THE INCORPORATION OF N^{15}-GLYCINE BY AVIAN ERYTHROCYTES AND RETICULOCYTES IN VITRO

BY V. ALLFREY AND A. E. MIRSKY

(*From the Laboratories of The Rockefeller Institute for Medical Research*)

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INTRODUCTION

The present report summarizes a group of tracer experiments designed to test (1) whether desoxyribonucleic acid (DNA) and its associated histone are in a state of continuous nitrogen turnover in the avian red cell, (2) whether the rate of glycine incorporation into hemoglobin of the nucleated reticulocyte exceeds that observed in the mature erythrocyte, and (3) whether a difference in rate of synthesis exists between the hemoglobin of the erythrocyte nucleus and hemoglobin in the cytoplasm.

The measure of DNA and histone turnover, and of hemoglobin synthesis, applied was the rate of incorporation of heavy nitrogen into these compounds, following the incubation of avian blood cells in the presence of N^{15}-labeled glycine. The incubation procedure used was essentially that recommended by London, Shemin, and Rittenberg (1), in which a suspension of freshly drawn blood (containing added N^{15}-glycine) is incubated under aerobic conditions at 38°C. Following incubation, the DNA and histone were isolated, and the hemoglobin was crystallized both from whole cells, and from nuclear and cytoplasmic fractions prepared in non-aqueous media (2). The N^{15} concentrations (atom per cent N^{15} excess) of these substances were determined in the mass spectrometer after the usual conversion of organic nitrogen to N_2 gas.

It was found that the incorporation of N^{15}-glycine into histone and DNA by *in vitro* suspensions of chicken erythrocytes and reticulocytes was negligible. The rates of glycine incorporation into hemoglobin indicate that the nucleated reticulocyte is more than twice as active in this respect as the erythrocyte. In both cases the rate of nitrogen incorporation into hemoglobin is low, and is only about a tenth of that measured in the heme prosthetic group. Because of the low over-all N^{15} uptake into hemoglobin, differences which might have existed between nuclear and cytoplasmic hemoglobins could not be detected.

EXPERIMENTAL

Blood was removed from capons and adult chickens by heart puncture, using heparin as an anticoagulant. The animals survived without ill effects when the volume

withdrawn was limited to 20 ml. per kilo body weight. Reticulocyte-rich cell suspensions used in the study of DNA turnover were obtained in the following way; 30 mg. phenylhydrazine per kilo body weight was dissolved in 10 ml. of saline and administered orally, followed after 72 hours by removal of about 20 ml. of blood per kilo body weight (which was discarded) and at 96 hours by removal of the reticulocyte-rich sample for incubation. For measurements of hemoglobin synthesis, reticulocyte-rich cell suspensions were obtained after four successive bleedings at 24 hour intervals (without prior phenylhydrazine administration), followed on the 5th day by removal of the final sample for incubation. Microscopic examination after staining with brilliant cresyl blue and Wright's stain showed that primitive red cells, largely reticulocytes, comprised approximately 70 per cent of the final cell population.

DNA and Histone Turnover.—Parallel experiments were performed with 85 to 100 ml. aliquots of normal blood and 170 ml. aliquots of reticulocyte-rich blood, placed in 1 and 2 liter bottles respectively. To each was added 4 ml. of saline containing enough N^{15}-glycine (34 atom per cent N^{15} excess), penicillin G potassium salt, and streptomycin sulfate to give a final concentration of 1 mg. glycine per ml. and 5 mg. per 100 ml. of each antibiotic. The bottles were mounted on their sides on motor-driven rollers and rotated slowly for 6 hours or 24 hours at 38°C. Following incubation the cells were collected by centrifugation and washed four times with about 5 volumes of cold 0.9 per cent NaCl. There was no hemolysis.

The washed cells were then hemolyzed by homogenization in the Waring blendor with half their volume of ice water. The homogenate was centrifuged at 2500 *G* for 30 minutes and the supernate was set aside for the preparation of hemoglobin as described below. The sediment was washed four times with 150 ml. portions of 0.9 per cent NaCl, and after discarding the washings it was divided into two equal parts for the preparation of histone and DNA.

The histone was extracted from one part by suspending it in 15 ml. of saline, adding an equal volume of 0.25 N HCl, and stirring for 30 minutes. The extract was clarified by high speed centrifugation (10,000 *G* for an hour) and dialyzed overnight against distilled water. The dialyzed extract was brought to pH 10.5 with 1 N NaOH and the precipitated histone collected by centrifugation. It was then redissolved in 40 ml. of 0.25 N HCl, dialyzed against distilled water, and reprecipitated at pH 10.5. The final precipitate was washed with 2:1 ethanol-H_2O, absolute ethanol, and ether, and dried at 106° for 15 minutes. About 130 mg. of histone was obtained from 100 ml. of normal blood by this procedure, which utilizes half of the total sediment for the preparation of DNA.

Desoxyribonucleic acid was extracted from the remaining one-half of the washed nuclear sediment by suspending it in 5 volumes of saline and adding an equal volume of 2 M NaCl. After 18 hours at 2°C., the suspension was centrifuged at 10,000 *G* for an hour. The clear extract was then poured into 6 volumes of distilled water and the fibrous nucleoprotein precipitate collected by winding it about a rod and transferring it to a clean centrifuge tube. It was redissolved in 1 M NaCl, brought to pH 9–10, and recentrifuged as before. The clear supernate was mixed in the blendor with an equal volume of 4:1 chloroform-octanol, the emulsion centrifuged at 2000 *G* for 4 hours, and the clear aqueous layer decanted. The solution was again blended

with 4:1 chloroform-octanol and centrifuged for an hour. The clear aqueous phase was collected and treated twice more with chloroform-octanol as described. The resulting DNA solution was dialyzed against cold 1 M NaCl for 48 hours and against distilled water for 6 hours. The final solution was poured into 3 volumes of ethanol and the precipitated DNA was collected by centrifugation. The nucleic acid was then washed with absolute ethanol and ether and dried at 106° for 15 minutes. About 150 mg. of DNA was obtained from 100 ml. of blood by this procedure.

Heme and Hemoglobin Synthesis.—Parallel experiments were performed with 30 ml. aliquots of normal blood and 50 ml. aliquots of reticulocyte-rich blood containing N^{15}-glycine, penicillin G potassium salt, and streptomycin sulfate in the concentrations listed above. The final isotopic glycine concentration of 1 mg. per ml. is that recommended by London, Shemin, and Rittenberg (1) as the saturating concentration for N^{15} incorporation in heme synthesis by duck erythrocytes. The cell suspensions were placed in 500 ml. bottles; these were mounted on motor-driven rollers and slowly rotated for 23 hours at 38°. Following incubation, the cells were collected by centrifugation and washed four times with about 5 volumes of cold 0.9 per cent NaCl. There was no hemolysis. The washed cells were then hemolyzed by homogenization in the blendor with 30 ml. of ice water. The homogenate was centrifuged at 2500 *G* for 30 minutes and the supernate was decanted. This solution (and the corresponding hemoglobin solutions obtained from cells incubated for 6 hours and 24 hours in studies of DNA and histone turnover) was then shaken with half its volume of toluene and allowed to stand for 18 hours at 2°. The aqueous layer was then clarified by high speed centrifugation and dialyzed against 3 volumes of warm 2.8 M phosphate buffer at pH 6.8 as recommended by Drabkin (3). The first crystals were extensively washed with 2.8 M phosphate buffer, redissolved in water, and dialyzed as before. The recrystallized hemoglobin was then washed, redissolved in water, and dialyzed against distilled water for 7 days. The final solution was lyophilized. About 1 gm. of hemoglobin was obtained in this way from 30 ml. of normal blood or 50 ml. of reticulocyte-rich blood.

Hemin was prepared from the recrystallized hemoglobins by dissolving 900 mg. portions of the latter in 15 ml. of water, adding an equal volume of 0.1 N HCl, and pouring the solution into 300 ml. of acetone containing 3 ml. of 1 N HCl (4). The precipitated globin (which still contains some attached hemin) was filtered off and discarded. Acetone was removed from the filtrate by vacuum distillation and the aqueous residue was lyophilized. About 36 mg. of hemin was obtained in this way from 900 mg. of hemoglobin.

Cell Fractionation.—Aliquots of normal blood (600 to 700 ml.) were incubated for 6 hours or 24 hours at 38° as described above. The final glycine concentration in all cases was 1 mg. per ml. The cells were washed four times with about 5 volumes of saline and then lyophilized. Nuclei were isolated from the dried cells in non-aqueous media by a modified Behrens procedure which is described in detail elsewhere (2). Microscopic examination of these nuclei after staining and counterstaining with acetoorcein-fast green, or hematoxylin-eosin, showed them to be free of cytoplasmic contamination. A cytoplasmic fraction was also prepared from the ground cells by selecting material of specific gravity 1.289–1.293. This fraction contained less than 1 per cent of nuclear contamination as determined by the Dische diphenylamine

reaction for DNA desoxypentose (5). 2 gm. of isolated nuclei was extracted with 25 ml. of saline and the extract was clarified by centrifuging at 10,000 *G* for 30 minutes. The supernate was dialyzed against 2.8 M phosphate buffer as described above for the crystallization of hemoglobin. About 140 mg. of recrystallized hemoglobin was obtained after extensive dialysis against water and lyophilization. 1½ gram portions of cytoplasm and whole cells were extracted with 15 ml. of water and the hemoglobin recrystallized in the usual way. About 530 mg. of hemoglobin was obtained from the cytoplasmic fraction and about 310 mg. from the whole cell preparation.

TABLE I

N^{15} Incorporation into Histone, DNA, Hemoglobin, and Heme of Avian Red Cells on Incubation with N^{15}-Labeled Glycine

Substance	Incubation period	Atom per cent N^{15} excess*
	hrs.	
Erythrocyte hemoglobin	6	0.005
	23	0.006
Erythrocyte hemin	23	0.086
Reticulocyte hemoglobin	6	0.013
	23	0.020
Reticulocyte hemin	23	0.188
Erythrocyte DNA	6	0.000
	24	0.000
Erythrocyte histone	6	0.000
	24	0.000
Reticulocyte DNA	6	0.000
Reticulocyte histone	6	0.000
Nuclear hemoglobin (erythrocyte) recrystallized	6	0.006
Cytoplasmic " recrystallized	6	0.006
Whole cell " recrystallized	6	0.006
Nuclear hemoglobin (crude preparation)	24	0.014
Cytoplasmic " " "	24	0.014

* Atom per cent N^{15} excess over N^{15} concentration of natural avian hemoglobin. Add 0.002 for N^{15} excess over tank nitrogen.

N^{15} Analyses.—The N^{15} concentrations of the isolated DNA, histone, hemoglobin, and hemin were determined in the mass spectrometer (Process and Instruments Company model) after the usual conversion of organic nitrogen to gaseous N_2 by Kjeldahl digestion and treatment with hypobromite. N^{15} concentrations were deter-

mined to within ± 0.001 atom per cent N^{15} (average deviation), and compared with tank nitrogen and natural avian hemoglobin as standards. The atom per cent N^{15} excesses over standard concentration are listed for the different preparations in Table I.

RESULTS

From the data summarized in Table I it can be seen that there was no detectable incorporation of N^{15} into either histone or desoxyribonucleic acid of erythrocytes or reticulocytes. This absence of turnover in the DNA of red cells is in contrast to results obtained in mammalian liver, in which small but definite incorporation of glycine-N^{15} into DNA purines is observed in a 28 hour period (6). The negligible turnover of DNA nitrogen in both mature and primitive nucleated red cells is in agreement with the absence of radiophosphorus turnover reported by Hevesy and Ottesen (7), although it should be emphasized that P^{32} turnover is uniformly low in the DNA of non-growing tissues, such as liver, and that definite incorporations of glycine-N^{15} do occur in non-growing liver in the absence of a corresponding P^{32} uptake (8, 9).

Both the erythrocyte and the reticulocyte utilize N^{15}-glycine for hemoglobin synthesis, and glycine incorporation into heme has been investigated in detail by London, Shemin, and Rittenberg (1, see also 10), who have also shown that the red cell utilizes labeled histidine in globin synthesis. A comparison of the data for N^{15} incorporation by erythrocytes and reticulocyte-rich cell suspensions after 24 hours' incubation shows, first, that the over-all incorporation into hemoglobin of the reticulocyte is about three times that observed in the mature red cell, and secondly, that the N^{15} uptake by the heme prosthetic group is 9 to 14 times the measured over-all incorporation into hemoglobin, and 12 to 20 times the calculated incorporation into the globin component of the molecule. This high N^{15} uptake by heme relative to globin in avian red cells recalls a similar situation in mammals, in which the uptake of C^{14} from methylene-labeled glycine into hemin was 6 to 9 times the incorporation into globin (11). A third point of interest is the observation that while the N^{15} incorporation into erythrocyte heme is nearly half (46 per cent) of that determined in the reticulocyte, the N^{15} concentration of erythrocyte globin is only about one-quarter of the corresponding uptake in the reticulocyte. It follows that maturation of the red cell results in a rapid decline in the rate of utilization of glycine nitrogen for both heme synthesis and globin synthesis, but the deceleration is much more marked in globin synthesis.

Nuclei isolated from avian erythrocytes in non-aqueous media have been shown to contain about 25 per cent of the hemoglobin concentration measured in the cytoplasm. To determine whether this nuclear hemoglobin is characterized by a different rate of synthesis, red cells were fractionated after 24

hours' incubation with N^{15}-glycine (by the procedures described under Experimental). Since the nuclear and cytoplasmic hemoglobins did not differ in N^{15} concentration after this period (Table I), the experiment was repeated using a 6 hour incubation; no differences were observed. It should be emphasized, however, that the over-all N^{15} incorporations into hemoglobin were quite low, and that the sensitivity of the experiments described is correspondingly limited.

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SUMMARY

A study of the incorporation of glycine-N^{15} by chicken red cells *in vitro* has shown that:

1. There is no detectable nitrogen turnover in the histone or desoxyribonucleic acid of erythrocytes or reticulocytes.
2. Hemoglobin synthesis in the nucleated reticulocyte proceeds at 2 to 3 times the rate observed in the mature erythrocyte.
3. The uptake of glycine-N^{15} by heme is 9 to 14 times the corresponding uptake into hemoglobin, and 12 to 20 times the calculated uptake into globin.
4. Maturation of the red cell results in a decline in the rate of synthesis of both heme and globin, but the deceleration is much more marked in globin synthesis.
5. No significant differences could be detected in the low N^{15} incorporations of nuclear and cytoplasmic hemoglobins.

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