Erythropoietin-Stimulated Incorporation of 1-^{14}C-Glucosamine into Glycolipids of Bone Marrow Cells in Culture

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Erythropoietin is the hormone that regulates the process of red blood cell formation (recent reviews: Goldwasser, 1966; Dukes, 1967a). The incorporation of labeled precursors *in vitro* by rat marrow cells is stimulated by the addition of erythropoietin to the culture medium. ^{59}Fe incorporation into heme (Erslev, 1962; Krantz *et al.*, 1963), the entry of ^{59}Fe into marrow cells (Hrinda & Goldwasser, 1966), ^{14}C-uridine incorporation into RNA (Krantz & Goldwasser, 1965), ^{14}C-formate incorporation into RNA (Pieber-Perretta *et al.*, 1965), ^{3}H-thymidine incorporation into DNA (Dukes, 1967b), ^{3}H-thymidine incorporation into erythroblasts, determined by nuclear grain count (Powsner & Berman, 1967) were all reported to be so affected. The incorporation of ^{14}C-glucosamine into acid insoluble stroma components, partially identified as neuraminic acids (Dukes *et al.*, 1964) and into soluble cellular constituents (Dukes & Goldwasser, 1965; Dukes, 1967b) was also reported to be stimulated by erythropoietin. Glucosamine has been reported to label glycolipids, specifically gangliosides, of the brain *in vivo* (Burton *et al.*, 1963; Suzuki, 1967). Gangliosides have been reported to be present in erythrocytes (Booth, 1963; Kuhn & Wiegandt, 1964a). It was therefore of interest to determine whether bone marrow cells in culture would also incorporate glucosamine into glycolipids and if so whether an erythropoietin effect on this process would be observable.

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In this communication I shall demonstrate that incorporation of ^{14}C-glucosamine into lipid fractions of marrow cells does indeed occur and that this incorporation is increased by the addition of erythropoietin to the culture medium. The stimulation occurs in the total lipid extract (TLE) as well as in non-dialyzable subfractions of it which were tentatively identified as glycolipids (gangliosides, hematosides, and globosides).

MATERIALS AND METHODS

Bone marrow cells from tibias and femurs of 6- to 8-week-old, male, Sprague-Dawley rats were collected and incubated in 50% $NCTC_{109}$, 50% calf serum in an atmosphere of 95% air, 5% CO_2 as described previously (Dukes & Goldwasser, 1965; Dukes, 1967b). Incubations were performed in 1 ml volumes containing about 15 x 10^6 nucleated cells. Radioactive label as 0.1 μC/ml of 1-^{14}C-glucosamine - HCl (8.7 mC/mmole) and 0.1 unit/ml erythropoietin, when indicated, were added at the beginning of the incubation.

The incubation period was terminated after 22 hours by chilling the samples for 10 minutes at 4°C. The cells were then treated as follows:

1. For determination of total incorporated radioactivity: Each cell suspension aliquot was washed over into a centrifuge tube with 3 times 2 ml of ice cold 0.001 M sodium phosphate, pH 6.8, buffered saline (PBS). The cells were centrifuged and washed twice more with 2 ml of PBS at 4°C. The washed cells were frozen and thawed, dissolved in 0.2 ml performic acid (formic acid: 30% H_2O_2, 5:1 V/V) and plated on preweighed stainless steel planchets. Dry weight was determined, and the samples were counted in a thin window, low background counter.

2. For determination of acid insoluble radioactivity: The washed cells were resuspended once more in 1 ml of PBS and mixed with 1 ml of ice cold 10% (W/V) trichloroacetic acid (TCA). After 10 minutes the precipitate was centrifuged and washed twice with 3 ml of H_2O. Dry weight and radioactivity were determined as described for washed cells.

Lipid extracts were prepared from 10 pooled incubation aliquots (15×10^7 incubated cells). Washing procedures were similar to the ones detailed above. Washed cells were transferred with 3 x 2 ml PBS and washed acid insoluble precipitate with 3 x 2 ml H_2O respectively to glass-stoppered 10 ml centrifuge tubes. After centrifugation the samples were frozen and thawed.

The lipids were extracted at room temperature by a method adapted from Miras et al. (1966). One ml of absolute methanol was added to each sample. The precipitate was scraped off the wall of the tube and mixed for 10 minutes, then 2 ml of $CHCl_3$ were added and the samples were extracted for 40 minutes. After centrifugation the supernatant extract was removed. Five more 20-minute extractions were performed with 0.5 ml portions of $CHCl_3$:CH_3OH, 2:1 (V/V) (C:M, 2:1) and all the extracts were pooled and designated TLE. Over 90% of the total extractable radioactivity was always found in the first extract and less than 1% in the last extract.

A partition dialysis method adapted from Folch et al. (1951) was used to eliminate low molecular weight labeled material by dialysis from the TLE and to fractionate the extract into polar glycolipids, mainly gangliosides, in the upper (aqueous) phase (U), and other lipids in the lower (organic) phase (L).

This was done as follows: Five ml aliquots of the pooled extracts (TLE) were put into dialysis bags, and dialyzed at 4°C over a 23-hour period against three 1 L changes of distilled H_2O. The contents of the dialysis bags, which had separated into 2 phases, were removed, thoroughly mixed, centrifuged, and the upper phase was removed. To the lower phase 1 ml of "pure solvents upper phase" prepared from C:M, 2:1 and H_2O was added. The phases were mixed and separated again. The upper phases were pooled and dialyzed again over a 23-hour period against two changes of 1 L of distilled H_2O. The last dialysate contained from 0 to at most 3% of the total dialyzable radioactivity. The dialysates were pooled, reduced in volume, and stored frozen (D). The dialyzed upper phases were lyophilized and redissolved in a small amount of C:M,

2:1 (U). The lower phase (L) sometimes required the addition of a few drops of absolute methanol to eliminate cloudiness.

In order to follow the course of extraction, dialysis, and fractionation, radioactivity was determined on aliquots of all fractions and pools. They were counted as infinitely thin samples on aluminum planchets.

Thin layer chromatography of lipids was done on commercially available plates coated with Silicagel G in the solvent system of Wherrett & Cumings (1963), containing $CHCl_3$:CH_3OH: 2.5 N NH_4OH, 60:35:8 (V/V/V). Substances were applied on a line 3 cm from one end of the 20 cm long plates. Solvent was allowed to ascend 14 cm from the origin. The chromatographic behavior of a large number of lipids of interest in this system was described by Honegger & Freyvogel (1963). The system was calibrated with a number of lipids and lipid mixtures as reference substances. In our hands it was found to yield comparable but not identical Rf values and a similar order of migration when compared with Honegger's data. All thin layer plates routinely had a cholesterol reference spot applied to them to make it possible to express the migration of all substances as R_{ch} (Jatzkewitz, 1964). Lipid spots were visualized by UV illumination, 2' , 7' dichlorofluorescein spray (Slipski, 1965) followed by resorcinol-HCl development (Wherrett & Cumings, 1963), which gives a purple color with gangliosides and free neuraminic acid. Alternatively, brom thymol blue (Jatzkewitz, 1964) or ninhydrin were also used to detect spots. Radioactivity on the thin layer plates was measured and recorded with a Packard Model 7201 scanner employing a windowless Geiger-Mueller detector.

Erythropoietin (Step III, lots 137 and 187) was generously provided by the United States Public Health Service Study Section on Hematology. N-acetylneuraminic acid, bovine brain gangliosides, sulfatides, cerebrosides, and sphingomyelin were purchased from Pierce Chemical Company. Brain extract Type VI and lecithin were obtained from Sigma Chemicals. Labeled glucosamine was bought from New England Nuclear Corporation. Merck, 10 x 20 cm,

Silicagel G, F-254, coated prescored glass thin layer plates were purchased from Brinkmann Instruments, Inc.

RESULTS AND DISCUSSION

A series of rat marrow cell incubations was performed to collect cells for lipid extraction. When 0.1 μ/ml of erythropoietin was added to the medium, the specific activity of the washed dried cells[3] increased by an average of about 90% (Table 1).

Table 1. Effect of Erythropoietin on the Incorporation of 1-^{14}C-Glucosamine into Bone Marrow Cells in Culture

	Specific Activity cpm/mg Dry Weight
Control	1228 ± 295 (6)*
Erythropoietin (0.1 u/ml)	2336 ± 492 (6)

*Mean ± S.D., number of experiments in parentheses.

Table 2 shows that components in both the upper and lower phases of the Folch partition-dialysis system became labeled. The addition of erythropoietin to the cultures did not change the distribution of radioactivity among the various fractions although total incorporation increased significantly.[3] This suggests that the glycolipid composition of the erythropoietin stimulated cells was not grossly different from that of those other cells of the culture which did incorporate glucosamine into lipids but were unaffected by erythropoietin. This was further born out by thin layer chromatography of fractions

[3]The dry weight of 10^6 washed rat marrow cells as used for total radioactivity determination or extraction was 70.9 ± 16.7 (8) μg; mean ± S.D., number of experiments in parentheses. The data in Table 2 are based on extractions of pools of about 150 x 10^6 cells corresponding to about 10.6 mg of material. From the data in Table 1 it follows that the average radioactivity of a pool was 13,060 cpm for controls and 24,843 cpm for erythropoietin-treated samples. Treatment of the labeled cells with TCA reduced their dry weight to 32.8 (2) μg/10^6 cells and left the specific activity of the residue approximately the same as that of the starting material.

Table 2. Comparison of the Distribution of Radioactivity in Lipid Extract Fractions of Marrow Cells Incubated With and Without Erythropoietin*

Fraction	Radioactivity			
	Control		0.1 u Erythropoietin	
	% of Total	% of TLE	% of Total	% of TLE
Total lipid extract (TLE)	18.17 ± 4.00 (10)**	100	18.01 ± 3.36 (10)	100
Lower layer (L)	4.58 ± 0.81 (6)	26.25 ± 6.34 (6)	5.15 ± 0.45 (6)	29.7 ± 7.26 (6)
Upper layer (U)	1.64 ± 0.49 (5)	9.60 ± 4.42 (5)	1.74 ± 0.39 (5)	9.74 ± 2.55 (5)
Dialysate (D)	6.04 ± 1.39 (5)	35.24 ± 2.00 (5)	6.60 ± 1.95 (5)	35.5 ± 4.08 (5)
Recovery of TLE L+U+D	12.26	71.09	13.49	74.94

*For the amount of total radioactivity incorporated into the marrow cells, see Table 1 and footnote in the text.

**Mean ± S.D., number of experiments in parentheses.

TLE, L and U: samples from controls and erythropoietin-treated cells showed identical radioactive peak patterns.

The total extract (TLE) of the TCA insoluble residue contained only one-third of the radioactivity found in the TLE of whole cells. Erythropoietin did not seem to have changed this distribution: control 6.0% (2) and 0.1 u erythropoietin 6.7% (2) of total radioactivity. Preliminary experiments suggest that practically no dialyzable radioactivity is found in the TLE from the TCA residue and that the L and U radioactivity is reduced by about a half. Some of the radioactivity lost may have resided in sialic acid residues split off during the conversion of multisialo- into monosialogangliosides caused by the acid treatment. Additionally, one may postulate that only some of the labeled glycolipids of the cells are attached to acid insoluble structures. Others are either detached from these structures by the TCA treatment or were in an acid and water soluble form to begin with.

Thin layer chromatography (Fig. 1) was undertaken to identify the labeled lipids in three of the fractions obtained. Because of the very small quantities of labeled lipids obtained so far their chemical identification has not yet been attempted.

Four radioactive areas (peaks) were observed. The first three fell within the range of migration of resorcinol-HCl positive spots of the controls (up to R_{ch} 0.17) suggesting free neuraminic acid and gangliosides (Wherrett & Cumings, 1963).

Peak 1 contained material that either did not move, or just barely moved off the origin. Proteins, peptides, mucopolysaccharides (Booth, 1963) nucleotide sugars and neuraminic acids have been reported to behave in this way. This peak predominated in the original extract (TLE). Dialyzed fractions (U, L) showed greatly reduced radioactivity in this area. (The composition of the dialysates (D) is under investigation.)

Peak 2 contained material with R_{ch} up to 0.07 suggestive of free neuraminic acid but possibly also some gangliosides. Radioactivity was found

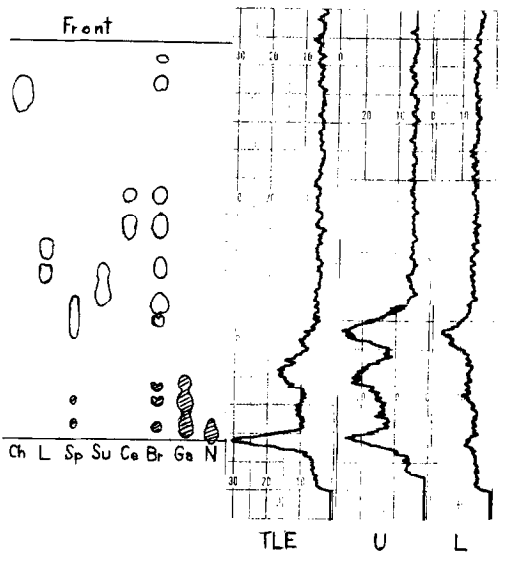

Figure 1. On the left, thin layer chromatograms of reference compounds and mixtures:

Ch = cholesterol (100 µg)
L = lecithin (50 µg)
Sp = sphingomyelin (50 µg)
Su = sulfatides (50 µg)
Ce = cerebrosides (50 µg)
Br = brain extract--bovine (150 µg)
Ga = gangliosides--bovine brain (50 µg)
N = N-acetylneuraminic acid (50 µg)

Shaded spots gave a purple color with resorcinol-HCl.

On the right, radioactivity scanner tracings of TLCs of labeled lipid fractions. For methods, see text.

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in this area of chromatograms of fractions TLE and U.

Peak 3 contained relatively slow moving material with R_{ch} up to 0.18. This labeled material appeared only in chromatograms of the original extract (TLE) and of the upper layer of the Folch distribution (U), was found in a position corresponding to a ganglioside of the reference chromatograms and migrated faster than neuraminic acid but slower than all other lipids. It can

therefore be suggested that it is a ganglioside or a mixture of gangliosides.

Peak 4 contained faster moving material with R_{ch} up to 0.29. This peak appeared in chromatograms of all three lipid fractions. In fraction L this was the only peak containing significant amounts of radioactivity. It could represent gangliosides with a lower neuraminic acid content than the ones found in the slower moving peaks or hematosides (N-acetylneuraminyllactosyl ceramides) (Kuhn & Wiegandt, 1964b; Hakomori & Murakami, 1968. Since however, some of the radioactivity on partition remained in the organic phase, it could also represent hydrophobic glycolipids, free of neuraminic acid, labeled in their hexosamine portion. Yamakawa & Yokayama (1963) and Statter & Shapiro (1963) have described such hexosamine containing glycolipids (globosides) from human red cell stroma.

These findings suggest that newly synthesized glycolipids play a role in conferring differentiated character on the cells responding to erythropoietin.

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