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*EXPERIMENTS ON HEMOGLOBIN BIOSYNTHESIS**

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We have been concentrating lately on the chemical characterization of the mechanism of peptide linking, the terminal reaction in the polymerization of amino acids. For this purpose, the laying down of peptide chains on ribosomes was studied with aminoacyl soluble ribonucleic acids (sRNA's), the "active" amino acids, as the donors. Unfortunately, in the much used systems from liver[1] and *E. coli*[2] the protein formed is rather ill-defined. In this predicament, we turned our attention to the hemoglobin-producing system in reticulocytes.[3] As is well known, these cells may be obtained from rabbits appropriately injected with phenylhydrazine. In the resulting phenylhydrazine anaemia, hemoglobin-producing, but already enucleated, red cell precursors, the reticulocytes, replace up to 90 per cent of the normal synthetically inert red cells. A hemoglobin-producing cell-free system has been prepared from reticulocytes;[4] it resembles those from liver and *E. coli*, with the important difference that it produces a well-defined protein.

After it had become clear that the aminoacyl-sRNA represents the activated amino acid from which the peptide link is formed, a rather amazing nonspecificity of aminoacyl-sRNA from various sources[5] aroused our interest. Apparently any amino acid-carrying sRNA would indiscriminately react with mammalian or microbial ribosomes; for example, it was reported[1] that ribosomes from rat liver or mouse neoplasm would utilize *E. coli*-derived aminoacyl-sRNA for synthesis of protein as easily as their own sRNA.

To confirm the general compatibility of any aminoacyl-sRNA, it was decided to attempt hemoglobin synthesis by cross-reacting *E. coli* aminoacyl-sRNA with rabbit reticulocyte ribosomes. Such experiments have been successful and it will largely be the purpose of this paper to report on them. Furthermore, a preliminary analysis is presented of the components of the aminoacyl-sRNA-ribosome system from reticulocytes.

Methods.—Preparation of E. coli aminoacyl-sRNA: Soluble-RNA from *E. coli* was prepared by the phenol procedure developed by Acs.[6] The bacterial paste was suspended in an equal volume of 0.001 *M* Tris-HCl buffer, pH 7.2, containing 0.01 *M* magnesium acetate. The heavy suspension was shaken with an equal volume of redistilled phenol at room temperature for at least 1 hour. The mixture was centrifuged at 10,000 × *g* for 30 minutes, the water layer was removed, and the phenol layer was washed once with 5 ml of 0.001 *M* Tris buffer, pH 7.2. To the combined water layers, one-tenth the volume of 20 per cent potassium acetate solution was added, and the

RNA was precipitated with 2 volumes of ethanol at $-20°$ and washed with 67 per cent ethanol. The precipitate mostly dissolved in 1 M NaCl solution, and a small residue was centrifuged off at 15,000 $\times$ g for 30 minutes. The supernatant was dialyzed overnight against distilled water and lyophilized.

To remove any attached amino acids, the sRNA preparation was incubated in 0.5 M Tris HCl, pH 8.8, for 45 minutes at 35°. For charging with amino acids, a typical reaction mixture contained in a final volume of 10 ml: an equimolar mixture of amino acids, 1 μmole of each amino acid, including uniformly labeled C^{14}-L-leucine (66 $\times$ 10^6 cpm/μM); 0.4 ml of dialyzed 105,000 $\times$ g supernatant from alumina ground *E. coli;* 100 mg of *E. coli* sRNA; 1,000 μM Tris HCl buffer, pH 7.2; 50 μM magnesium acetate; 30 μM ATP; 200 μM PEP; and 0.4 mg of pyruvate kinase. After incubation for 10 minutes at 37°, the reaction was stopped by the addition of 10 ml of redistilled phenol and the charged sRNA was isolated as described. Aminoacyl-sRNA in the amount of 85 mg was obtained, which had a specific activity of 55,000 cpm/mg sRNA, or 0.83 mμM leucine/mg of sRNA.

Preparation of ribosomes from rabbit reticulocytes: Essentially the method of Schweet *et al.*[4] was used. After lysing the washed cells with 4 vol of 0.005 M magnesium acetate for 5 minutes, 1 vol of 1.5 M sucrose-0.15 M potassium chloride was added and the 10,000 $\times$ g precipitate discarded. The supernatant was centrifuged for 1 hour at 105,000 $\times$ g and the ribosome pellet washed once with 0.35 M sucrose, 0.035 M $KHCO_3$-5% CO_2, 0.004 M $MgCl_2$, and 0.025 M KCl, pH 7.4 (Medium A).

Preparation of deoxycholate-washed (DOC) particles for transfer factor studies: Ribosomes, washed once in Medium A, were homogenized in a 0.5 per cent solution of DOC, centrifuged at 15,000 $\times$ g for 10 minutes, and the precipitate rejected. The ribosomes were collected by centrifuging at 105,000 $\times$ g for 3 hours. The supernatant was carefully decanted; the pellets were flushed five times with Medium A and redissolved by homogenizing lightly. Aggregated material was removed by a 10-minute spin at 15,000 $\times$ g. The final solution of ribosomes was made up to approximately 20 mg/ml in Medium A; it could be frozen, and stored well at $-20°$. The concentration of RNA was estimated by optical density determination at 260 mμ, assuming 1 mg/ml to be equivalent to an optical density of 24. Protein content was determined by the Lowry method[7] with bovine serum albumin as standard. Hemoglobin was estimated spectrophotometrically as cyanmethemoglobin.[8]

Assay for radioactivity: The protein precipitated in 5 per cent TCA was treated at 90° for 20 minutes with 5 per cent TCA, washed twice with 5 per cent TCA, once with ethanol/ether 3:1, and finally with ether. The dry material was dissolved in concentrated formic acid, transferred to planchets, weighed after evaporation of the formic acid, and counted. Appropriate corrections for self-absorption were made.

Materials.—ATP and GTP were products of Pabst Laboratories, Milwaukee. Phosphoenolpyruvate (PEP) silver barium salt and pyruvate kinase were obtained from C. F. Boehringer & Soehne, Mannheim, Germany. Uniformly labeled C^{14}-leucine with a specific activity of 88.5 $\mu C/\mu M$ was obtained from Volk Radiochemical Company, Chicago. Carboxymethyl (CM) cellulose was a product of Serva Entwicklungslabor, Heidelberg, Germany, and had a capacity of 0.72 mEq/gm. Trypsin was 2 $\times$ crystallized, salt-free protein, and was obtained from Worthington Biochemicals, Freehold, New Jersey.

CHARACTERISTICS OF THE AMINOACYL-SRNA-RIBOSOME SYSTEM

It has been found with liver ribosomes[5, 9] that the absolute amount of transfer depends largely on the concentration of ribosome; if the proportion between the amino acid-carrying sRNA and the ribosome is optimal, a very efficient transfer occurs. Occasionally it has been observed in liver preparations of this kind that up to 80 per cent of the added amino acid charged to the RNA may be transferred. The efficiency of transfer in the reticulocyte ribosome system is rather similar. As shown in Table 1, up to 66 per cent of the added leucyl-sRNA appears in the protein after 1 hour's incubation if the amount of added aminoacyl-sRNA is well tuned to the amount of particles. A fourfold increase in the amount of aminoacyl-

sRNA results in only a 1.4-fold increase in over-all incorporation.

In contrast to the liver system, under favorable conditions the reticulocyte particle releases most of the newly formed protein. The data in the last two columns of Table 1 indicate that the relative amount of hemoglobin shed into the supernatant depends on the absolute amount of aminoacyl-sRNA added. At a low level, percentage transfer may be high and relatively little is released, while at the highest level used, 80 per cent of the newly formed protein is liberated into the surrounding medium. The release of hemoglobin is obviously favored if the ratio of aminoacyl-RNA to ribosomes is high. This lends support to the conclusion by Bishop *et al.*,[10] and the even more convincingly presented conclusion by Dintzis[11] that globin chains form on the particle by a terminal addition of amino acids. It would then be expected that the number of chains that are completed and can be released will depend on the amount of aminoacyl-sRNA available. An increasing release with increasing availability of active amino acids therefore had to be expected and indeed was found. Morris and Schweet[12] have shown recently that the release of radioactive protein from previously labeled reticulocyte ribosomes is dependent on the presence of soluble enzymes and ATP in the complete system necessary for the incorporation of free amino acids. Neither ATP nor soluble enzymes, other than those present on the washed ribosomes, are required to release soluble hemoglobin in our experiment starting from *E. coli* aminoacyl-sRNA.

TABLE 1

TRANSFER OF INCREASING AMOUNTS OF AMINO ACIDS FROM *E. coli* sRNA TO HEMOGLOBIN

No.	C^{14}-leucine-bound sRNA added		Over-all incorporation		Ribosomes		Supernatant	
	cpm	mμmole	cpm	%	cpm	%	cpm	%
1	880	0.1	530	61	372	70	158	30
2	26,200	3.0	17,300	66	5,250	30	12,050	70
3	105,000	12.0	30,350	29	6,150	20	24,200	80

Incubation conditions were the same as those in Table 3, except that 5 mg ribosomes, once washed in Medium A were used which did not require supernatant factor for activity. Increasing amounts of *E. coli* sRNA containing all amino acids including C^{14}-leucine were added; the specific activity was 8,800 cpm/mμ*M* leucine per mg of sRNA. The incubation was for 1 hour at 35°.

IDENTIFICATION OF HEMOGLOBIN DERIVED FROM *E. coli* AMINOACYL-SRNA

To test the synthesis of hemoglobin from *E. coli* sRNA-bound amino acids, we chose conditions where the larger amount of hemoglobin is separated from the particles. Obviously, for purification and further analysis, the free hemoglobin in solution is much more suitable than that which may be bound to particles. The following large-scale experiment was carried out: 50 mg of reticulocyte ribosomes washed once with Medium A, and 59 mg of *E. coli* sRNA charged, as described under *Methods*, with 18 amino acids and 49 mμ*M* C^{14}-L-leucine having a radioactivity of 3.24×10^6 cpm, were incubated for 60 minutes at 35° in 10 ml containing 1000 μ*M* Tris HCl, pH 7.4, 500 μ*M* potassium chloride, 50μ *M* magnesium acetate, 5 μ*M* GTP, 100 μ*M* PEP, 0.4 mg of pyruvate kinase, 80 μ*M* GSH, and 1.0 μ*M* hemin. A complete amino acid mixture was included containing the 19 free amino acids in the proportions described by Borsook *et al.*[13] and also including 10 μ*M* of nonradioactive L-leucine to dilute out any liberated radioactive leucine. The reaction mixture was cooled after incubation and 4.5 ml of $105,000 \times g$ re-

ticulocyte lysate supernatant were added to provide carrier hemoglobin. The ribosomes were separated by centrifugation at 105,000 × g for 1 hour. Of the added sRNA-bound leucine, 55 per cent was incorporated; about 20 per cent was bound to the sedimented particles and 80 per cent was in the supernatant. The supernatant only was used for analysis.

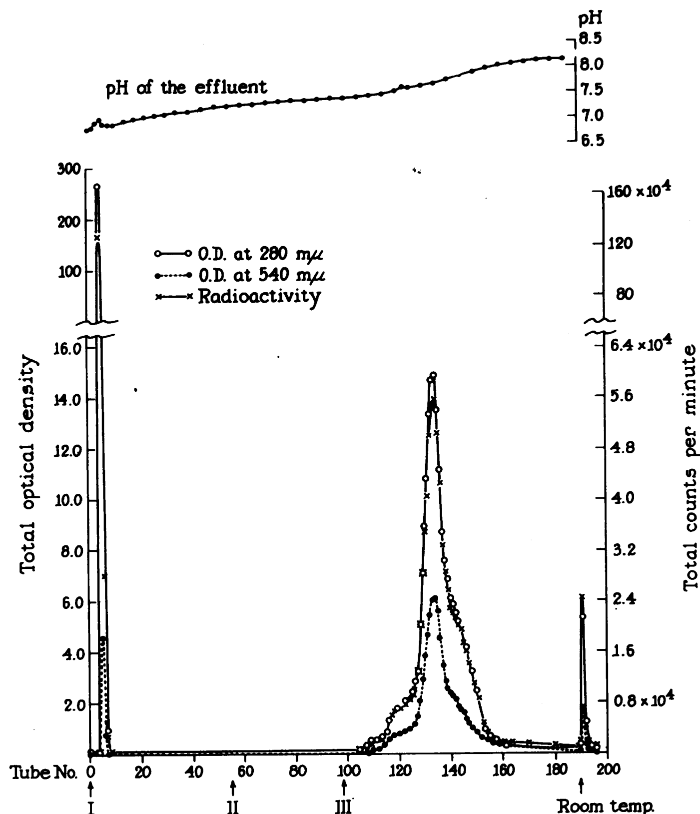

FIG. 1.—Effluent diagram of rabbit CO-hemoglobin synthesized *in vitro* by rabbit reticulocyte ribosomes and C^{14}-leucine containing *E. coli* sRNA. Conditions as described by Gutter *et al.*[14] Buffers: I. 0.01 M Na phosphate at pH 7.0 in 200 ml mixing chamber. Gradient to 0.01 M Na phosphate at pH 7.3 started immediately; II. Gradient to 0.01 M Na phosphate at pH 7.5; III. Gradient to 0.01 M Na_2HPO_4.

Hemoglobin was converted to carbon monoxyhemoglobin by blowing CO over the solution with gentle shaking for 5–10 minutes; the solution was then mixed with 0.05 vol of 1 M $MnCl_2$. A precipitate, comprising largely RNA, was removed by centrifugation and the supernatant dialyzed against 0.1 M Tris buffer, pH 7.5, containing 0.005 M EDTA. A small precipitate formed and was discarded. Then, the hemoglobin was precipitated by 0.9 saturation with solid ammonium sulfate, and the precipitate was dissolved in and dialyzed against 0.01 M sodium phosphate buffer, pH 7.0. The dialyzed solution was placed on a column of 50 × 0.9 cm CM-cellulose and eluted as noted in Figure 1. Fractions of 5–7 ml were collected

and optical densities at 280, 418, and 540 mμ, and the radioactivity were recorded for each fraction. Radioactivity distribution in the progress of purification is seen in Table 2. The fraction used for chromatography contained 57 per cent of the incorporated C^{14}-leucine. As seen in Figure 1, 75 per cent of this radioactivity

TABLE 2

PURIFICATION OF HEMOGLOBIN CONTAINING C^{14}-LEUCINE

Fraction	Protein content (mg)	Hemoglobin content (mg)	Total radio-activity (cpm)	Specific radioactivity (cpm/mg protein)	Per cent
Incubation mixture after addition of carrier hemoglobin	235	172	1.78×10^6	7,570	100
Hemoglobin solution before column	158	112	1.01×10^6	6,400	57
First peak on column	46	6	0.126×10^6	2,740	7.1
Second peak on column	94	94	0.778×10^6	8,280	44
Third peak on column	4	2	0.032×10^6	8,000	1.8

The hemoglobin was synthesized *in vitro* by rabbit reticulocyte ribosomes and C^{14}-leucine-containing *E. coli* sRNA containing 3.24×10^6 cpm. See text for incubation mixture.

coincided with the main hemoglobin peak. No non-heme protein was present in this peak as indicated by the constant ratio of optical densities at 540, 418, and 280 mμ throughout the peak, the ratios being 540 mμ/280 mμ = 0.4; 418 mμ/280 mμ = 4.5. The specific radioactivity (cpm/mg of hemoglobin) remained constant within the experimental error throughout the main peak. Of the 25 per cent of the radioactivity appearing in two minor peaks, the very early one obviously was not adsorbed. A second peak was eluted when, at the end, the column was warmed to room temperature; its specific activity was similar to that of the major peak, whereas the first peak contained a large amount of RNA and a small amount of hemoglobin mixed with non-heme protein of a lower radioactivity. The protein concentration of the early peak was determined by the Lowry method[7] because of the high optical density at 280 mμ due to the high nucleic acid content. This peak probably corresponds to the fraction designed "V" by other workers, [14, 15] and the last peak probably corresponds to the A_2-peak reported with hemoglobin from other species. Altogether, 91 per cent of the total protein on the column was recovered, and 93 per cent of the radioactivity.

The hemoglobin in tubes 128–140 was concentrated by the addition of ammonium sulfate to 0.9 saturation, redissolved in a small amount of water, and dialyzed against water for 2 days. The dialyzed hemoglobin was digested with trypsin and the peptides separated by the method of Ingram.[16] A 2-ml solution containing 18 mg of labeled hemoglobin was brought to pH 8.24 with dilute NaOH in an auto-titrator and denatured in salt free solution by heating in a water bath at 90° for 6 minutes. It was cooled immediately and stirred in an atmosphere of nitrogen at 38°; trypsin, 0.02 ml of a 0.5 per cent solution in 0.001 *M* HCl was added, and the pH kept at 8.2 by automatic addition of 0.1 *N* NaOH. After 65 minutes, a further aliquot of trypsin was added and the digestion continued for an additional 40 minutes. The reaction was stopped by adjusting the pH to 6.5 with 1 *N* HCl and the precipitate was removed by centrifugation. Of the original solution, 0.2 ml was dried *in vacuo* over sulfuric acid and soda lime, dissolved in 0.020 ml of water, and the peptides separated on Whatman No. 3 MM paper cut as described by Ingram.[16]

Electrophoresis was carried out in one dimension between polished glass plates

resting on the two buffer vessels, using pyridine/glacial acetic acid/water in the proportion 10:0.4:90 by volume, at pH 6.4. A potential of 19V/cm was applied for 150 minutes. The paper was dried, and ascending chromatography in the second dimension carried out for 15 hours in *n*-butanol/glacial acetic acid/water, 3:1:1 by volume. The paper was dried again and the peptide spots were developed by dipping the paper into 0.25 per cent ninhydrin in acetone, allowing the color

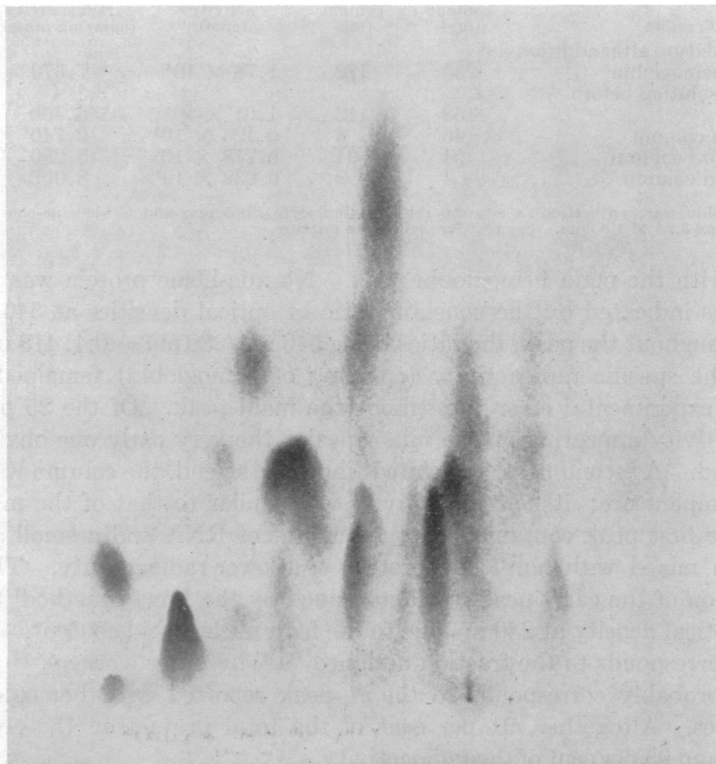

FIG. 2.—Ninhydrin stain of the fingerprint of rabbit hemoglobin obtained from tube 128–140 of the chromatogram shown in Fig. 1. See text for experimental conditions.

development to take place at room temperature. A photograph was taken after 24 hours when maximum intensity was reached (Fig. 2).

A radioautogram was made by exposing X-ray film for 10 weeks to the one-dimensional electropherogram and to the "fingerprint." In the two-dimensional pherogram. or fingerprint, 19 labeled peptides can be seen (Fig. 3). All labeled peptides are superimposable upon the authentic ninhydrin-positive peptides of the nonlabeled carrier hemoglobin. Too little is known with certainty about the structure of these peptides from rabbit hemoglobin, as, for example, whether the two more heavily labeled peptides contain two or more leucine residues per peptide, to warrant a further evaluation of these data. It seems safe, however, to conclude from the labeling pattern, by comparing it with data on human hemoglobin, that both the α and β chains of the rabbit hemoglobin are synthesized from the amino acids bound

to *E. coli* sRNA. The fact that the nonlabeled free leucine present in large amounts in the incubation mixture did not dilute out the C^{14}-leucine sRNA, makes us feel sure that the RNA-bound C^{14}-leucine was indeed incorporated and excludes incorporation by way of reactivation of free C^{14}-leucine liberated by hydrolysis of

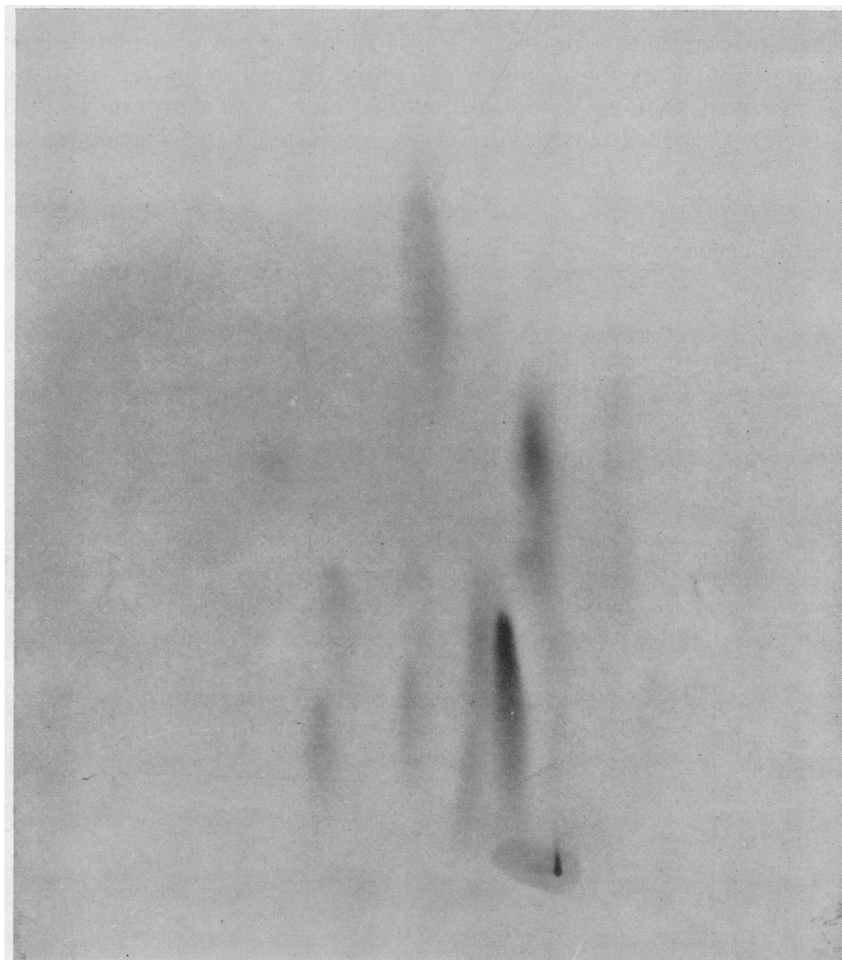

FIG. 3.—Radioautogram obtained by exposing the fingerprint shown in Fig. 2 to X-ray film for 10 weeks.

the *E. coli* aminoacyl-sRNA, which is unlikely anyway in view of the absence of ATP.

PRELIMINARY ANALYSIS OF THE COMPONENTS OF THE TRANSFER SYSTEM

During the last few years, a considerable amount of effort has been directed towards characterizing the components of the system forming peptide bonds with aminoacyl sRNA as the immediate substrate. Nathans has reported on the isolation of enzyme fractions which catalyzed the transfer from aminoacyl-sRNA to liver ribosomes[1] or *E. coli* ribosomes.[2] In order to obtain a good assay for the transfer factor, it was necessary to treat the liver particles with 0.5 per cent DOC

to remove the membrane fraction. The factor is strongly absorbed to the cruder microsomal fraction spun down at 105,000 × *g*. The same is true for a similar reticulocyte fraction. Washing three times with Medium A does not remove the factor and generally no difference could be found with and without addition of supernatant.[17] In the experiments reported in the preceding paragraphs, such microsomal preparations were used. The reticulocytes can be depleted, however, in the same manner as the microsomal fraction from liver by repeated washing with 0.5 per cent DOC as described under *Methods*. As shown in Table 3 and Fig. 4, the DOC-washed particles are nearly completely dependent on addition of

TABLE 3

COFACTOR REQUIREMENT OF AMINO ACID TRANSFER FROM *E. coli* sRNA TO HEMOGLOBIN BY RABBIT RETICULOCYTE DOC PARTICLES

Conditions	C^{14}-leucine incorporated cpm
Complete system	6,110
−PEP, kinase, GTP	122
−GTP	1,530
−PEP, kinase	3,042
−GSH	5,010
−Supernatant factor	856
+1 μM chloramphenicol	5,950
+0.5 μM puromycin	232

The reaction mixture consisted of: 0.25 μmoles of GTP, 10 μmoles of PEP, 40 μg of pyruvate kinase, 8 μmoles of GSH, 0.1 μmole of hemin, 50 μmoles of KCl, 5 μmoles of $MgCl_2$, 100 μmoles of Tris HCl buffer pH 7.4, a balanced mixture of all amino acids[13] including 1 μM of C^{14}-leucine, 2 mg of DOC particles, 0.1 mg of purified supernatant factor, and 77 μg of *E. coli* sRNA containing all amino acids including C^{14}-leucine, 10,000 cpm (specific activity 66,300 cpm/1 mμM leucine) in a total volume of 1 ml. The incubation was for 15 minutes at 37°.

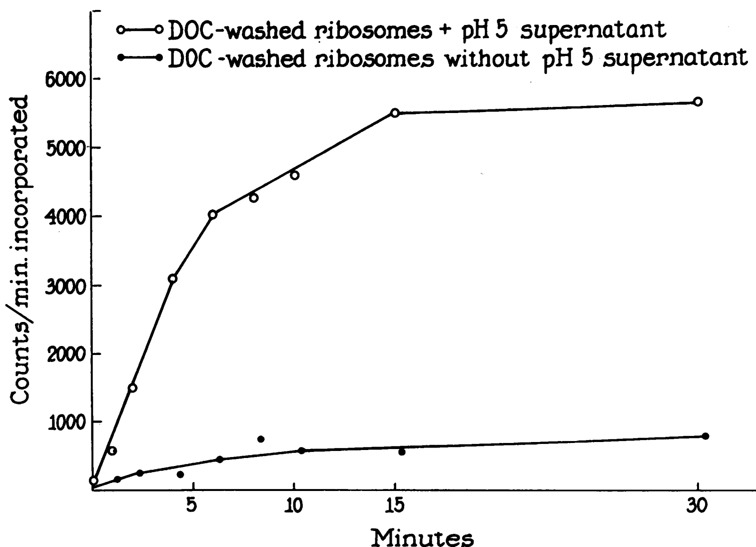

FIG. 4.—Time curve of C^{14}-leucine transfer from *E. coli* sRNA to hemoglobin. Two tubes containing 10 ml of the reaction mixture as given in Table 3 were incubated at 37°. The pH 5 supernatant from reticulocytes was used as the source of transfer factor in one tube while the other tube did not contain any transfer factor. At each time point, 1.0 ml was pipetted into 5 per cent TCA and the precipitate treated as described under *Methods*.

the supernatant and require the usual cofactors for full activity. It is of interest that the rat liver supernatant fraction could be used in place of the comparable reticulocyte fraction. The transfer factor from reticulocytes was partially purified; the pH of the 105,000 $\times$ g supernatant was adjusted to 5.2 and the factor precipitated from the supernatant by 0.55 saturation with ammonium sulfate followed by passage through a short column of CM-cellulose. A further purification may be obtained following roughly the procedure of Nathans.[2, 17] Recently, Bishop and Schweet[18] also reported a similar transfer factor.

Comments.—The result of this study has rather encouraged us to believe that in the system used here, consisting of aminoacyl-sRNA and the ribosomal fraction, we are dealing with the polymerization mechanism in hemoglobin synthesis. The fact that hemoglobin can be synthesized from the amino-acyl-sRNA derived from a microorganism, *E. coli*, makes us believe more strongly in the generality of what one now considers as the amino acid code represented in the amino acid specific sRNA. Comparing the components of this system with the analogous one obtained from *E. coli*, described in a recent paper,[2] GTP and a soluble enzymatic fraction are shown to be needed and both systems are inhibited by puromycin. The resistance of mammalian systems to chloramphenicol, however, in contrast to the inhibition of the analogous microbial system, is quite noteworthy. On no occasion have we observed a reliable effect of chloramphenicol with a mammalian system, even at concentrations of 0.01 molar. Differences between the mechanisms of chloramphenicol and puromycin interference have emerged from experiments with the *E. coli* system. These suggest that chloramphenicol blocks a partial reaction preceding that blocked by puromycin.[19] We are beginning to feel that the difference between the mammalian system and the microbial system must indicate a special, probably additional, feature in the microbial system which precedes the polymerization of amino acids, the mechanism of which we presently presume to be the same in all cells.

We should like to thank Dr. V. M. Ingram for introducing us to the method of fingerprinting.

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† Helen Hay Whitney Foundation Fellow.

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"REVERSIBLE" DNA

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By chemical modification of DNA, we have produced materials whose helix-coil transitions are totally reversible. This communication concerns the properties of this "reversible" DNA, which we have made by two distinctly different processes: (1) Reaction with the chemical mutagen, sodium nitrite, at pH 4.2 (HNO_2-DNA), and (2) Reaction with the cytotoxic bifunctional alkylating agent, bis(β-chloroethyl)methylamine hydrochloride at pH 7.1 (HN2-DNA).

The spectrophotometric and density-gradient centrifugation experiments reported below provide evidence that the chemical reactions leading to these products need not substantially change the secondary structure of double-helical, native DNA. This suggests the hypothesis that the reversibility of denaturation in these chemically modified DNA's is controlled by covalent bonds which link complementary strands of the double helix.

Materials and Methods.—The DNA used in these experiments was isolated from salmon testes by the method of Simmons.[1] *Pseudomonas fluorescens* DNA, used as a density reference in CsCl density-gradient centrifugation, was prepared by a method to be described elsewhere.[2]

Ultraviolet absorbance measurements were made in the following manner. A DNA solution, in a stoppered cuvette, is heated to a given temperature in the thermostated cell compartment of a spectrophotometer and the absorbance (A) at 259 mμ is measured after equilibrium is attained. The cuvette is then removed, plunged into ice to insure rapid cooling (quenching), re-equilibrated in a second spectrophotometer maintained at 25°C, and the absorbance is measured again. This heating and quenching cycle is now repeated at successively higher temperatures. Although an entire denaturation curve can be performed on a single sample, a certain saving of time is achieved by using two cells simultaneously. Measurements at a series of temperatures yield the curves shown in Figures 1 and 3. Absorbances measured at the elevated temperatures yield the curves marked (*d*), while measurements, at 25°C, on quenched solutions yield the curves marked (*i*). Appropriate control measurements are made to verify that the properties of DNA at the highest temperatures are not substantially affected by previous cycles of heating.

Preparation of HNO_2-DNA: Salmon DNA (0.1%) in 0.01 M NaCl was mixed with 4 volumes of 1.25 M $NaNO_2$, 0.3 M Na acetate, pH 4.20, and equilibrated at 25°C. After a specified reaction time, the mixture was placed in ice and 0.5 volumes 2 M Na_2HPO_4 added to neutralize (to pH 6.5 approx.). There followed 24 to 48 hours' dialysis against several changes of 0.01 M NaCl at 1°C.

Preparation of HN2-DNA: HN2, DNA in 0.01 M NaCl and phosphate buffer were mixed to give: 4.3 μ moles DNA-P, 2.4 μ moles HN2, 0.07 M phosphate pH 7.1, total volume 7.7 ml. After 45 minutes' incubation at 25°C, the mixture was cooled in ice and 1 ml added to 9 ml 8.0 M $NaClO_4$, pH 7.05 for the thermal denaturation experiment, shown in Figure 3.

Results and Discussion.—The reversibility of the thermal denaturation of HNO_2-