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Inhibition of Heme Synthesis in Bone Marrow Cells by Succinylacetone: Effect on Globin Synthesis

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The effects of 4,6-dioxoheptanoic acid (succinylacetone, SA), an inhibitor of δ-aminolevulinic acid dehydratase, on total iron uptake, heme synthesis, and globin synthesis were studied in rat marrow cells in culture in order to examine the coordination of heme and globin synthesis. SA inhibited heme synthesis in both control and erythropoietin-stimulated cells in a dose-dependent fashion; at 10^{-3} M, inhibition was complete, whereas at 10^{-7} M, there was no significant effect. Inhibition of total iron uptake was also dose-dependent although, at 10^{-3} M, it was not complete. The inhibition of heme synthesis by SA was partially overcome by addition of 10^{-4} M porphobilinogen or protoporphyrin IX. SA caused an almost complete suppression of globin formation in both erythropoietin-stimulated and unstimulated cells as early as five hours after the addition of the inhibitor. When inhibition of heme synthesis was incomplete, globin synthesis was partially inhibited. These results indicate that heme synthesis is required for erythropoietin-mediated induction of globin synthesis in cultured bone marrow cells.

Key words: erythropoietin, succinylacetone, hemoglobin synthesis, heme, globin

In the differentiating red cells of rabbit bone marrow, the synthesis of the components of hemoglobin are closely coordinated; heme causes the stimulated synthesis of α- and β-globin chains and the conversion of αβ-dimers to the hemoglobin tetramer. Heme also acts to coordinate the synthesis and assembly of α- and β-chains [1]. Dabney and Beaudet [2] have shown that addition of hemin to mouse erythroleukemia cells cultured in the presence of dimethyl sulfoxide resulted in a greater than tenfold stimulation of globin synthesis and, in fully induced cells, the inhibition of δ-aminolevulinic acid dehydratase caused the reduction of globin synthesis to the uninduced level. This inhibition was fully prevented by the simultaneous addition of hemin to the cultures, suggesting that exogenous hemin can promote globin synthesis and that endogenously synthesized heme is required for globin synthesis. Hemin has also been shown to promote the synthesis of globin by reticulocytes [3]. Ponka et al [4] have shown that isonicotinic acid hydrazide, which inhibits

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heme synthesis, also inhibits globin synthesis secondarily and reversibly in reticulocytes, and hemin added to the cells relieves the inhibition of globin synthesis. More recent work by Ponka has shown that SA causes the inhibition of globin synthesis in reticulocytes [5]. Succinylacetone, which is formed by decarboxylation of the β-keto acid succinylacetoacetate, an unusual metabolite found in patients with hereditary tyrosinemia [6], has been shown to be a potent inhibitor of δ-aminolevulinic acid (ALA) dehydratase [7–9].

The present study was undertaken to determine the effect of inhibiting heme synthesis on globin formation in vitro in erythropoietin(epo)-stimulated and control marrow cells that were isolated from rats with suppressed erythropoiesis. Specifically, we deal with the question of whether heme synthesis is required for induced globin synthesis to proceed. We have made use of the fact that succinylacetone can block heme synthesis completely in these cells by inhibiting δ-aminolevulinic acid dehydratase.

MATERIALS AND METHODS

Animals

Male Long Evans rats (Charles River Breeding Laboratories, Inc, Wilmington, MA), 8–12 wk old, were used in these experiments. To reduce the number of mature erythroid cells in the bone marrow, the animals were first made polycythemic by placing them in a decompression chamber at 0.5 atm for 3–4 wk, as previously described [10]. The drinking water was supplemented with iron by adding 0.3 ml of a 25 mg/ml iron solution (Fer-In-Sol, Mead Johnson and Co, Evansville, IN) per 450 ml of drinking water. After the rats were taken out of the chamber, they were kept at normal pressure for 5–6 days to allow differentiated erythroid cells to mature and leave the marrow. Only animals that had hematocrits greater than 65% after the rest period were considered to be suppressed and used as a source of marrow cells for all experiments, except those dealing with the question of reversal of inhibition due to SA, where normal marrow was used.

Cell Culture Techniques

Cells were flushed from the femora and tibiae of polycythemic rats with medium and the final nucleated cell count was adjusted to 20×10^6 cells/ml [11]. The composition of the medium was 65% NCTC 109, 30% fetal calf serum and 5% rat serum containing 73 nmol of ferric nitrate per ml [12]. The sera were heat-inactivated at 56°C for 30 min before use. Complete medium contained 50 μg/ml of gentamicin (Garamycin, Schering Corporation, Kenilworth, NJ) as an antibiotic. NCTC 109 was obtained from Gibco Laboratories (Grand Island Biological Company, Grand Island, NY), fetal bovine serum, from KC Biological, Inc (Lenaxa, KS), and rat serum, from Pel-Freez Biologicals (Rogers, AR).

Erythropoietin (prepared in this laboratory), where present, was added at a final concentration of 0.1 U/ml. The specific activity of the erythropoietin was 56,100 U/mg of protein (approximately 80% pure). Succinylacetone (United States Biochemicals Corp, Cleveland, Ohio), in all except the dose-response experiment, was added at a final concentration of 10^{-3} M from a 0.5 M stock solution in deionized water. All incubations were in a humidified incubator at 37°C and 3% CO_2 in air.

Determination of Total Iron Uptake and Heme Synthesis

Two-tenth-milliliter cell suspensions were aliquoted into the wells of disposable culture trays (Costar 3524, Cambridge, MA) and, at the times specified for each experiment, 20 μl of rat serum containing radioiron (approximately 10^5 cpm) were added and the cultures terminated 5 hr later. The method used to label transferrin with ^{59}Fe was as outlined previously [11] with the following modifications: 4.5 ml of NCTC 109 and 0.5 ml of 7.5% $NaHCO_3$ were added to 5 ml of frozen rat serum and 0.1 ml of ^{59}Fe (supplied as $^{59}FeCl_3$ in 0.1 N HCl, 1 mCi/ml, about 100 μg of Fe/ml, by Amersham-Searle Co, Arlington Heights, IL) was used. After thawing and mixing, the rat serum was incubated for 30 min at 37°C before storing overnight at 4°C. Total ^{59}Fe uptake and ^{59}Fe incorporation into heme were determined as described previously [11]. In the statistical analysis of these and other data the Student's t-test was used.

Sephacryl S-200 Chromatography of Marrow Cell Lysates

The effect of SA on heme synthesis can be determined by measuring the amount of ^{59}Fe in the hemoglobin peak, since almost all of the heme extractable from marrow cells after acid treatment is derived from hemoglobin [10,13]. Hemoglobin was separated by gel permeation chromatography on S-200 (Pharmacia, Uppsala, Sweden). Five-milliliter cultures, containing 20 × 10^6 cells/ml, were incubated in 25-cm^2 plastic tissue culture flasks (Costar 3050, Cambridge, MA) with and without epo and with and without SA. One group was incubated with 0.5 ml of rat serum containing ^{59}Fe added at zero time and label was added to the second group at 19 hr of incubation. The cultures were terminated 5 hr later (at 5 and 24 hr, respectively) by washing over into centrifuge tubes with ice-cold phosphate buffered saline (PBS:0.145 M NaCl, 0.013 M phosphate pH 7.3). The cells were centrifuged at 1,200 rpm for 10 min at 4°C. The supernatant fluid was discarded and the pellet washed three times with 12 ml of cold PBS. The cells in the final pellet were lysed by addition of 0.5 ml of ice-cold deionized water and vortexed intermittently. After 5 min, isotonicity was restored by adding 27 μl of 3 M KCl. The lysate was centrifuged at 12,000g for 15 min at 4°C. The supernatant solution was saved and the pellet was resuspended in 1 ml of cold PBS. After a similar centrifugation, the supernatant solutions were combined and applied to an S-200 column (1.5 × 50 cm). PBS was pumped through the column at a rate of 0.6 ml/min and 2.0 ml fractions were collected. The ^{59}Fe in each fraction was counted and absorbance at 280 and 415 nm measured.

Determination of the Rates of Total RNA and Protein Synthesis

Aliquots of cells (0.2 ml, at 20 × 10^6 cells/ml) were incubated in disposable culture trays (Costar 3524) and pulsed for 1 hr with 1.0 μCi of ^{3}H-uridine (30 Ci/mmol, Amersham, Arlington Heights, IL) or 0.5 μCi of ^{3}H-leucine (52.2 Ci/mmol, New England Nuclear, Boston, MA) beginning at 4, 9, 23, and 47 hr in vitro. The cells were then washed out of the wells with cold PBS into 13 × 100-mm tubes and centrifuged at 1,200 rpm for 10 min at 4°C. The supernatant fluid was discarded and the cell pellet was resuspended in 1.5 ml of cold 5% TCA. The precipitate was collected by centrifugation at 2,500 rpm for 10 minutes 4°C and resuspended once

more in 1.5 ml of 5% trichloroacetic acid (TCA) and centrifuged. The final pellet was dissolved in 0.2 ml of formic acid by heating in a boiling water bath for 10 min. After cooling, this solution was washed out of the tubes with 10 ml of Aquasol (New England Nuclear, Boston, MA) into scintillation vials and radioactivity determined.

Measurement of the Rate of Globin Synthesis

Globins labeled with ^{3}H-leucine were isolated by the method of Clegg et al [14,15]. Duplicate or triplicate 1-ml cultures of suppressed cells, at 20 × 10^6 cells/ml, were incubated for 5 hr with 20 μCi of ^{3}H-leucine. After the 5-hr period, the cells were harvested, washed twice with cold PBS, and lysed with 1.0 ml of sterile water. Fifteen milligrams of ^{14}C-leucine-labeled hemoglobin derived from reticulocytes were added to each lysate and the entire sample was precipitated with 20 vol of 2% HCl in acetone on ice. After washing the precipitate twice with acetone and once with ether, the pellet was dried and dissolved in 3.0 ml of a buffer consisting of 5 mM Na_2HPO_4, 15mM NaH_2P_4, 8 M urea and 50 mM 2-mercaptoethanol, pH 6.65. An aliquot was counted directly in Biofluor (New England Nuclear, Boston, MA) and the rest dialyzed for 5–6 hr at room temperature against 500 ml of the same buffer. The dialyzed sample was applied to a 1 × 10-cm column of carboxymethyl cellulose (CM-52 Whatman Inc, Clifton, NJ), equilibrated with the same buffer; after washing the column with 30–40 ml of buffer, a linear 240-ml gradient was applied by mixing equal volumes of the original buffer with 17.5 mM Na_2HPO_4, 52.5mM NaH_2PO_4, 8 M urea and 50 mM 2-mercaptoethanol, pH 6.65, in a gradient mixer. Three-milliliter fractions were collected and counted directly by adding an 800-μl aliquot of each to 10 ml of Biofluor. The samples were counted in a Searle Mark III Liquid Scintillation Counter.

RESULTS

The Effect of SA on Total Iron Uptake and Heme Synthesis

Tables I and VI show the dose-dependent inhibition by SA of both total cellular iron uptake and hemoglobin synthesis by control and epo-stimulated cells over a period of 48 hours in vitro. At a concentration of 10^{-3} M, there was complete inhibition of heme synthesis but only partial inhibition of total iron uptake; decreasing concentrations of SA had smaller inhibitory effects, and at 10^{-7} M, there was no significant inhibition.

Gel permeation chromatography of cell lysates from suppressed marrow cells labeled with ^{59}Fe-transferrin confirms that hemoglobin synthesis was completely inhibited by 10^{-3} M SA at 0–5 and 19–24 hr in vitro. The radioactive peak that cochromatographed with hemoglobin was virtually eliminated when SA was present during incubation; at the same time, SA had an enhancing effect on ^{59}Fe incorporation into the fraction which elutes in the void volume and is probably ferritin since the radioactivity is completely precipitated with antiferritin (Fig. 1).

At 0–5 hr, epo stimulated hemoglobin synthesis by about 15% and at 19–24 hr, by 55%; and SA inhibited hemoglobin synthesis by 90% at the earlier time and 95% at the later time. When epo and SA were present together, the stimulatory effect of epo was almost completely abolished: induced hemoglobin synthesis was inhibited by 85% both in the first 5 hr and at 19–24 hr.

TABLE I. Effect of Varying Concentrations of Succinylacetone (SA) on Total Iron Uptake and Heme Synthesis in Erythropoietin (Epo)-Stimulated and Unstimulated Cells*

	0–5 hr		19–24 hr		43–48 hr	
	CPM ± SD	% Inhibition	CPM ± SD	% Inhibition	CPM ± SD	% Inhibition
Total iron uptake						
Control	3,410 ± 140		1,350 ± 56		430 ± 26	
10^{-5} M SA	2,480 ± 140	27	960 ± 49	29	320 ± 9	26
10^{-6} M SA	3,240 ± 150	5	1,190 ± 56	12	380 ± 20	12
10^{-7} M SA	3,570 ± 120	0	1,350 ± 33	0	430 ± 26	0
Epo	3,840 ± 170		2,120 ± 140		970 ± 42	
10^{-5} M SA	2,830 ± 160	26	1,320 ± 82	38	550 ± 45	43
10^{-6} M SA	3,560 ± 90	7	1,980 ± 100	7	860 ± 31	11
10^{-7} M SA	3,910 ± 110	0	2,280 ± 150	0	980 ± 57	0
Heme synthesis						
Control	1,320 ± 70		280 ± 2		14 ± 4	
10^{-5} M SA	340 ± 18	74	36 ± 4	87	1 ± 1	93
10^{-6} M SA	1,120 ± 26	15	210 ± 18	25	5 ± 1	64
10^{-7} M SA	1,380 ± 48	0	260 ± 26	7	14 ± 2	0
Epo	1,430 ± 120		520 ± 52		190 ± 10	
10^{-5} M SA	350 ± 18	76	80 ± 4	84	30 ± 8	83
10^{-6} M SA	1,140 ± 34	20	380 ± 14	27	130 ± 22	32
10^{-7} M SA	1,420 ± 48	1	540 ± 26	0	190 ± 22	0

*Suppressed marrow cell cultures were incubated with or without 0.1 U/ml epo and with the indicated amounts of SA. The total ^{59}Fe uptake and ^{59}Fe incorporation into heme was determined as described in Materials and Methods. Each value is the mean of six replicates.

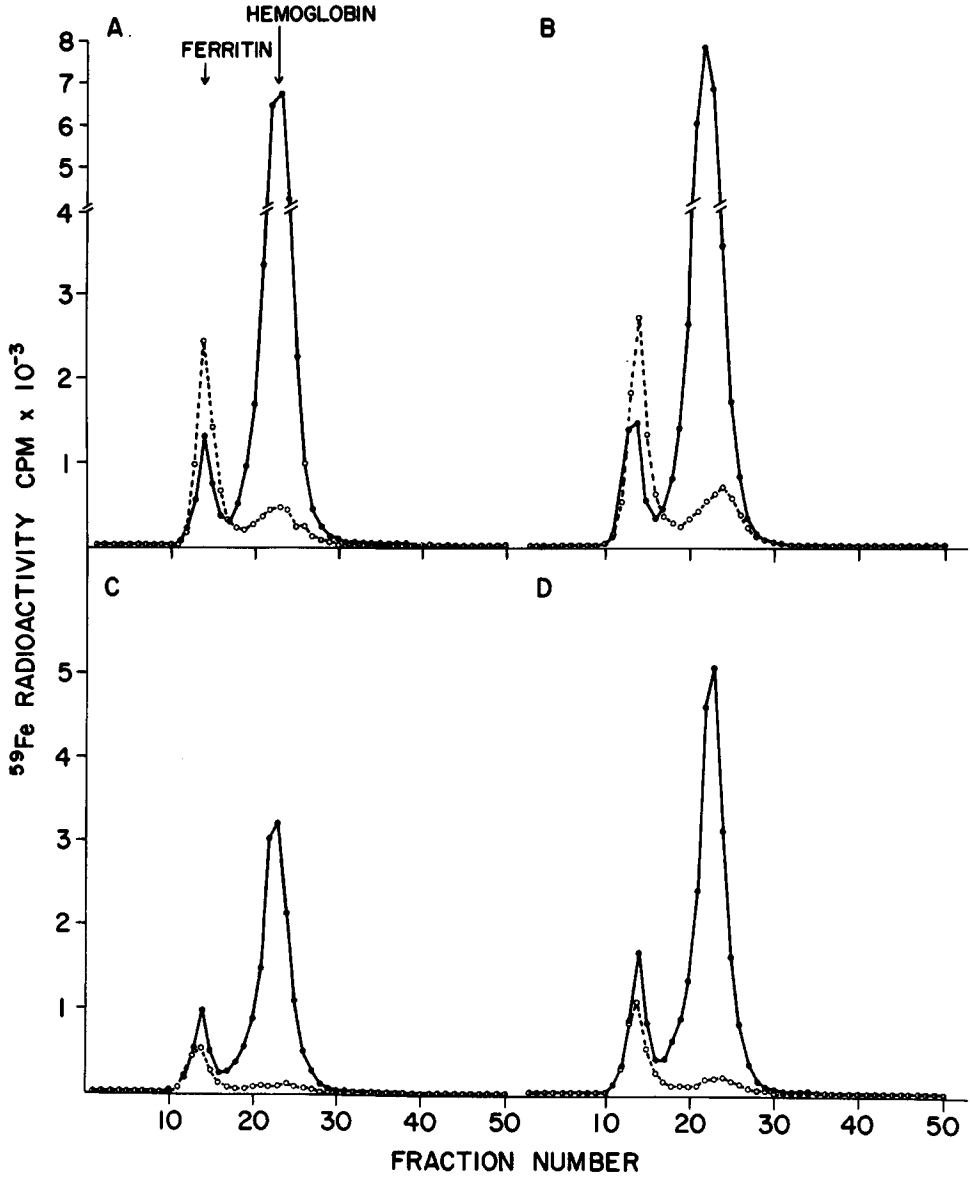

Fig. 1. The effects of erythropoietin and succinylacetone on hemoglobin synthesis: The effects of erythropoietin and succinylacetone on hemoglobin synthesis were determined by S-200 chromatography of suppressed marrow cell lysates. Erythropoietin (panels B and D) was added at a final concentration of 0.1 U/ml and SA at 10^{-3} M. The cells were pulsed with ^{59}Fe-transferin at 0–5 hr (A,B) or 19–24 hr (C,D). ●———●, controls; ○———○, succinylacetone-inhibited.

The Reversal of SA Inhibition

SA inhibits δ-aminolevulinic acid dehydratase; addition of intermediates later than ALA in the heme biosynthetic pathway should overcome the inhibition by SA if they are taken up by the cells. To determine whether this was the case, porphobilinogen (PBG) or protoporphyrin IX were added to marrow cells that were incubated in the presence of 10^{-3} M SA. The results of two experiments are shown in Table II.

At 5 hr, PBG caused a small, significant increase in heme synthesis by both control and epo-treated cells. It also caused a very small but statistically significant increase in heme synthesis by cells grown in 10^{-3} M SA in the presence or absence of epo, although the level of heme synthesis in these did not approach the level of heme synthesis in uninhibited cells. At 24 hr, there was no significant increase in heme synthesis due to PBG; nor was there any release of inhibition of the inhibition as seen at the earlier time.

When protoporphyrin IX was added at a final concentration of 10^{-4} M, the inhibition of heme synthesis by SA was overcome to a much greater degree than when PBG was added (Table II). It is noteworthy that cells treated with protoporphyrin IX made less heme than controls as measured by ^{59}Fe incorporation. This might be due to a cytotoxic effect of protoporphyrin IX at the concentrations used. Both protoporphyrin IX and heme can partially reverse the inhibitory action of SA on globin synthesis in this system (data not shown).

The Effect of SA on RNA and Protein Synthesis Rates

The effects of SA on rates of total RNA and protein synthesis by suppressed rat marrow cells were studied in order to determine whether the profound inhibition of heme and globin synthesis might have been due to a general inhibition of cell function. Table III shows the effect of SA on leucine incorporation and Table IV shows its effect on uridine incorporation. Epo-treated cells showed marked increases in both the rates of RNA and protein synthesis, especially 24 and 48 hr after the start of incubation. The increase in labeled uridine found in RNA due to epo was due to an increased rate of RNA synthesis and not to increased specific activity of the uridine triphosphate (UTP) pool in marrow cells [16]. SA caused significant inhibition in the rate of total protein synthesis (12–20%) at 24 hr in vitro, but the rate of protein synthesis in SA-treated cells was the same as that in control cells at 48 hr. In cells treated with SA and epo, there was much less inhibition at 5 hr and, by 48 hr, there was an increase over control values. This is not surprising as epo by this time has been shown to have a stimulatory effect on the synthesis of proteins other than globin [17].

The effect of SA on the rate of RNA synthesis is more difficult to interpret. There was very little change at 5 and 10 hr but, at later times, there was an increase, reaching 200–300% of control levels by 48 hr of incubation. This effect could be due to an effect of SA in decreasing the UTP pool size but no data are yet available on this point.

Analysis of Globin Synthesis and the Effect of SA on Globin Synthesis

Peripheral red cells isolated from rats contain eight hemoglobins and seven distinct globin chains [18]. Carboxymethyl cellulose (CMC) chromatography of marrow cell lysates resolved four major (αI, αII, βII, βIII) and three minor globins (βo, αo, βI) that cochromatographed with ^{14}C-leucine-labeled globins isolated from reticulocytes of phenylhydrazine-treated rats.

TABLE II. Effect of Protoporphyrin IX on Inhibition of Heme Synthesis by Succinylacetone†

	0–5 hr				21–26 hr			
	No additions		10^{-4} M protoporphyrin IX		No additions		10^{-4} protoporphyrin IX	
	CPM ± SD	%	CPM ± SD	%	CPM ± SD	%	CPM ± SD	%
Control	960 ± 40	100	740 ± 40*	100	200 ± 80	100	170 ± 2	100
Epo 0.10μ/ml	1,070 ± 50	112	840 ± 60*	114	670 ± 100	335	360 ± 20	212*
10^{-3} M SA	17 ± 5	2	390 ± 20*	53	5 ± 0.1	3	60 ± 2	35*
10^{-3} M SA + Epo	12 ± 3	1	400 ± 10*	54	3 ± 0.8	2	120 ± 10	71*

†Culture conditions were the same as in Table I except that 10^{-4} M protoporphyrin was added.
*Where values have been indicated with an asterisk, the effect of protoporphyrin IX is significant at $P < 0.001$.

TABLE III. The Effect of SA on the Rate of Total Protein Synthesis†

	5 hr		10 hr		24 hr		48 hr	
	CPM ± SD	% of control	CPM ± SD	% of control	CPM ± SD	% of control	CPM ± SD	% of control
Control	30,750 ± 470		28,230 ± 1,530		21,730 ± 980		7,590 ± 1,140	
Epo	33,000 ± 880*	107	33,610 ± 930*	119	25,220 ± 650*	116	10,640 ± 580*	140
SA	27,070 ± 1,080*	88	24,740 ± 1,700*	88	17,060 ± 1,580*	79	8,170 ± 360	108
Epo + SA	29,440 ± 1,040*	96	26,500 ± 1,230	94	20,230 ± 870*	93	9,110 ± 1,210*	120

†0.2 ml suppressed cell suspensions, at 20×10^6 cells/ml, were pulsed with 1 μCi of [^{3}H]-leucine for 1 hr and cultures were terminated at the indicated times. Each value is the mean of six replicates. Epo was added at a final concentration of 0.10 units/ml and the concentration of SA was 10^{-3} M.
*Values indicated with an asterisk are significantly different from controls at $P < 0.05$.

TABLE IV. Effect of SA on the Rate of Total RNA Synthesis†

	5 hr		10 hr		24 hr		48 hr	
	CPM ± SD	% of control	CPM ± SD	% of control	CPM ± SD	% of control	CPM ± SD	% of control
Control	49,720 ± 960		31,700 ± 1,640		7,080 ± 800		980 ± 8	
Epo	50,460 ± 1,540	101	32,880 ± 1,140	104	8,870 ± 170*	125	1,150 ± 90*	117
SA	44,170 ± 1,500	89	30,510 ± 1,710	96	8,010 ± 120*	113	2,450 ± 830*	250
Epo + SA	47,260 ± 2,300*	95	32,960 ± 1,130	104	8,680 ± 580*	123	3,050 ± 870*	311

†0.2 ml suppressed cell suspensions, at 20×10^6 cells/ml, were pulsed with 1 μCi of [^{3}H]-uridine for 1 hr and cultures were terminated at the indicated times. Each value is the mean of six replicates. Epo was added at a final concentration of 0.10 units/ml and the concentration of SA was 10^{-3} M.
*Values indicated with an asterisk are significantly different from controls at $P < 0.05$.

The effects of epo and 10^{-3} M SA on globin synthesis by suppressed marrow cells during a 48-hr period in vitro are summarized in Figure 2 and Table V. At 0–5, 5–10, 19–24, and 43–48 hr, epo caused a 42%, 32%, 218%, and 2,970% increase in globin synthesis relative to control cells incubated for the same times. SA, at a concentration of 10^{-3} M, caused an immediate and almost complete inhibition of globin synthesis in both control and epo-treated cells. At 5, 10, and 24 hr, globin synthesis was inhibited by approximately 90%.

The inhibitory effect of SA was not only on control heme and globin synthesis; it also had a profound effect on epo-stimulation of those processes. In this experiment, suppressed marrow cells were incubated with varying concentrations of SA for 24 hr at the end of which the rates of heme and globin synthesis were determined. As seen in earlier experiments, when SA was present at concentrations less than 10^{-3} M, the level of inhibition of heme synthesis was decreased until, at 10^{-7} M, there was no significant effect. Inhibition of globin synthesis followed the same pattern: Decreasing levels of heme synthesis were accompanied by corresponding decreases in globin synthesis (Table VI). Moreover, the stimulatory effect of epo on heme and globin synthesis was only slightly affected by SA at a concentration of 10^{-7} M. However, when the concentration of SA was increased, the stimulatory effect of epo was progressively reduced until, at 10^{-3} M, it was completely abolished (Table VI).

TABLE V. Comparison Between Leucine Incorporation Into Total Protein and Incorporation Into Globin*

	Total DPM × 10^{-5}	Globin DPM × 10^{-5}	Nonglobin DPM × 10^{-5}
0–5 hr			
Control	9.9	3.15	6.75
Epo	11.1	4.5	6.6
SA	7.1	0.02	7.08
Epo + SA	7.3	0.035	7.27
5–10 hr			
Control	12.9	3.06	9.84
Epo	13.9	4.05	9.85
SA	7.8	0.026	7.77
Epo + SA	8.5	0.045	8.45
19–24 hr			
Control	6.6	0.95	5.65
Epo	9.7	3.2	6.5
SA	4.4	0.002	4.4
Epo + SA	5.0	0.01	4.99
43–48 hr			
Control	3.0	0.005	3.0
Epo	4.9	1.38	3.5
SA	2.1	0	2.1
Epo + SA	2.1	0	2.1

*^{3}H-leucine incorporated into total protein was determined as outlined in Materials and Methods. ^{3}H-leucine incorporated into globin was calculated from the globin peaks shown in Figure 2. SA where present was at a final concentration of 10^{-3} M. Epo where present was added at 0.1 U/ml.

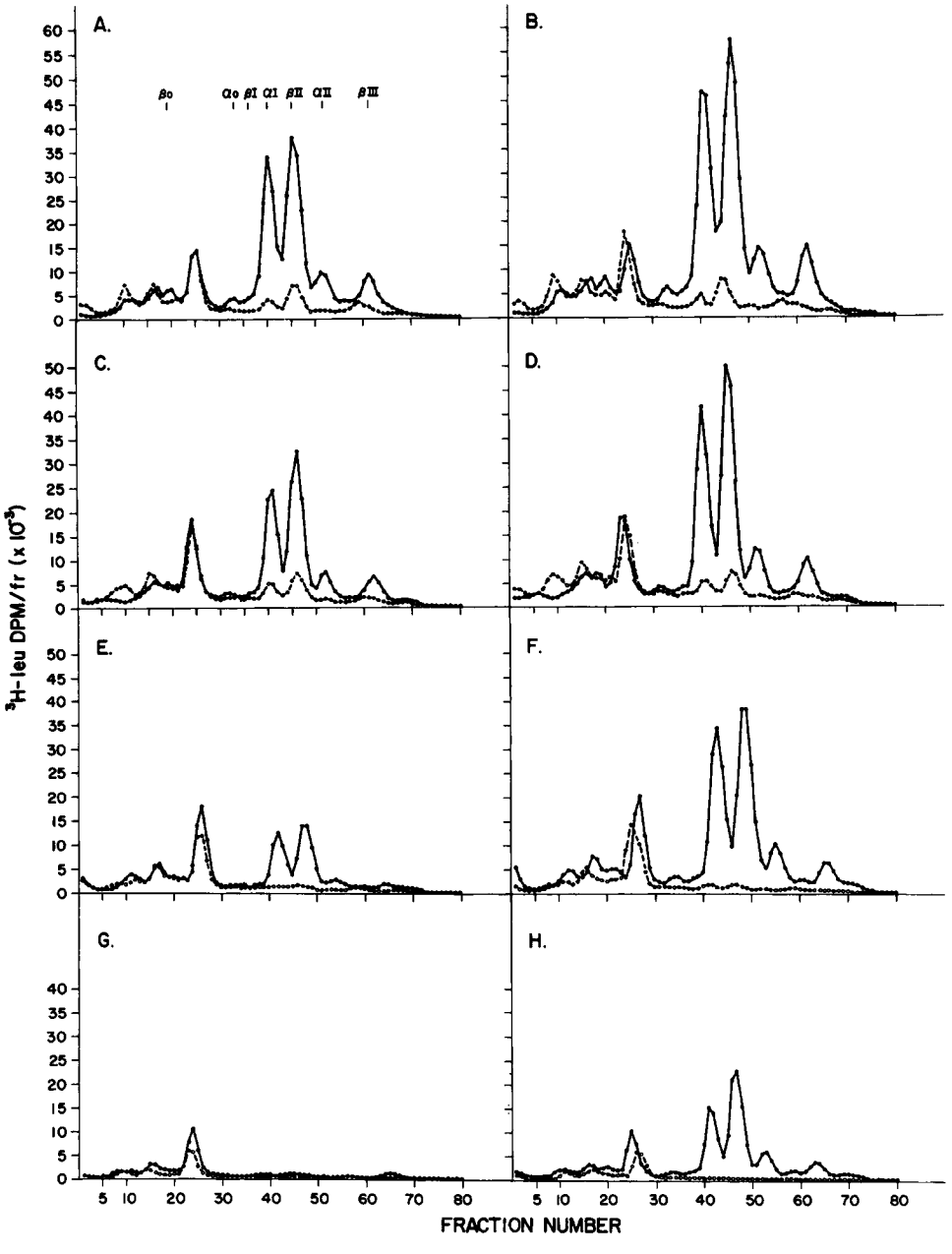

Fig. 2. The effects of erythropoietin and succinylacetone on globin synthesis. Suppressed marrow cells were incubated with (panels B,D,F,H) or without (panels A,C,E,G) 0.1 U/ml erythropoietin and with or without succinylacetone. At 0-5 (A,B), 5-10 (C,D), 19-24 (E,F), and 43-48 (G,H) hr, duplicate 1-ml cell suspensions were labeled with 20 μCi ^{3}H-leucine. Cell lysates were precipitated with acid-acetone and chromatographed on carboxymethyl cellulose. Each panel shows a represenative result of duplicate analysis. ●————●, controls; ○- - - - -○, succinylacetone-inhibited.

TABLE VI. The Effect of SA on Heme and Globin Synthesis*

		Heme synthesis			Globin synthesis		
SA (M)	Epo	CPM ± SD	Δ	% Inhibition Δ	DPM × 10^{-3}	Δ	% Inhibition Δ
0	−	96 ± 8	—	—	117	—	—
0	+	296 ± 20	200	—	383	266	—
10^{-7}	−	96 ± 18	—	—	131	—	—
10^{-7}	+	256 ± 4	160	20	361	230	14
10^{-6}	−	77 ± 6	—	—	102	—	—
10^{-6}	+	201 ± 22	124	33	285	183	31
10^{-5}	−	35 ± 2	—	—	61	—	—
10^{-5}	+	94 ± 4	59	71	105	44	83
10^{-4}	−	4 ± 1	—	—	25	—	—
10^{-4}	+	8 ± 2	4	98	33	8	97
10^{-3}	−	5 ± 2	—	—	14	—	—
10^{-3}	+	3 ± 0.4	0	100	18	4	99

*Suppressed rat marrow cells, at 20 × 10^6 cells/ml were incubated with the indicated concentrations of SA for 24 hr. At the end of this period, heme and globin synthesis were determined and the epo effect calculated. Heme synthesis was measured in six replicates and globin synthesis in duplicate samples. Epo when present was at a concentration of 0.1 U/ml.

DISCUSSION

The data we have presented show that SA, which is a specific inhibitor of δ-aminolevulinic acid dehydratase, also inhibits heme and globin synthesis by suppressed rat marrow cells in vitro. At a concentration of 10^{-3} M, inhibition by SA was almost complete and was rapid; heme synthesis was inhibited by greater than 98% and globin synthesis by 90% in the first 5 hr of incubation with SA. The low level of globin synthesis seen at early times, despite the complete inhibition of heme synthesis, might have been due to a small pool of heme made prior to the addition of SA.

The effects of SA on total iron uptake, total protein, and total RNA synthesis were also studied. At 10^{-3} M SA, total iron uptake was not completely inhibited; total protein synthesis was affected only slightly and the decrease could be accounted for by the absence of globin synthesis (Table V). The effect of SA on total RNA synthesis did not fit this pattern. There was slight, but statistically significant, inhibition early, but after 10 hr of incubation, transcription was stimulated. This surprising effect reached a 2.5–3-fold stimulation by 48 hr. The mechanism of this action of SA is still unclear, and we are currently studying the nature of the transcripts.

The effect of SA on the action of epo with respect to heme, globin, total protein, and total RNA synthesis was also studied. Stimulation of heme synthesis by epo was seen in the first 10 hr and, in fact, in most experiments increased heme synthesis was seen within 5 hr of the addition of epo. It should be noted here that we are dealing with a declining system and that stimulation does not necessarily mean an increase over zero time values but rather an increase relative to control values at the same times. This time-dependent loss is probably due to the loss of late erythroid cells in the cultures. Although control hemoglobin synthesis declines with time in this system, the absolute magnitude of synthesis induced by epo does not, indicating that the epo-

responsive cells are functional even at the later times. Total protein synthesis was also significantly stimulated by epo to a small but significant extent (10%) within 5 hr after its addition. At 10, 24, and 48 hr, the increase was approximately 15%, 15%, and 30%, respectively. At all four time points, most of the increase in protein synthesis was due to the increase in globin synthesis. For the period up to 10 hr, there was no discernible effect of epo on total RNA synthesis, but at 24 and 48 hr, there were small but significant increases.

The inhibitory effect of SA on both unstimulated and epo-induced heme and globin synthesis is dose-dependent. At 10^{-7} M SA, neither process was affected; when the concentration of SA was increased, however, there was a corresponding increase in inhibition. Complete inhibition of the epo effect was found at 10^{-3} M.

From the data we have presented, it is clear that SA inhibition of heme synthesis resulted in a specific inhibition of globin synthesis by cultured rat marrow cells. Protein synthesis other than globin was only slightly affected as seen by the following results: The complete inhibition of heme synthesis by SA is accompanied by a rapid and almost complete inhibition of globin synthesis. Partial inhibition of heme synthesis at intermediate concentrations of SA is accompanied by a corresponding partial inhibition of globin synthesis. The decrease in total protein synthesis due to SA can be accounted for by the absence of globin synthesis. Moreover, there are at least three nonglobin protein components identified by chromatography on carboxymethyl cellulose that are unaffected by SA.

The specific inhibition of globin synthesis in bone marrow cells is in contrast to other systems where heme has been implicated in the regulation of overall protein synthesis. Protein synthesis in reticulocytes and reticulocyte lysates has been reported to be regulated by heme [3,19–21]. Zucker and Schulman [20] reported that in an unfractionated cell-free system from rabbit reticulocytes, hemin is specifically involved with an initiation process resulting in continued synthesis of new nascent chains and that polysomal integrity is dependent on this function. It has been suggested that heme controls the rate of globin synthesis by preventing inhibition of protein chain initiation [22–24] by way of interaction with the translational inhibitor protein characterized in reticulocytes which affects peptide chain initiation generally and not globin chain synthesis specifically [25]. Ranu et al [23] and Beuzard and London [26] have reported that inhibition of protein synthesis in reticulocytes caused by hemin deficiency can be partially overcome by the specific initiation factor (eIF-2) involved in the binding of the initiator Met-$tRNA_f$ to the 40s subunit. Our findings indicate that there is a more specific, direct relationship between heme synthesis and globin synthesis both in control and epo-stimulated bone marrow cells. The mechanism by which this coordinate effect occurs is not yet clear.

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