THE RELATIONSHIP OF RIBONUCLEIC ACID TO THE *IN VITRO* INCORPORATION OF RADIOACTIVE GLYCINE INTO THE PROTEINS OF RETICULOCYTES

by

SEYMOUR B. KORITZ* AND HUBERT CHANTRENNE

Laboratory of Animal Morphology, Université libre, Brussels (Belgium)

INTRODUCTION

The high content of ribonucleic acid in tissues which are in a state of rapid protein synthesis has led to the assumption that these substances are somehow involved in the biosynthesis of proteins[1,2]. However, there has been little information bearing on the mechanisms involved. In an attempt to throw some light on this problem, it was thought that the reticulocyte system might be an appropriate one. It consists of a highly specialized, enucleated cell which is capable of protein synthesis as reflected in its ability to incorporate amino acids into its proteins at an early stage of its development, but not in its mature stage[3,4,5].

That the maturation of the reticulocytes consists of more than only a differentiation process to the exclusion of the synthesis of new proteins is also indicated from the work of NIZET AND ROBSCHEIT-ROBBINS, who found that reticulocytes from dogs suffering from both anemia and hypoproteinemia required a mixture of amino acids for maturation *in vitro*[6].

HOLLOWAY AND RIPLEY[28] have found that with increasing reticulocytosis in rabbits an increase in the RNA content and in the rate of incorporation of labelled leucine into the proteins of the blood cells took place.

By measuring the extent of ^{14}C-glycine incorporation into the reticulocyte proteins, the content of ribonucleic acid, and the formation of specific proteins, all as a function of a developing and disappearing reticulocytosis, it was thought that some insight into the role of ribonucleic acid in protein biosynthesis could be obtained.

EXPERIMENTAL

To induce a reticulocytosis, mature rabbits were injected daily for two successive days with 1.0 ml of a 2.5% neutralized solution of phenylhydrazine. Blood samples were withdrawn from an ear vein daily until the reticulocytosis had largely disappeared. The first blood sample (day 0) was withdrawn prior to the first phenylhydrazine injection. Heparin was used to prevent clotting. The blood cells were separated from the plasma by centrifugation, washed at least twice with isotonic

* Post-doctorate fellow of the American Cancer Society on recommendation by the Committee on Growth of the National Research Council. Present address, the Department of Zoology and Entomology, The Ohio State University, Columbus, Ohio.

References p. 215.

NaCl and resuspended in a volume of isotonic NaCl approximately equal to the original volume of the whole blood. This preparation will be referred to as the washed cells suspension. Erythrocyte counts were made on both the whole blood and the washed blood cells suspension by a standard method. Reticulocyte counts on the basis of 1000 cells counted were made on whole blood after staining with brilliant cresyl blue followed by washing the smear with methyl alcohol and counterstaining with Giemsa solution. In this way the granules of the Heinz bodies were not stained.

At the end of incubations with carboxyl labelled ^{14}C-glycine, 10% trichloroacetic acid (TCA) was added and the resulting precipitate centrifuged down. The precipitate was washed twice with water, twice with ethyl alcohol, three times with a hot alcohol: ether mixture (3:1 v/v), once with ethyl alcohol, and twice with water. Three ml of neutralized 0.4 *M* thioglycolic or thiomalic acid were added and the precipitate was left at room temperature for at least two hours. It was then washed twice with water and hydrolyzed with 6 *N* HCl in sealed tubes at 135° for at least 12 hours. For counting purposes, the hydrolyzates were treated according to VAN SLYKE[7], the liberated CO_2 trapped in NaOH and precipitated as $BaCO_3$. This was washed repeatedly with boiling water and alcohol, plated, weighed, and counted with a thin window Geiger counter. All radioactivity determinations are expressed as counts/min/μM CO_2 and are corrected for self-absorption. All determinations, analytical and experimental, were made at least in duplicate.

Unless otherwise noted in the text, all determinations and assays were made with aliquots from the washed blood cells suspension. Ribonucleic acid was determined by the method of SCHNEIDER[8] as modified by MILLER *et al.*[9]. Hemoglobin was estimated essentially according to the procedure of COHEN AND SMITH[10]. Dipeptidase activity was estimated by incubating lysed blood cells at 38° with DL-alanylglycine (Hoffman-La Roche) in 0.15 *M* borate buffer at pH 8.0. After incubation, 10% TCA was added and the supernatant analyzed by the ninhydrin method of MOORE AND STEIN[11]. Proteinase activity was estimated by incubating lysed blood cells at 38° with casein in 0.1 *M* phosphate buffer adjusted to a final pH of 7.4. The casein used was purified by the method of REMMERT AND COHEN[12]. After the incubation period 10% TCA was added and the supernatant analyzed by the phenol reagent of FOLIN AND CIOCALTEU[13]. Carbonic anhydrase activity was determined essentially according to the colorimetric method of WILBUR AND ANDERSON[14].

Units: Carbonic anhydrase activity was defined by the slope of the initial linear portion of the curve obtained by plotting ml of enzyme against $t' - t$ where t' is the reaction time of the blank and t is the reaction time of the solution containing the enzyme. Activities are expressed as slope per 10^6 cells. Peptidase was determined as γ of glycine liberated in one hour by 10^9 cells. The reaction was run up to 4 hours, and the curves obtained by plotting γ of glycine liberated for both the 1 and 4 hour incubation against days after the initial phenylhydrazine injection had the same shape. Hemoglobin was defined as the optical density of the hemoglobin solution per 10^6 cells.

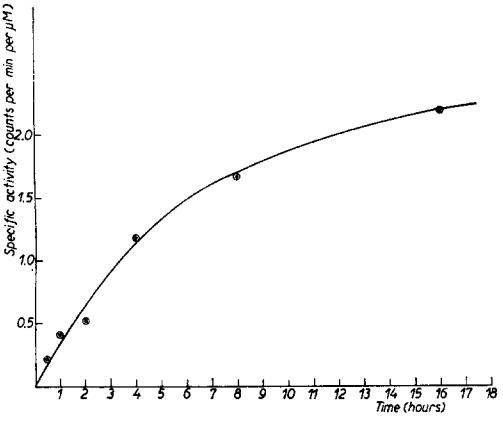

Fig. 1. Rate of incorporation of carboxyl ^{14}C-glycine into proteins of the washed cell suspension. The incubation mixture contained 0.10 ml of 0.145 *M* glucose made up in 0.1 *M* phosphate buffer at pH 7.50, 0.122 ml of carboxyl-labelled ^{14}C-glycine containing 40 μM of glycine per ml, 0.10 ml of an antibiotic solution containing 1.5 mg each of streptomycin and penicillin G per ml, 0.2 ml of 0.15 *M* NaCl and 1.0 ml of the washed cell suspension. The specific activity of the ^{14}C-glycine was 3125 counts per minute per μM. The incubation was at 38° in air, with agitation of the flasks.

RESULTS

It may be seen from Fig. 1 that the incorporation of labelled glycine into the proteins of the washed cell preparation is approximately linear for four hours. The decrease in incorporation with time is also a test of the effectiveness of the antibiotics in the prevention of bacterial growth. In the presence of plasma the rate of incorporation is increased as has been reported by BORSOOK *et al.*[3]

When determinations are made of both the ability to incorporate ^{14}C glycine into proteins and the content of RNA of washed cell suspensions withdrawn on successive days from rabbits which had been injected with phenylhydrazine, curves such as those

References p. 215.

presented in Fig. 2 are obtained. It can be seen that the peak of incorporation of the labelled glycine into proteins occurs 1 to 2 days before the RNA content of the cells reaches its peak. This type of experiment was repeated three times with identical results. These results would appear to indicate that RNA is not the limiting factor in the process of the primary incorporation of amino acids into protein substances. A possible explanation for the delay in the RNA peak involves the assumption that RNA may be concerned with the differentiation of a relatively undifferentiated "primary" protein into the final stereospecific, biologically active form.

If this hypothesis has any validity the formation of various specific proteins should coincide with the RNA peak rather than with the ^{14}C-glycine incorporation peak. Accordingly, carbonic anhydrase, peptidase, proteinase and hemoglobin were determined as a function of the development and disappearance of the reticulocytosis. As may be seen from Fig. 3, the content of carbonic

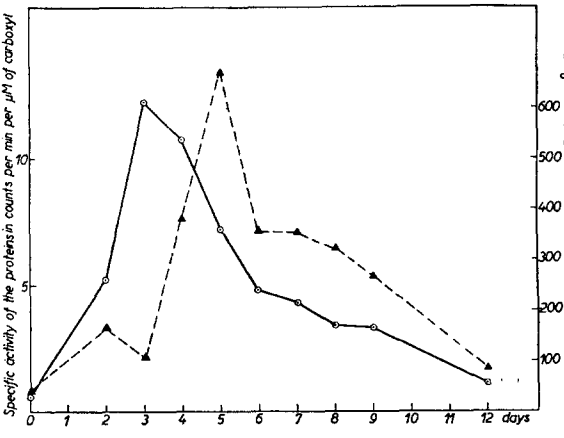

Fig. 2. Relationship of specific activity and RNA content of rabbit blood cells to time after first phenylhydrazine injection. Incubation mixture and conditions were the same as those for Fig. 1. Incubation time was 16 hours. ▲ – RNA, ◉ – specific activity.

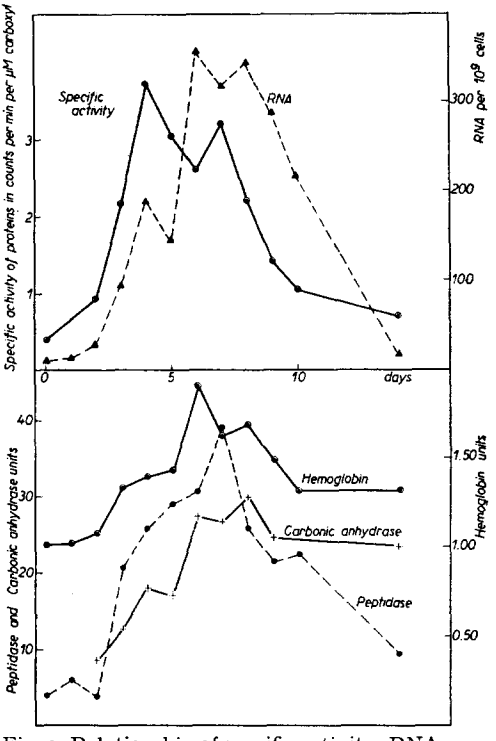

Fig. 3. Relationship of specific activity, RNA, hemoglobin content, carbonic anhydrase and peptidase activity of rabbit blood cells to time after first phenylhydrazine injection. Hemoglobin values were determined on the whole blood. Incubation mixture and conditions were the same as those for Fig. 1. Incubation time was 8 hours. Units: see text.

anhydrase, peptidase and hemoglobin on a per cell basis was found to reach a maximum in good agreement with that of the RNA curve. No proteinase activity was found under the conditions of assay employed. Some comment should be made on the double peaks observed in Fig. 3. During the course of this experiment unusually large amounts of blood were taken from the rabbit to run certain subsidiary experiments. In all probability a secondary reticulocytosis due to anaemia was thus induced. This would also account for the humps observed in the curves in Fig. 2. It is of interest that the second peaks of Fig. 3 show the expected time relationship with the RNA peak coming a day later than the incorporation peak.

References p. 215.

The data presented in Table I indicate that there is no obvious direct relationship tionship between the total reticulocyte content of the blood and either the peak of the incorporation curve or the RNA curve. That an elevated reticulocyte content of the blood is necessary for both phenomena to be observed is apparent from both experimental treatment of the rabbits and the occurence of an elevated reticulocyte count concurrent with the peak in incorporation rate and RNA content of the washed cell suspension. In addition, the presence of RNA in the reticulum of young red cells has been observed directly[215].

The direct observation by radioautography[30] of the presence of the majority of the ^{14}C in the reticulocytes makes it clear that the phenomenon reported here is the result of reticulocytosis.

TABLE I

PERCENT RETICULOCYTES OF WHOLE BLOOD ON DAYS AFTER FIRST PHENYLHYDRAZINE INJECTION

Days	*2*	*3*	*4*	*5*	*6*	*7*	*8*	*9*	*12*
% Reticulocytes	14	32	35	44	41	36	31	31	18

The reticulocyte counts refer to the experiment reported in Fig. 3.

It was considered to be of interest to determine the morphological relationships between the incorporation of labelled glycine into proteins and the ribonucleic acid content, as revealed by centrifugal fractionation. Accordingly, cells which had been incubated with ^{14}C-glycine in the usual manner were washed free of the radioactive medium with isotonic NaCl, hemolyzed with distilled water, and the hemolyzate separated into three fractions in a Spinco preparative ultracentrifuge. Pellet I was obtained by centrifuging the original hemolyzate at 2600 *g* for 15 minutes. This pellet was fairly bulky and grey in colour. Pellet I was obtained by centrifuging the clear red supernatant from the first centrifugation at 135,000 *g* for 45 minutes. This pellet was quite small and a transparent red in appearance. The third fraction was the clear red supernatant from the second centrifugation. As may be seen from the data presented in Table II, the specific activity of the proteins and the content of ribonucleic acid are associated, both being high in pellet I, low in pellet II, and intermediate in the supernatant.

TABLE II

SPECIFIC ACTIVITY OF PROTEIN, AND RNA CONTENT OF VARIOUS CENTRIFUGED FRACTIONS FROM WASHED CELL SUSPENSIONS INCUBATED WITH ^{14}C-GLYCINE

Fraction	*Specific activity counts per μM*	*γ RNA*
Pellet I	4.66	321
Pellet II	1.72	35
Supernatant	2.64	74

DISCUSSION

It is apparent from the data presented that the ribonucleic acid content is not the limiting factor for the incorporation of amino acids into reticulocyte protein. This is

References p. 215.

true for a wide range in variation of speed of incorporation and of ribonucleic acid content. On the other hand, the close parallelism between the ribonucleic acid curve and the hemoglobin, peptidase and carbonic anhydrase curves makes it reasonable to consider that ribonucleic acid may be involved in protein differentiation.

A more precise interpretation of the data obtained is made difficult because of the cellular heterogeneity of the system utilized. There is no obvious direct correlation between the per cent reticulocytes and the incorporation curve or the ribonucleic acid curve (Table I and Fig. 3). Similar observations have been made by LONDON *et al.*[4] who found that no close correlation between the reticulocyte count and the extent of incorporation of ^{15}N-glycine into heme existed. It is thus probably the *type* of reticulocytes rather than the absolute number which determines the observed phenomena. GAVOSTO AND RECHENMAN[30] in this laboratory have obtained recently direct evidence of this: using a refined radioautographic technique, they observed with nuclear plates the tracks of the electrons emited by ^{14}C-atoms of blood cells previously incubated with ^{14}C-glycine under conditions similar to those of the experiments reported above. Their results show quite clearly that the average reticulocytes are nearly ten times as radioactive as the mature red blood cells, and that among the reticulocytes the activity decreases from class I (young reticulocytes) through class II and III to class IV (the classes being defined according to HEILMEYER AND BEGEMANN[31]). In the light of these results, it would appear that in the course of maturation the reticulocytes increase their ribonucleic acid content subsequent to losing their amino acid incorporating capacity.

The results presented may be interpreted to give the following working hypothesis. The biosynthesis of proteins may be divided into at least two major processes. The primary process probably involves the incorporation of amino acids into high molecular weight substances insoluble in trichloroacetic acid and susceptible to ninhydrin degradation after acid hydrolysis. This material may be a relatively undifferentiated "primary" protein. Ribonucleic acid is not directly involved in this process. Secondly, this primary protein is differentiated into its final biologically active form through the mediation of perhaps stereospecific ribonucleic acids. It is conceivable that intracellular peptidases and proteinases may be involved in this second step to open strategic peptide bonds and thus aid in the rearrangement of the molecule. The observed increase in peptidase and the reports by FREIBERG[26], LINDERSTRØM-LANG AND HOLTER[27] and by ROBINSON AND BROWN[28] that proteinase and polypeptidase activity increases in stems and roots of plants which are increasing their protein nitrogen would suggest this possibility.

The concept of the differentiation of a "primary" protein into a final form as a step in protein synthesis has been proposed by ALCOCK[16] and by NORTHROP[17]. Recent work by DANIELSSON[18] on the ripening process in peas favors the idea of high molecular weight protein precursors. DALY AND MIRSKY[20] have come to the conclusion, from studies on the protein content of pancreas during a cycle of secretion and restitution of the cellular enzymes, that a protein to protein transformation takes place in the synthesis of these enzymes. PETERS[19] has obtained convincing evidence that serum albumin is not formed directly from amino acids but arises from the transformation of a protein-like precursor which differs from serum albumin in its physical and serological properties. The results obtained by CAMPBELL AND WORK[29] on milk protein formation are also in line with the same idea.

The non-participation of ribonucleic acid in the primary incorporation of amino acids into proteins is also to be inferred from the results obtained by HOKIN[21], who

References p. 215.

found that doubling the rate of amylase synthesis in the pancreas slices did not result in increased uptake of ^{32}P by ribonucleic acid, but that a stimulation of amylase secretion resulted in a corresponding increase of the ^{32}P uptake by ribonucleic acid. It has also been found by TAKATA[22] that high levels of ribonucleic acid persist in the regenerating lens after regeneration is essentially completed and he has suggested that ribonucleic acid may be involved in protein differentiation.

A role of ribonucleic acid in protein differentiation would imply that there are many ribonucleic acids of various stereospecificities. While there is little conclusive evidence to indicate this, the report by FAIRLY *et al.*[23] indicating that the guanine to adenine ratio of ribonucleic acid isolated from the large granule fraction of homogenized pancreas differed from the ratio for the organ as a whole, suggests this may be the case. It is of interest to note that CHARGAFF[24] has observed that the ribonucleic acids from the same organ of different species were more similar to each other than those from different organs of the same animal species. The report of HERSHEY AND CHASE[25] to the effect that most of the sulfur containing protein of the T_2 bacteriophage does not enter the bacterial cell and that most of the desoxyribonucleic acid does enter also suggests that the structure of the specific proteins synthesized is controlled by a nucleic acid organizer.

SUMMARY

1. The rate of incorporation of ^{14}C-glycine into the proteins of reticulocytes and their RNA content have been determined as a function of the development and disappearance of a phenylhydrazine induced reticulocytosis in rabbits.

2. The peak of labelled amino acid incorporation occurred 1 to 2 days before the RNA peak.

3. The formation of carbonic anhydrase, hemoglobin and peptidase corresponds quite closely with the RNA curve rather than with the ^{14}C glycine incorporation curve.

4. These results are interpreted to indicate that RNA is not involved in the initial incorporation of amino acids into proteins but rather mediates the differentiation of a "primary" protein into its final stereospecific, biologically active form.

RÉSUMÉ

1. Le développement et la régression de la réticulocytose provoquée par l'injection de phénylhydrazine à des lapins s'accompagnent de variations du taux d'incorporation de glycocolle marqué dans les protéines des réticulocytes et de la teneur de ceux-ci en acide ribonucléique.

2. Le maximum de la vitesse d'incorporation du glycocolle dans les protéines s'observe un deux jours avant que la teneur en acide ribonucléique atteigne son maximum.

3. Les quantités d'anhydrase carbonique, d'hémoglobine et de peptidase varient parallèlement à la quantité d'acide ribonucléique, et non au taux d'incorporation du glycocolle marqué.

4. Ces résultats suggèrent que l'acide ribonucléique ne jouerait aucun rôle dans l'incorporation initiale d'acides aminés dans les substances protéiques, mais qu'il interviendrait plutôt dans la transformation de matières protéiques primitives en protéines parfaites douées d'activités biologiques spécifiques.

ZUSAMMENFASSUNG

1. Die Geschwindigkeit des Einbaus von mit ^{14}C markiertem Glycin in die Proteine der Reticulocyten und ihr RNS-Gehalt wurde als eine Funktion des Entstehens und Verschwindens einer mit Phenylhydrazin induzierten Reticulocytose in Kaninchen bestimmt.

2. Das Maximum des Einbaus von markierter Aminosäure tritt 1 bis 2 Tage vor dem RNS-Maximum auf.

3. Die Bildung von Kohlensäureanhydrase, Hämoglobin und Peptidase stimmt viel besser mit der RNS-Kurve als mit der Kurve für den Einbau von ^{14}C-Glycin überein.

4. Diese Ergebnisse werden dargelegt um zu zeigen, dass die RNS nicht bei dem Einbau der Aminosäuren in Eiweissstoffe beteiligt ist, sondern vielmehr die Differenzierung eines "primären" Proteins in seine endgültige stereospezifische, biologisch aktive Form vermittelt.

References p. 215.

REFERENCES

1 J. BRACHET, *Chemical Embryology*, Interscience, New York, London, 1950.
2 T. O. CASPERSSON, *Cell Growth and Cell Function*, W. W. Norton & Co., New York, 1950.
3 H. BORSOOK, C. L. DEASY, H. J. HAAGEN-SMIT, B. KEIGHLEY AND P. H. LOWY, *J. Biol. Chem.*, 196 (1952) 669.
4 I. M. LONDON, D. SHEMIN AND D. RITTENBERG, *J. Biol. Chem.*, 183 (1950) 749.
5 D. SHEMIN, I. M. LONDON AND D. RITTENBERG, *J. Biol. Chem.*, 183 (1950) 757.
6 A. NIZET AND F. S. ROBSCHEIT-ROBBINS, *Blood*, 5 (1950) 648.
7 D. VAN SLYKE, D. A. MACFADYEN AND P. HAMILTON, *J. Biol. Chem.*, 141 (1941) 671.
8 W. C. SCHNEIDER, *J. Biol. Chem.*, 161 (1945) 293.
9 G. L. MILLER, E. E. MILLER AND R. H. GOLDEN, *Federation Proc.*, 9 (1950) 206.
10 B. COHEN AND A. H. SMITH, *J. Biol. Chem.*, 39 (1919) 487.
11 S. MOORE AND W. H. STEIN, *J. Biol. Chem.*, 176 (1948) 367.
12 L. F. REMMERT AND P. P. COHEN, *J. Biol. Chem.*, 181 (1949) 431.
13 O. FOLIN AND V. CIOCALTEU, *J. Biol. Chem.*, 73 (1929) 627.
14 K. M. WILBUR AND N. G. ANDERSON, *J. Biol. Chem.*, 176 (1948) 147.
15 P. DUSTIN, *Bull. Acad. roy. Belg. (Classe des Sciences)*, 28 (1942) 223.
16 R. S. ALCOCK, *Physiological Rev.*, 16 (1936) 1.
17 J. H. NORTHROP, M. KUNITZ AND R. M. HERRIOTT, *Crystalline Enzymes*, Columbia University Press, New York, 1948.
18 C. E. DANIELSSON, *Acta Chem. Scand.*, 6 (1952) 149.
19 T. PETERS Jr., *J. Biol. Chem.*, 200 (1953) 461.
20 M. M. DALY AND A. E. MIRSKY, *J. Gen. Physiol.*, 36 (1952) 243.
21 L. E. HOKIN, *Biochim. Biophys. Acta*, 8 (1952) 225.
22 K. TAKATA, *Experientia*, 8 (1952) 217.
23 J. L. FAIRLEY Jr., H. L. SEAGRAM AND H. S. LORING, *Federation Proc.*, 9 (1950) 169.
24 E. CHARGAFF, *Experientia*, 6 (1950) 201.
25 A. D. HERSHEY AND M. CHASE, *J. Gen. Physiol.*, 36 (1952) 39.
26 S. A. FRIEDBERG, *Science*, 115 (1952) 674.
27 T. PETERS, *J. Biol. Chem.*, 200 (1953) 461.
28 B. W. HOLLOWAY AND S. H. RIPLEY, *J. Biol. Chem.*, 196 (1952) 695.
29 P. N. CAMPBELL AND T. S. WORK, *Biochem. J.*, 52 (1952) 217.
30 F. GAVOSTO AND R. RECHENMAN, *Biochim. Biophys. Acta*, in the press.
31 L. HEILMEYER AND H. BEGEMANN, *Blut und Blutkrankheiten*, Springer Verl. 1951.

Received July 31st, 1953