PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 146, 759–763 (1974)

Heme Synthesis by Cultured Human Marrow Cells in Response to Cyanate-treated Erythropoietin[1] (38188)

ANTHONY S. GIDARI,[2] MARK H. COHEN, AND RICHARD D. LEVERE
State University of New York, Downstate Medical Center, Department of Medicine, 450 Clarkson Avenue, Brooklyn, New York 11203

Sickle cell disease (homozygous hemoglobin S) results from the presence of an abnormal hemoglobin ($\beta 6$ val → glu) in the red blood cells (1) which, upon deoxygenation, causes the erythrocytes to sickle. Cerami and Manning (2) have demonstrated that treatment *in vitro* with potassium cyanate (KCNO) can prevent the sickling of erythrocytes obtained from patients with sickle cell disease. Moreover, sickle cell erythrocytes which have been treated *in vitro* with cyanate exhibit an increase in their mean 50% survival time, as measured by radiochromium, when they are returned to the patient (3). Although cyanate may result in the carbamylation of the ϵ-amino groups of lysine residues in proteins (4), there was no detectable carbamylation of the lysine residues of hemoglobin S in these studies. Thus, these actions of cyanate appear to be mediated by the specific carbamylation of the amino terminal valine residues of the hemoglobin S molecule. More recently, Manning *et al.* (5) have noted that hemoglobin is carbamylated by injected ^{14}C-cyanate to a much greater extent than the serum proteins. This specificity has been attributed to the low pKa of the amino terminal residues of oxyhemoglobin.

Cyanate has the potential, however, to modify the free amino groups of many proteins, since the carbamylation of these groups is irreversible and nonspecific (6). In this regard, deFuria *et al.* (7) have demonstrated a decrease (approximately 25%) in the activity of the enzyme pyruvate kinase in sickle cells treated with cyanate. Additionally, Crist *et al.* (8) have reported that intravenously administered cyanate results in the carbamylation of brain, blood, and liver proteins. Sodium cyanate *in vitro* (10 m*M*) has also been reported to adversely affect the activity of bovine luteinizing hormone and thyroid stimulating hormone as determined by bioassay (9). Thus, the relatively nonspecific aspect of this reaction along with the potential therapeutic use of cyanate in sickle cell disease as an orally administered agent prompted us to examine its effect on the glycoprotein hormone erythropoietin (Ep), the principal regulator of erythropoiesis in man (10).

Materials and Methods. Morphologically normal human bone marrow cells obtained by aspiration from the sternum or iliac crest of patients undergoing this procedure as part of the clinical evaluation of their disease states, were cultured according to a previously reported (11) modification of the method of Krantz (12). The aspirated bone marrow cells were immediately placed in cold NCTC-109 culture medium (adjusted to pH 7.2 with 7% $NaHCO_3$) containing 200 units heparin and 50 units of penicillin G per ml. The cells were washed twice with the cold NCTC-109 solution and finally suspended at a concentration of 2–5 × 10^6 nucleated cells/ml in an incubation medium consisting of 60% NCTC-109 solution and 40% heat-inactivated (56° for 30 min) human plasma, type AB.

Samples (0.8 ml) of the marrow cell

[1] Supported by N.I.H. Grants AM-09838 and HL-15170.

[2] National Institutes of Health Post-doctoral Trainee (Grant AM 05601).

suspension were placed into 35 × 10 mm plastic tissue culture dishes (Falcon Plastics) and then treated with Ep or cyanate-treated Ep. After these additions, the cultures were incubated at 37° in a humidified atmosphere of 5% CO_2 in air for 72 hr. Six hr before the cells were harvested, 0.5 μCi ^{59}Fe citrate was added in 0.1 ml of heat inactivated AB plasma. At the termination of the cultures, the cells were washed three times with cold, isotonic saline. The cells were lysed by freeze-thawing in distilled H_2O (1 ml) and Drabkins' reagent (1 ml). The next day, the heme was extracted into 3 ml of 2-butanone according to the method of Teale (13). Radioheme was determined in an Autowell II gamma counter (Picker Nuclear, Inc.). A minimum of 3 plates was employed for each determination in each experiment. The statistical significance of the data was determined by the use of the Student's *t* test for small samples.

Potassium or sodium cyanate, recrystallized from ethanol-water, or ^{14}C-labeled potassium cyanate (New England Nuclear, specific activity 10 mCi/mmole), or ^{14}C-labeled sodium cyanate (kindly provided by Dr. Peter N. Gillette) was employed as the carbamylating agent. Step III sheep plasma Ep (3.47 IU/mg protein) was obtained from Connought Medical Research Laboratories, Toronto, Canada. In a typical experiment, a solution of 200 m*M* KCNO was prepared just prior to use in each experiment. This solution (0.4 ml) was diluted to 50 m*M* with an Ep preparation (24 IU/ml isotonic saline), adjusted to pH 7.2 with 1–2 drops of 7% $NaHCO_3$, and incubated for 1–1.5 hr at 37°. Following the incubation step, the material was collected into a dialysis sac and dialyzed against cold (4°), distilled H_2O for 1 day. The dialyzed material was used immediately or frozen until just prior to use in the culture system. In each experiment, several control values were determined on samples in which a 0.9% NaCl solution replaced the 200 m*M* cyanate solution. Similar experiments with NaCNO gave essentially identical results.

^{14}C-labeled cyanate and polyacrylamide gel electrophoresis were used to provide evidence that the cyanate-treated hormone had been carbamylated. Thus, 10–40 μl of an Ep preparation which had been incubated with K^{14}CNO, as described above, was electrophoresed at 5 mA/gel according to the procedure of Weber and Osborn (14) with the exception that the gel buffer was used at half-strength. The gels were stained with Coomassie Brilliant Blue R and the absorbance of the stained material at 570 nm determined. The gel was then cut into 0.9 mm sections which were each dissolved by incubation with 0.1 ml 30% H_2O_2 at 50°. The dissolved gel fractions were counted in 10 ml Aquasol in a Beckman LS-250.

Results. The results of three separate experiments are presented in Table I. In each experiment, the treatment of each of the bone marrow cell cultures with 0.4 IU Ep resulted in a significant increase (>200%, $P < 0.01$) in the amount of radioiron incorporated into heme. Heme synthesis in cultures treated with Ep which had been previously incubated with cyanate was also significantly stimulated (>200%, $P < 0.01$), when compared with that in the untreated controls. Moreover, in each experiment there was no significant differ-

TABLE I. Effect of Cyanate-treated Erythropoietin on Heme Synthesis in Normal Human Bone Marrow Cell Cultures.

	CPM Radioheme/ml of 2-butanone extract[a]			
Experiment	Control untreated	Ep	Dialyzed Ep	Cyanate-treated Ep
1	17.26 ± 1.38	70.29 ± 3.50	60.18 ± 5.72	42.71 ± 6.68
2	10.43 ± 0.51	25.05 ± 2.52	24.70 ± 2.55	32.15 ± 1.75
3	88.43 ± 7.24	197.28 ± 11.27	163.48 ± 5.97	181.00 ± 8.51

[a] Mean net counts ± 1 standard error of mean; background CPM ≃ 200. Ep-erythropoietin.

ence ($P > 0.05$) between the values obtained for the Ep stimulated cultures and those for cultures exposed to the cyanate-treated Ep. This demonstrates that the exposure of Ep to 50 m*M* cyanate for 1 hr at 37° did not inhibit the biological activity of the hormone. Additionally, the biological activity of the cyanate-treated hormone, as measured by its ability to stimulate heme synthesis in these cultures, did not usually differ significantly from that of the dialyzed hormone. In one experiment #3, however, a slight but significant ($P < 0.05$) loss of activity was noted in the dialyzed, non-cyanate treated, Ep preparation.

Discussion. Cyanate reacts irreversibly with the amino groups of proteins (6). Thus, despite its potential beneficial effect in sickle cell disease, cyanate has been reported to decrease the activity *in vitro* of erythrocyte pyruvate kinase (7), bovine luteinizing hormone (9) and thyroid stimulating hormone (9). In addition, some investigators (15) have reported a decrease in the over-all activity of the hexose monophosphate shunt and of glucose-6-phosphate dehydrogenase in erythrocytes which have been incubated with cyanate for one hour. Moreover, an inhibition of globin synthesis in human bone marrow cells exposed to cyanate (5 m*M*) has been observed (16).

deFuria *et al.* (7), however, have shown that there is no significant reduction in the major rate-limiting enzymes of the erythrocyte glycolytic pathway. Other studies (9) have shown that the exposure, *in vitro,* of bovine growth hormone, human chorionic gonadotropin, or bovine follicle stimulating hormone to 10 m*M* NaCNO does not effect their biological activities. The latter hormone, however, lost approximately one-third of its activity after exposure to a higher concentration of cyanate (100 m*M*). Moreover, rats receiving 100 mg/kg of sodium cyanate daily show no differences in pituitary weights and pituitary luteinizing hormone concentrations when compared to those of control animals (17). These observations are in accord with the suggestions of some investigators (18, 19) that the only significant change in animals treated daily with cyanate is an increase in the oxygen affinity of the blood. In this regard, deFuria *et al.* (7) have shown that hemoglobin oxygen affinity is a sensitive index of the degree of carbamylation. The results of the present study show that the exposure of Ep to 50 m*M* cyanate for one hour at 37° does not adversely affect the biological activity of the hormone as tested by this system. Thus, the cyanate-treated Ep preparation was able to stimulate heme synthesis in normal bone marrow cell cultures as effectively as native Ep. Cerami and Manning (2) have shown that the concentration of cyanate employed in these experiments is able to irreversibly inhibit, *in vitro,* the sickling of sickle-cell erythrocytes.

Only minor differences were noted, probably reflecting the different degrees of carbamylation of the several proteins in this preparation, between the electrophoretic mobility in polyacrylamide gels of the cyanate-treated preparation when compared with that of the untreated hormone. Several distinct protein bands were present in each preparation. The apparent heterogeneity of more purified Ep (500–2,300 IU/mg of protein) subjected to either polyacrylamide gel electrophoresis or isoelectric focusing has suggested that the active hormone may be a "family" of closely related molecules (10).

In this regard, it is not unequivocally clear that the active hormone has necessarily been carbamylated in these experiments since we employed a reaction volume of 1.6 ml containing 28.8 IU Ep in 8.3 mg of protein. Thus, utilizing the reported (20) specific activity of pure Ep ($\simeq$9,000 IU/mg), it is apparent that only about 3 μg of protein in our preparation is Ep. We are not able to determine what percent of this amount has been carbamylated. Therefore, although an absolute answer to this question will have to await the availability of highly purified Ep, it has been shown that cyanate, as employed here, does not interfere with the biological action of the hormone. The polyacrylamide gel electrophoretic pattern (Fig. 1) of the ^{14}C-cyanate-treated Ep suggests that several proteins in this Ep preparation have been carbamylated by the labeled cyanate. In order to ascer-

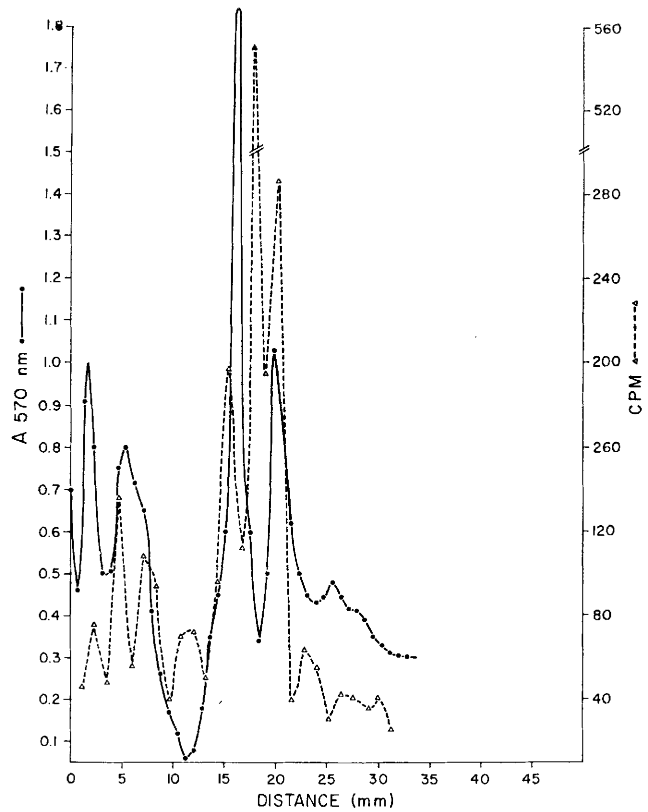

FIG. 1. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of cyanate-treated erythropoietin. Gross CPM have been plotted; the background was less than 10 CPM.

tain that Ep has been carbamylated, we are currently attempting to isolate a more purified ^{14}C-labeled Ep preparation utilizing a preparative polyacrylamide gel.

Cyanate reacts reversibly with sulfhydryl, imidazole, tyrosyl and carboxyl groups of proteins; only its reaction with amino groups is irreversible. In this connection, in these experiments it is likely that only the free amino groups of the molecule have been carbamylated. This suggests that these groups may not be necessary for the biological activity of the hormone.

The excellent technical assistance of Mrs. Amal N. Ibrahim is gratefully acknowledged.

1. Pauling, L., Itano, H. A., Singer, S. J., and Wells, I. C., Science **110**, 543 (1949).

2. Cerami, A., and Manning, J. M., Proc. Nat. Acad. Sci., USA **68**, 1180 (1971).

3. Gillette, P. N., Manning, J. M., and Cerami, A., Proc. Nat. Acad. Sci. USA **68**, 2791 (1971).

4. Stark, G. R., Stein, W. M., and Moore, S., J. Biol. Chem. **235**, 3177 (1960).

5. Manning, J. M., Cerami, A., Gillette, P. M., deFuria, F. G., and Miller, D. R., *in* "Hemoglobin and Red Cell Structure and Function" (G. J. Brewer, ed.), p. 253. Plenum Press, New York and London (1972).

6. Means, G. E., and Feeney, R. E., "Chemical Modification of Proteins," p. 254. Holden-Day, Inc., San Francisco (1971).

7. deFuria, F. G., Miller, D. R., Cerami, A., and Manning, J. M., J. Clin. Invest. **51**, 566 (1972).

8. Crist, R., Grisolia, S., Bettis, C., Diederich, A., Fed. Proc. **31**, 231 (1972) (Abstract).

9. Graziano, J. H., Allen, T. A., Cerami, A., Fed. Proc. **31**, 271 (1972) (Abstract).

10. Gordon, A. S., and Zanjani, E. D., *in* "Regulation of Hematopoiesis" (A. S. Gordon, ed.), Vol. 1, p. 413. Appleton-Century-Crofts, New York (1970).

11. Mizoguchi, H., and Levere, R. D., J. Exp. Med. **134**, 1501 (1971).
12. Krantz, S. B., Life Sci. **4**, 2393 (1965).
13. Teale, F. W. M., Biochem. Biophys. Acta **35**, 543 (1959).
14. Weber, K., and Osborn, M. J., Biol. Chem. **244**, 4406 (1969).
15. Gladen, B. E., and Conrad, M. E., Nature New Biology **237**, 336 (1972).
16. Habib, M., Watson, V., and Schwartz, E., Blood **41**, 635 (1973).
17. Cerami, A., New Eng. J. Med. **287**, 807 (1972).
18. Gillette, P. N., Peterson, C. M., Manning, J. M., and Cerami, A., *in* "Hemoglobin and Red Cell Structure and Function" (G. J. Brewer, ed.), p. 261. Plenum Press, New York and London (1972).
19. Zalusky, R., Zanjani, E. D., Lifrak, I. L., and Ross, J., Program 16th Annu. Meet. Amer. Soc. Hematol., p. 114 (1973) (Abstract).
20. Goldwasser, E., and King, C. K.-H., Proc. Nat. Acad. Sci. USA **68**, 697 (1971).

Received Jan. 12, 1974. P.S.E.B.M., 1974, Vol. 146.