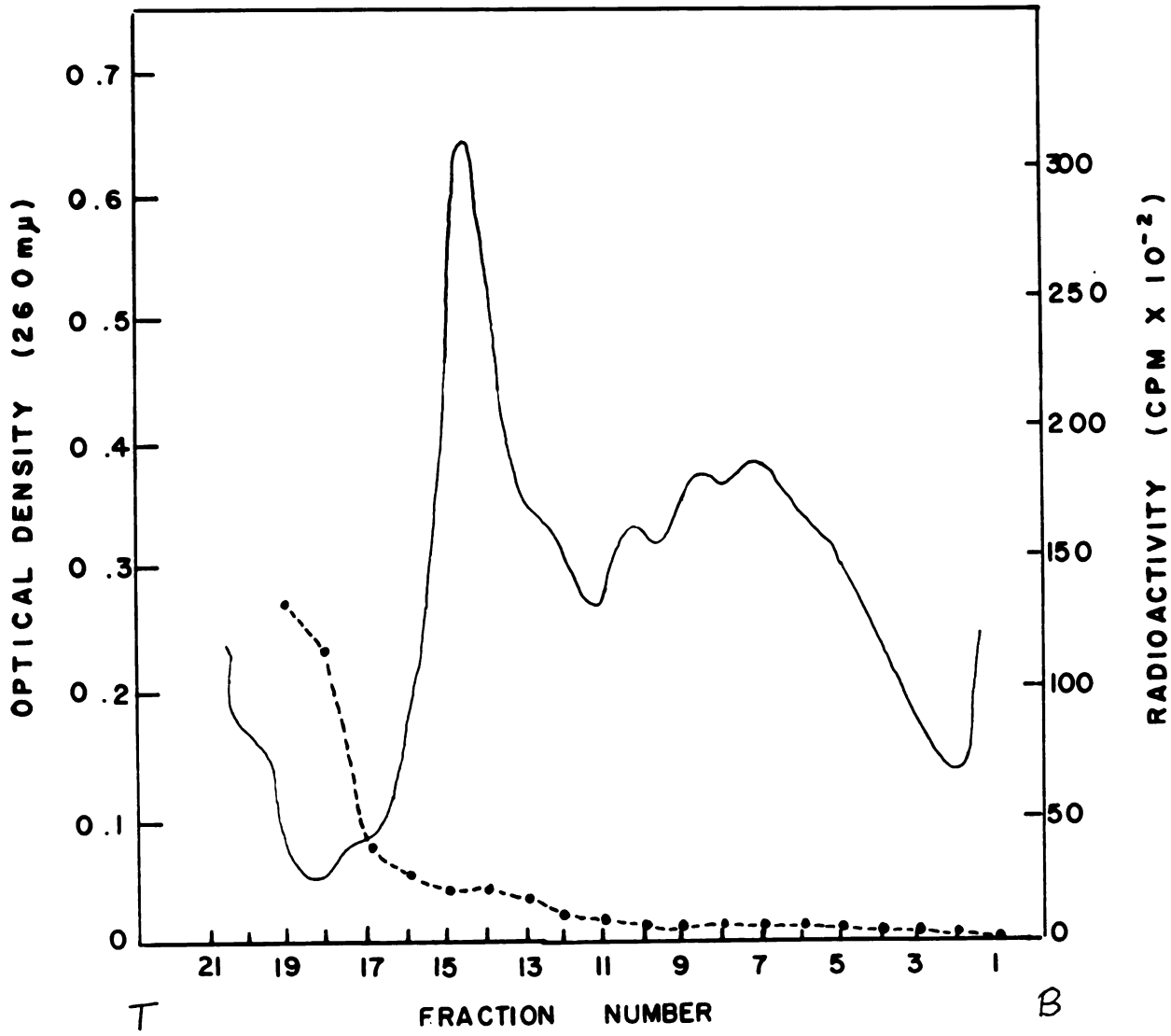

OPTICAL DENSITY (260mμ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
RADIOACTIVITY (CPM X 10⁻²)
300
250
200
150
100
50
0
21
19
17
15
13
11
9
7
5
3
1
T
FRACTION NUMBER
B

FIGURE 3

SUCROSE DENSITY GRADIENT ANALYSIS OF RIBOSOMES FROM A RABBIT RETICULOCYTE LYSATE INCUBATED WITH C^{14} δ-ALA FOR 40 MINUTES

(Designations same as in Figure 2)

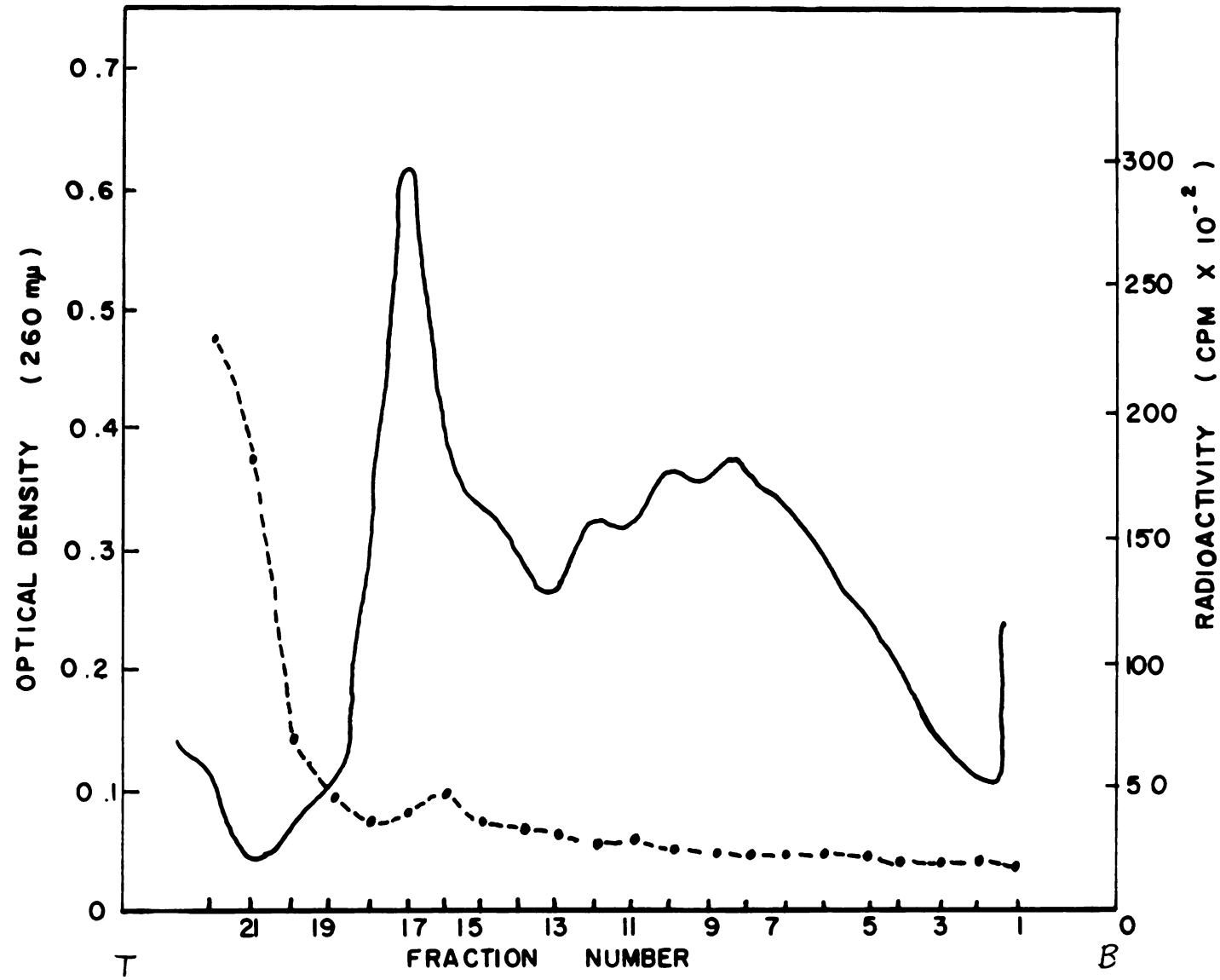

OPTICAL DENSITY (260 mμ)
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
RADIOACTIVITY (CPM X 10⁻²)
300
250
200
150
100
50
0
21
19
17
15
13
11
9
7
5
3
1
T
FRACTION NUMBER
B

TABLE III

POLYACRYLAMIDE GEL ELECTROPHORESIS ANALYSIS OF SUPERNATANTS FROM RABBIT RETICULOCYTES

Incubation Time of Reticulocyte Lysate (min.)	Specific Activities of Supernatants (Counts/min./O.D. 409)
5	258
10	283
20	332

FIGURE 4

RADIOACTIVITY ASSOCIATED WITH RIBOSOMES AFTER
SUCCESSIVE WASHINGS BY RESUSPENSION
AND RESEDIMENTATION

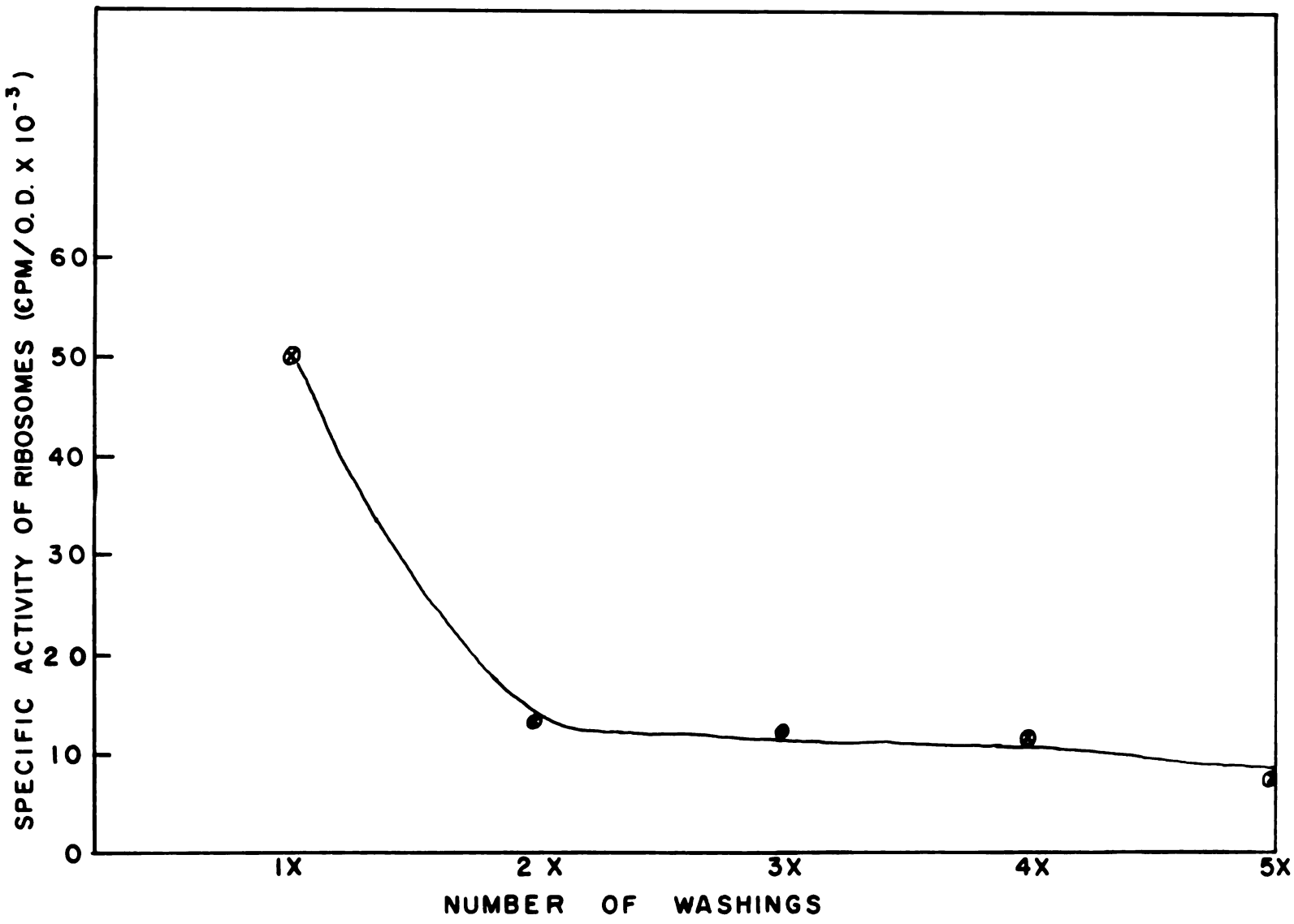

SPECIFIC ACTIVITY OF RIBOSOMES (CPM/O.D. X 10⁻³)
0
10
20
30
40
50
60
1X
2 X
3X
4X
5X
NUMBER OF WASHINGS

TABLE IV

RELEASE OF PROTEIN BOUND RADIOACTIVITY FROM C^{14}-ALA RIBOSOMES

a) 5 Minute Prelabelled Ribosomes (3X)*

Conditions		Radioactivity in Supernatant (counts/min.)	% Release of Total Radioactivity
Complete,	0 min.	4295	31.0
Complete,	5 min.	4791	34.6
Complete,	10 min.	4386	31.6
Complete,	20 min.	4355	31.5
Complete,	40 min.	4200	30.4
Complete minus energy,[a]	40 min.	4360	31.4

*2 mg. ribosomes contained 13,835 counts/min. total radioactivity

b) 5 Minute Prelabelled Ribosomes (2X)**

Complete,	10 min.	2257	43.2
Complete minus energy,	10 min.	2288	43.8
Complete,	40 min.	2607	50.0
Complete minus energy,	40 min.	2440	46.7

**2 mg. ribosomes contained 5,213 counts/min.

c) 40 Minute Prelabelled Ribosomes (2X)***

Complete,	10 min.	939	42.4
Complete minus energy,	10 min.	959	43.1
Complete,	40 min.	900	40.5
Complete minus energy,	40 min.	906	40.8

***2 mg. ribosomes contained 2,220 counts/min.

[a] No energy indicates omission of ATP, GTP, phosphoenolpyruvate and pyruvate kinase.

studies showed a 40-45% energy dependent release of the total radioactivity of the nascent protein from the ribosomes into the soluble phase.

It can be seen that the C^{14} associated with the polysomes was not released with the growing peptide chains and hence appears to be nonspecifically bound to the ribosomes.

In an attempt to reduce the amount of nonspecifically bound proteins on the ribosomes, ribosomes were treated with 0.5% deoxycholate, washed with medium B, resedimented and incubated under similar conditions of protein synthesis. The results, in Table V, indicate that the ribosomes still contained substantial amounts of bound radioactivity.

The radioactivity released from ribosomes after deoxycholate treatment was slightly less than that of the controls (cf Table IV b) possibly because these ribosomes were washed and resedimented one time more than the control ribosomes (cf Table IV a).

Puromycin has been shown to cause the release of nascent polypeptide chains from ribosomes (18, 19, 20). The effect of puromycin on the release of polypeptide chains from C^{14} δ-ALA ribosomes incubated in the presence of protein synthesis was investigated in order to determine whether or not the C^{14} labelled materials bound to ribosomes were also released (Table VI). The results indicate that puromycin had no effect on the release of C^{14} protein bound material from C^{14} δ-ALA ribosomes.

The possibility was considered that heme intermediates attach to globin chains only when reticulocytes are incubated with C^{14} δ-ALA in the presence of active protein synthesis.

Reticulocytes were thus prelabelled with C^{14} δ-ALA in the presence of a complete system of amino acid incorporation (14). Ribosomes

TABLE V

RELEASE OF PROTEIN BOUND RADIOACTIVITY FROM DEOXYCHOLATE TREATED C^{14} α-ALA RIBOSOMES

Conditions	Radioactivity in Supernatant (counts/min.)	% Release of Total Radioactivity
Complete, 5 min.	870	34
Complete minus energy, 5 min.	787	31
Complete, 40 min.	866	34
Complete minus energy, 40 min.	755	30

2 mg. ribosomes contained 2521 counts/min.

TABLE VI

EFFECT OF PUROMYCIN ON THE RELEASE OF PROTEIN BOUND RADIOACTIVITY FROM C^{14} α-ALA RIBOSOMES

Conditions		Radioactivity in Supernatant (counts/min.)	% Release of Total Radioactivity
Complete,	40 min.	734	28.6
Complete plus Puromycin (10^{-3}M)	40 min.	754	29.4

2 mg. ribosomes contained 2582 counts/min.

were isolated and incubated in a release assay similar to those described previously. Results in Table VII indicate that the radioactivity associated with ribosomes preincubated with C^{14} δ-ALA in the presence of protein synthesis is not attached to the globin chains. The slightly higher percent of release by ribosomes in a complete system compared to that incubated without energy was found to be insignificant by later results.

In order to further demonstrate that the radioactivity was not attached to globin chains, ribosomes were dissociated in 0.5% sodium dodecyl sulfate and analyzed on a sucrose density gradient (15). With the conditions of this gradient, soluble RNA sediments to a point about mid-point in the centrifuge tube and soluble globin remains near the top of the tube (23). Results of such analyses of dissociated ribosomes are shown in Figure 5.

There was a considerable amount of radioactivity associated with the sRNA peak and a similar amount of radioactivity associated with the globin peak. In addition, radioactivity was found in between the sRNA and the globin peaks. The presence of radioactivity in the sRNA peak raised the possibility of heme intermediates attached to globin chains which are attached to sRNA on the polysomes. However, the length of time required for the centrifugation produced a broadening of the peaks due to diffusion and an unequivocal interpretation of the data could not be made.

In order to obtain a more concise evaluation of the possible role of heme-peptidyl-sRNA, C^{14} δ-ALA ribosomes were further analyzed for radioactivity associated with sRNA in ECTEOLA cellulose column

TABLE VII

RELEASE OF PROTEIN BOUND RADIOACTIVITY FROM C^{14} α-ALA RIBOSOMES PREINCUBATED IN THE PRESENCE OF PROTEIN SYNTHESIS

Conditions		Radioactivity in Supernatant (counts/min.)	% Release of Total Radioactivity
Complete,	5 min.	1224	41
Complete minus energy,	5 min.	1117	38
Complete,	40 min.	1250	42
Complete minus energy,	40 min.	1132	38

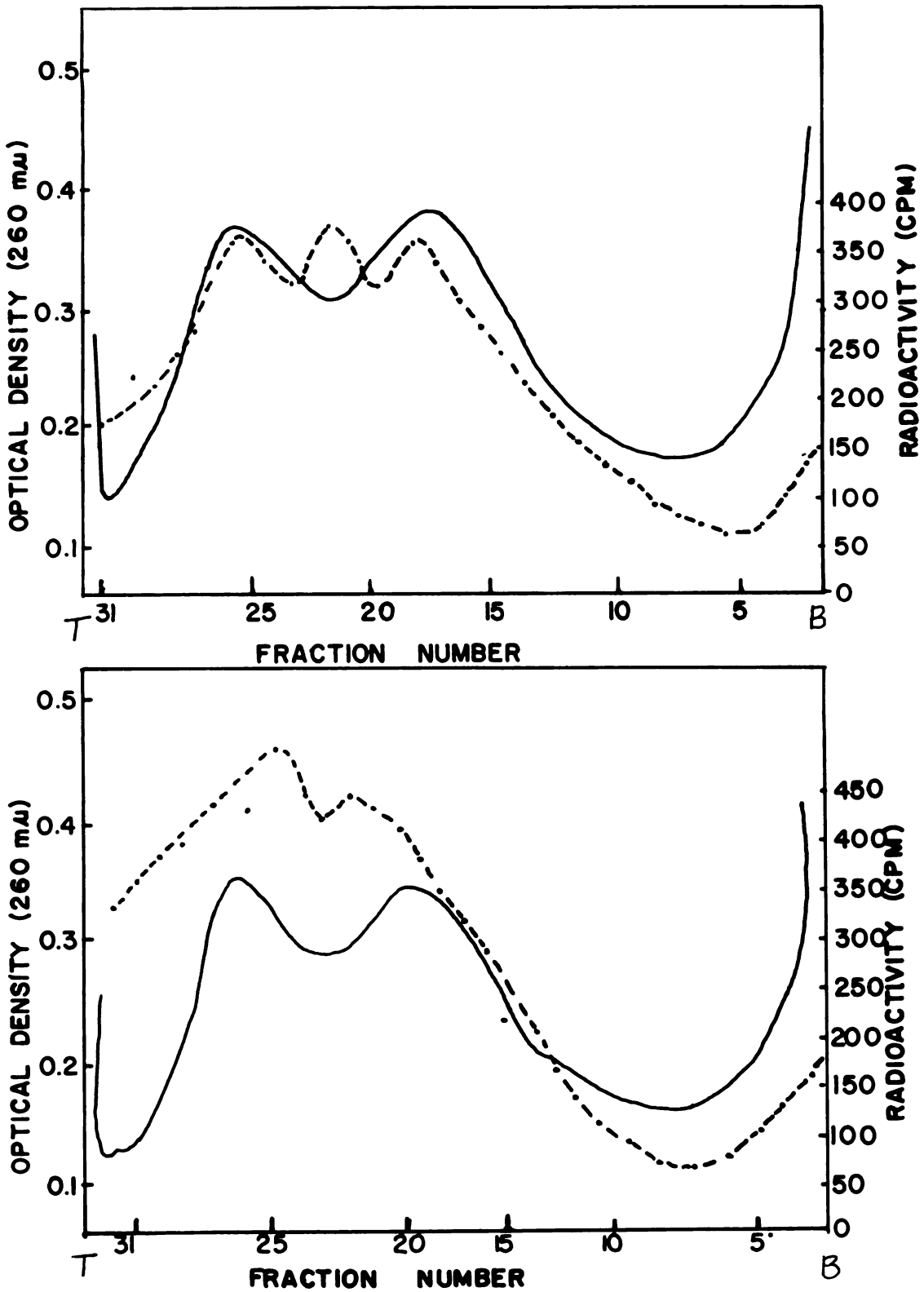
2 mg. ribosomes contained 2972 counts/min.

FIGURE 5

SUCROSE DENSITY GRADIENT ANALYSES OF SOLUBLE RNA FROM C^{14} -ALA RIBOSOMES

a) 2 minute prelabelled ribosomes
b) 5 minute prelabelled ribosomes

(Solid lines denote optical density at 260 mμ; dotted lines denote radioactivity in counts/min.; carrier sRNA peaks at fraction 17 and carrier globin peaks at fraction 26)


OPTICAL DENSITY (260 mμ)
0.5
0.4
0.3
0.2
0.1
RADIOACTIVITY (CPM)
400
350
300
250
200
150
100
50
0
T
31
25
20
15
10
5
B
FRACTION NUMBER
OPTICAL DENSITY (260 mμ)
0.5
0.4
0.3
0.2
0.1
RADIOACTIVITY (CPM)
450
400
350
300
250
200
150
100
50
0
T
31
25
20
15
10
5
B
FRACTION NUMBER

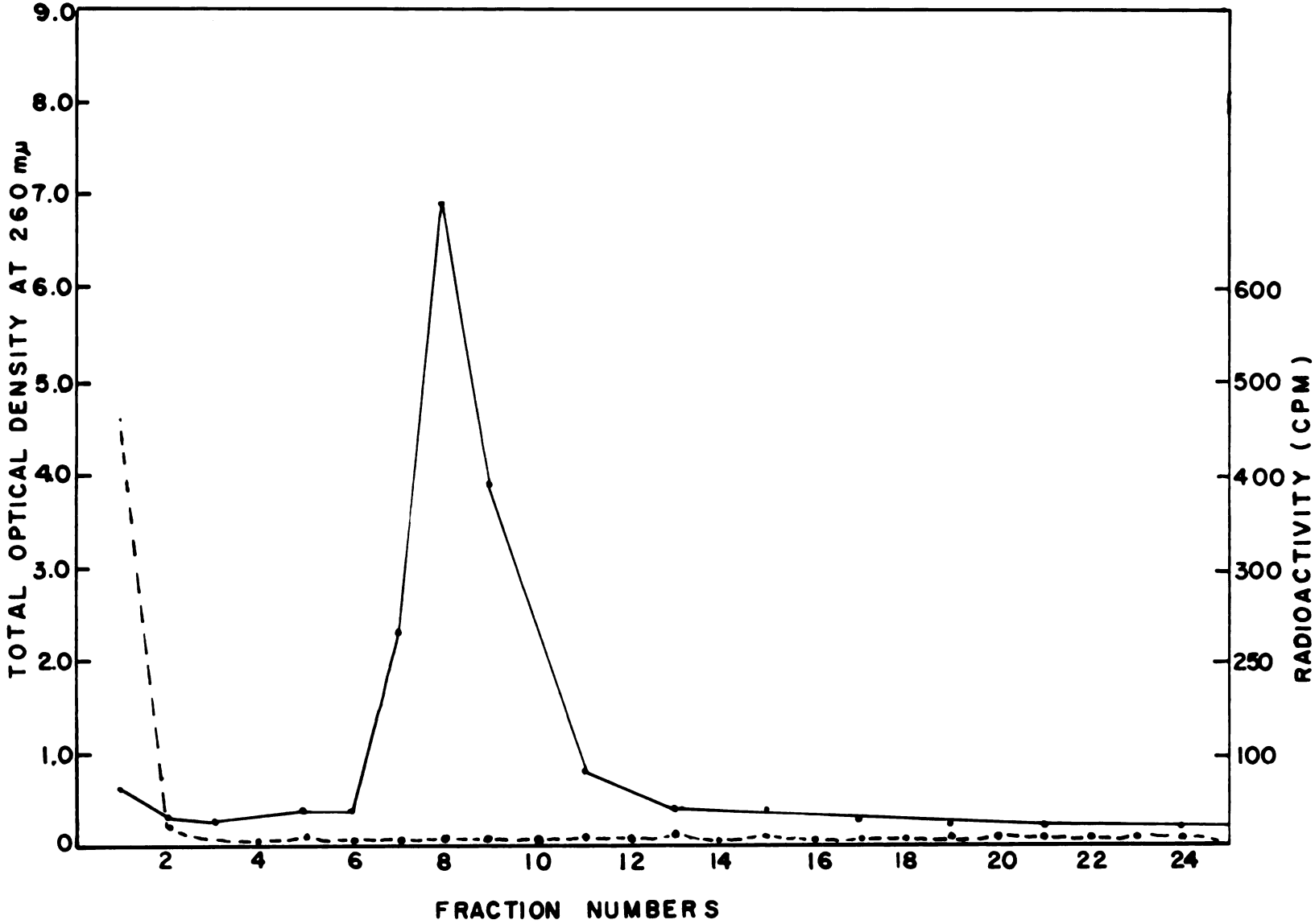
chromatographs, A modification of the procedure according to Ganoza and Nakamoto (24) was used. Soluble RNA and peptidyl-sRNA were eluted from the column at approximately 0.6M NaCl, while soluble methemoglobin was eluted at 0.1M NaCl.

Figure 6 shows the analysis of C^{14} ∝-ALA ribosomes in an ECTEOLA cellulose column. Clearly, all of the radioactivity from the ribosomes was associated with the fraction corresponding to globin and no radioactivity was associated with the sRNA (peptidyl-sRNA) peak. These results provided more conclusive evidence that heme intermediates are not attached to globin chains at the polysomal stage of globin synthesis.

FIGURE 6

SCTEOLA-CELLULOSE COLUMN CHROMATOGRAPHY
OF C^{14} α-ALA RIBOSOMES

(Solid lines denote optical density at 260 mμ.
Dotten lines denote radioactivity in counts/min.)


9.0
8.0
7.0
6.0
5.0
4.0
3.0
2.0
1.0
0
TOTAL OPTICAL DENSITY AT 260 mμ
600
500
400
300
250
100
RADIOACTIVITY (CPM)
2
4
6
8
10
12
14
16
18
20
22
24
FRACTION NUMBERS

DISCUSSION

The present studies have shown that heme or heme intermediates are not combined with globin chains when the latter are being synthesized on polysomes. The attachment, therefore, is believed to occur at a later stage in the synthesis of hemoglobin. The above conclusion is based on the following data:

1) The absence of a release of the C^{14} α-ALA labelled material bound to ribosomes when completed globin chains attached to these ribosomes were released from the ribosomes.
2) The absence of a release of the C^{14} labelled material when nascent polypeptide chains were released from the ribosomes in the presence of puromycin.
3) The absence of heme-peptidyl-sRNA by direct analysis of peptidyl-sRNA.

It is therefore suggested that heme interacts with globin after the latter has been released from the polysome. Winterhalter and Huehns (21) have reported the presence of free globin in the red cell supernatant. They have also shown (22) that heme can promote the conversion of α plus β globin dimers of globin to the stable hemoglobin tetramer. In the light of the present studies, such a role of heme may be postulated for its regulation of globin synthesis. However, it does not seem likely that heme enhances the release from ribosomes of globin chains by coiling around them and causing conformational changes on the polysome as proposed by Gribble and Schwartz (6).

Karibian and London (25) reported that hemin inhibits the incorporation of glycine into heme and postulated a control mechanism which involves a feedback inhibition by heme in the conversion of glycine to δ-ALA. Granick and Levere (26) have reported in their studies with chicken blastoderm that heme synthesis is regulated by the first enzyme in its biosynthetic pathway, δ-aminolevulinic acid synthetase (δ-ALA synthetase).

If indeed heme inhibits δ-ALA synthetase and free globin is present in the soluble fraction of the cell, a mechanism of the regulation of heme synthesis by globin can be postulated. Globin which is released from ribosomes combines with heme to form hemoglobin, thus making heme unavailable to inhibit δ-ALA synthetase and thereby inhibit heme synthesis. However, the reverse mechanism in which heme regulates globin synthesis is not known.

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