Biochemical Pharmacology, Vol. 29, pp. 1825–1831.
Pergamon Press Ltd. 1980. Printed in Great Britain.

EFFECT OF HEME DEPLETION ON GROWTH, PROTEIN SYNTHESIS AND RESPIRATION OF MURINE ERYTHROLEUKEMIA CELLS

DONALD P. TSCHUDY,* PAUL S. EBERT,* RICHARD A. HESS,* BRUCE C. FRYKHOLM* and EUGENE C. WEINBACH†

*National Cancer Institute and †National Institute of Allergy and Infectious Diseases, Bethesda, MD 20205, U.S.A.

(*Received* 24 *September* 1979; *accepted* 6 *December* 1979)

Abstract—4,6-Dioxoheptanoic acid (succinylacetone, SA) profoundly inhibits δ-aminolevulinic acid (ALA) dehydratase, the second enzyme of the heme biosynthetic pathway. When murine eyrthroleukemia (MEL) cells were grown in defined medium containing 0.3–1.0 mM SA and gelatin as the protein source, cellular heme concentrations decreased markedly and growth was inhibited. Growth inhibition was partially reversed when hematin (300 pmoles/ml) was added with SA on day 0, but no reversal of growth inhibition was seen when this concentration of hematin was added on day 4. Complete reversal of growth inhibition produced by 0.3 mM SA occurred when hematin was added in concentrations of 1–2 nmoles/ml on day 0. Cellular heme content and growth rates indicate that exogenously added hematin was taken up from defined medium containing only gelatin as the protein component. The decrease of cellular heme concentration was accompanied by a decrease of protein synthesis, which, like growth inhibition by SA, was partially reversed by addition of hematin to the medium. Although no significant impairment of endogenous cellular respiration was observed in these studies, there was a decrease in NADH-stimulated oxygen consumption in heme-depleted cells, suggesting diminished respiratory reserve in these cells.

4,6-Dioxoheptanoic acid (succinylacetone, SA), which has been identified in the urine of patients with hereditary tyrosinemia, appears to cause increased excretion of δ-aminolevulinic acid (ALA) through its inhibition of ALA dehydratase, the second enzyme of the heme biosynthetic pathway [1]. Since this compound appears to be more specific and more active as an inhibitor of heme biosynthesis than those studied previously (unpublished data), it is now possible to examine more readily the effects of heme depletion on various cell functions, including cell growth. Heme is well known to be involved in a number of cell functions, including mitochondrial and microsomal electron transport (respiration and drug metabolism), hydrogen peroxide degradation, peroxidative functions, tryptophan catabolism, and, more recently, initiation of protein synthesis [2–4]. Inhibition of heme biosynthesis might produce different effects in various cell types, depending on the relative activities of these heme-derived functions in different cells. Through its potent inhibition of ALA dehydratase [5], SA provides a method for studying the effects of inhibition of heme synthesis on these various cell functions. It has been shown previously that SA inhibits growth of murine erythroleukemia (MEL) cells in culture after approximately two cell divisions and also inhibits hemoglobin synthesis after stimulation by various inducing agents such as dimethylsulfoxide and butyrate [5]. The present study was designed to examine the effects of heme depletion on protein synthesis and certain aspects of cellular respiration, and also to clarify further the quantitative relationships between cellular heme content and growth in MEL cells.

MATERIALS AND METHODS

Cells and culture procedures. MEL cells from clone 745 were routinely maintained in RPMI 1640 (GIBCO, Grand Island, NY) containing 10% heat-inactivated fetal bovine serum as described previously [5, 6]. Cells were conditioned to low protein concentration by maintaining them for 2 days in RPMI Medium containing 1% heat-inactivated fetal bovine serum. Following this conditioning period, the cells were centrifuged and resuspended at a final concentration of 2–4 × 10^5 cells/ml in RPMI 1640 medium containing 1% gelatin (DIFCO, Detroit, MI), penicillin–streptomycin–neomycin (10:10:20 μg/ml), 2 mM glutamine, 100 μm $FeSO_4$ and 30 nM insulin (Sigma Chemical Co., St. Louis, MO). SA (Proteochem Inc., Denver, CO) and hematin (prepared in our laboratory) were added as indicated in the legends to the figures. Viable cells were determined in a hemacytometer by the trypan blue exclusion procedure. When cell density reached a concentration of 10^6 live cells/ml, the cultures were divided, i.e. cells at a density of about 2 × 10^5/ml were added to fresh medium which sometimes contained hematin and SA, as indicated in the figures. Since growth rates in control cultures often differed from those treated in various ways, cell cultures grown under different conditions were not always divided on the same day.

Protein synthesis. Aliquots of 2 × 10^6 viable cells were centrifuged and resuspended in 1 ml RPMI 1640 stock solution (with none of the above additions) containing 0.5 μCi of ^{14}C-amino acid hydrolyzate (New England Nuclear, Boston, MA). The

1 ml suspensions of MEL cells were incubated in test tubes for 30 or 60 min at 37° with shaking. The reactions were stopped by placing the tubes in ice and adding 5 ml of cold 10% trichloroacetic acid (TCA). The incorporation of label into protein was linear for at least 60 min. Following centrifugation, the precipitates were sequentially washed twice with cold 5% TCA, once with 5% TCA at 90° for 20 min, three times with ethanol at 65° for 5 min each, and once with ether [7]. The resultant precipitates were dissolved in 1 ml Protosol (New England Nuclear) by heating at 55° for 12 hr and counted with Aquasol in a liquid scintillation spectrometer. The few samples which were colored by the digestion were quench-corrected by internal standardization. Data on protein synthesis in Figs. 1 and 2 are expressed as activity/10^6 viable cells. The reason that it is not expressed as activity/mg protein is because dead cells would contribute protein but virtually no incorporation of label. This would create an artifact which would appear to represent progressively decreasing protein synthesis as cell viability decreased.

Heme determination. The term "heme" is used for the endogenous substance and "hematin" is used for exogenously added material. Heme was determined in duplicate on 4×10^5 cells by the oxalic acid–fluorometric procedure of Sassa *et al.* [8] with minor modifications. The cells were washed three times in 10 mM Tris (pH 7.4)–0.15 M NaCl, suspended in 0.5 ml water, and frozen prior to heme determination. Heme concentrations are expressed per mg protein rather than in terms of viable cells because dead cells are presumed to contain heme also. Expression of the heme concentration in terms of viable cells would create an artifact which would give artificially high values, since the heme content of dead cells would be included in this value.

Respiration measurement. Oxygen uptake was determined polarographically with the Clark oxygen electrode (Yellow Springs Instrument Co., Inc., Yellow Springs, OH). One milliliter of the cell suspension (1.5×10^7 viable cells in 1 ml of stock RPMI 1640 medium) was equilibrated with air by preincubation at 35° in a glass cuvette mounted in a plastic thermostated chamber and fitted with a magnetic stirrer. After a 3-min equilibration period, the electrode was inserted, forming an air-tight seal, and the recording was begun. Additions to the suspension of volumes not exceeding 50 μl were made with microliter syringes through a capillary port in the cuvette [9]. Statistical comparisons were made by means of Student's *t*-test. Differences with a $P < 0.01$ were considered significant.

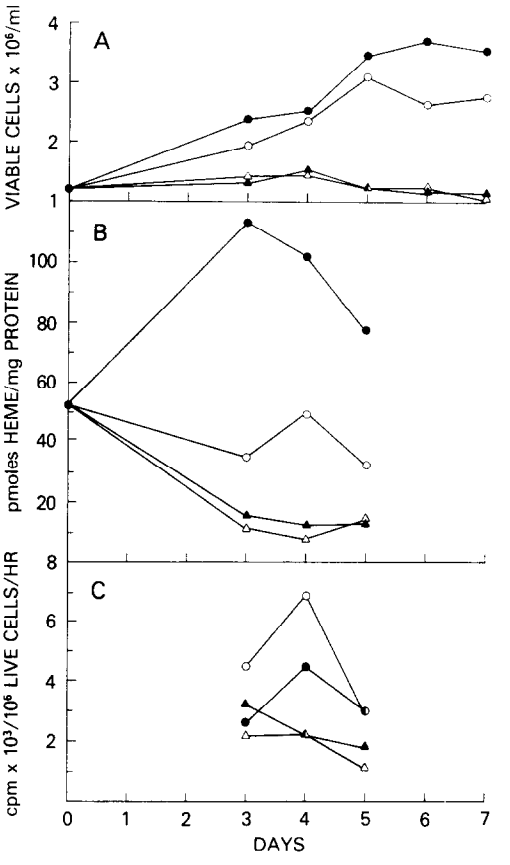

Fig. 1. Growth characteristics, heme concentrations and rates of protein synthesis in MEL cells grown in defined medium with and without SA. MEL cells were grown in RPMI 1640 medium containing 1% gelatin. Growth curves beyond day 3 are corrected to show continuous growth. This was done because the cultures were subdivided and cell counts were corrected to reflect total viable cells based on the cell number from day 0. Key: (●——●) control; (○——○) 0.1 mM SA; (▲——▲) 0.5 mM SA; and (△——△) 1.0 mM SA. Panel A shows the growth pattern of MEL cells grown in medium with three concentrations of SA and without SA. Panel B shows the heme concentrations of cells grown in medium with and without SA. Panel C shows the rates of protein synthesis on days 3–5 of viable cells grown in three concentrations of SA and without SA. The rationale for the presentation of heme concentrations per mg protein and protein synthesis per 10^6 viable cells is presented for Figs. 1–3 in the Materials and Methods section for these measurements. Viabilities in SA-treated cells declined progressively where growth inhibition is indicated in the figures. In the presence of high concentrations of SA, viabilities declined to values as low as 17 per cent, whereas uninhibited cells usually had viabilities in the range of 68–96 per cent.

RESULTS

The effects of three concentrations of SA (0.1, 0.5 and 1.0 mM) on growth rate, heme content and protein synthesis in MEL cells grown in defined medium (1% gelatin in place of fetal bovine serum) are shown in Fig. 1. SA at concentrations in the medium of 0.5 and 1.0 mM markedly inhibited growth and decreased cellular heme concentrations to values which ranged from approximately 8 to 18 per cent of control values on day 3 and afterwards.

Total cellular protein synthesis was not inhibited on day 3 by 0.5 and 1.0 mM SA, but some inhibition was observed on days 4 and 5 at these concentrations. Although it was not observed in this experiment, some increase of cell growth was observed in a number of other experiments when cells were grown in 0.1 mM SA. Thus, the moderate increase of protein synthesis seen in this experiment when cells were grown in 0.1 mM SA is compatible with the frequent observation of moderate growth stimulation when

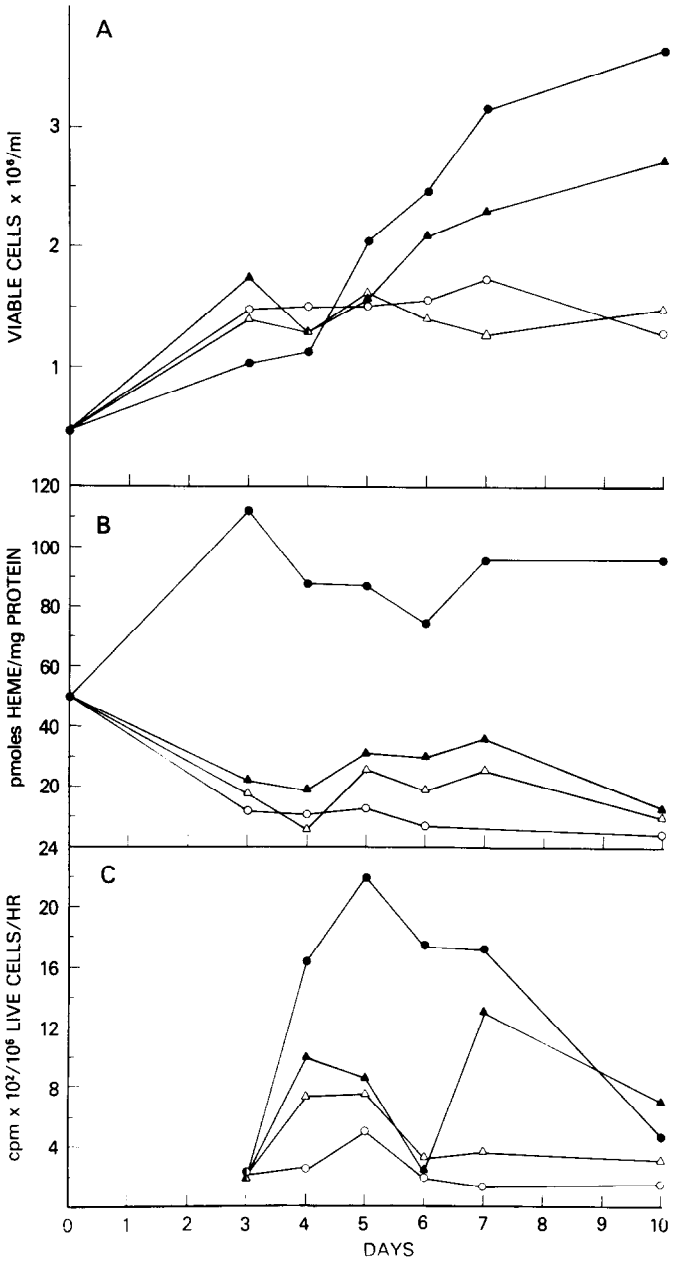

Fig. 2. Growth characteristics, heme concentrations, and rates of protein synthesis in MEL cells grown in defined medium without SA, with SA alone, and with SA in the presence of hematin added on day 0 or day 4. MEL cells were incubated in RPMI 1640 medium containing 1% gelatin. Growth curves beyond day 3 are corrected to show continuous growth. Key: (●——●) control; (○——○) 0.3 mM SA; (▲——▲) 0.3 mM SA + hematin (300 pmoles hematin/ml) added on day 0; and (△——△) 0.3 mM SA + hematin (300 pmoles hematin/ml) added on day 4. Panel A shows the growth pattern. Panel B shows the cellular heme concentrations. Panel C shows the rates of protein synthesis of viable cells on days 3–10.

cells are grown in low SA concentrations (0.1 mM).

The effects of SA, with and without added hematin, on growth, heme content and protein syntheses of MEL cells grown in defined medium are shown in Fig. 2. When hematin was added to the medium on day 0, partial reversal of growth inhibition by 0.3 mM SA was observed, but addition of hematin on day 4 produced no significant reversal of growth inhibition. These findings correlate with the observed heme concentrations in that cellular heme concentrations increase in the order (a) SA alone, (b) SA with hematin added on day 4, and (c) SA with hematin added on day 0. In other experiments (not shown), addition of higher concentrations (1–2 nmoles/ml) of hematin on day 0 in the presence of 0.3 mM SA completely reversed growth inhibition.

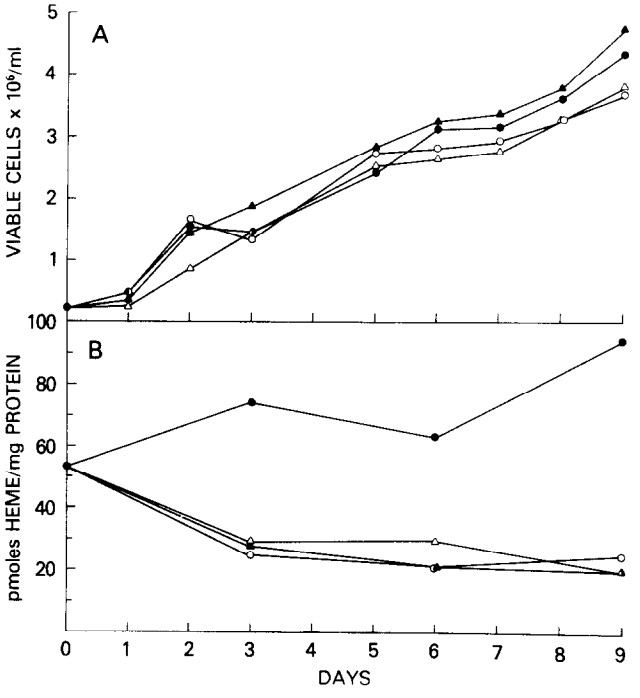

Fig. 3. Growth characteristics and heme concentrations of MEL cells with three concentrations of SA in medium containing 1% fetal bovine serum. Growth curves beyond day 3 are corrected to show continuous growth. Key: (●——●) control; (▲——▲) 0.2 mM SA; (○——○) 0.5 mM SA; and (△——△) 1 mM SA. Panel A shows the growth characteristics of MEL cells. Panel B shows the heme concentrations of cells grown with and without SA.

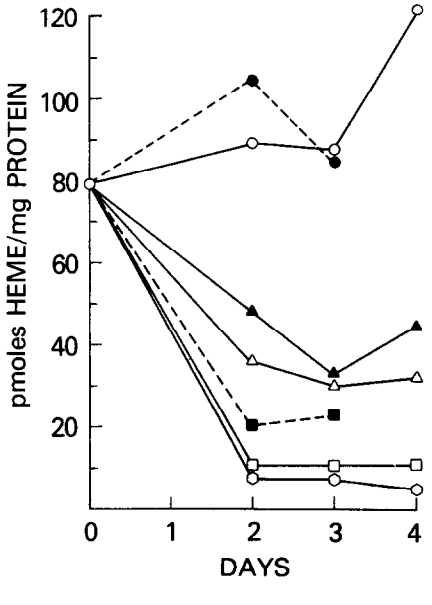

Fig. 4. Heme concentrations of MEL cells grown in medium containing 1% gelatin or 0.2% fetal bovine serum with and without SA. Key: (○——○) control cells in gelatin; (□——□) 0.5 mM SA in gelatin; (◇——◇) 1.0 mM SA in gelatin; (▲——▲) 0.5 mM SA + hematin (300 pmoles/ml) in gelatin; (△——△) 1.0 mM SA + hematin (300 pmoles/ml) in gelatin; (●- - -●) control cells in fetal bovine serum; and (■- - -■) 1.0 mM SA in fetal bovine serum.

It appears that hematin uptake occurs in the absence of albumin or hemopexin.

Protein synthesis was markedly inhibited in the presence of 0.3 mM SA after day 3. An increasing degree of reversal of this inhibition by SA occurred when hematin was added on day 4 and day 0, respectively. In Fig. 2, where cells (controls, heme-depleted and heme-depleted with hematin supplementation) were grown in defined medium, there was a general correlation among heme content, protein synthesis and growth rate. In cells grown in defined medium without exogenous hematin supplementation, there is also a general correlation among heme content, protein synthesis and growth rate, with the exception of protein synthesis in cells grown in the presence of 0.1 mM SA (Fig. 1). However, there was no correlation of growth rate with heme content in cells grown in medium containing fetal bovine serum (Fig. 3). The explanation for this is presumably that, while SA caused a marked decline in cellular heme content, heme uptake from the medium prevented cellular heme levels from declining to the critical concentrations that appear to be growth inhibitory in this medium.

The effects of SA on growth and heme content of cells grown in medium containing 1% fetal bovine serum are shown in Fig. 3. Concentrations of SA that markedly inhibited growth in defined medium, which contained no heme, produced no significant inhibition in medium supplemented with 1% fetal bovine serum, which does contain heme [5]. Although cellular heme concentrations in the presence of inhibitor were markedly decreased in

Table 1. Respiration of MEL cells*

No.	Sample treatment in culture	Additions	Oxygen uptake Day 3	4	5	6	7	10
1	None (control)	None	16	42	38	38	42	45
2	0.3 mM SA	None	26	38	19	29	22	35
3	0.3 mM SA + hematin, day 0	None	22	38	48	26	38	22
4	0.3 mM SA + hematin, day 4	None		42	38	51	38	19
1	None (control)	Menadione Initial	157	234	182	102	355	115
	None (control)	Final	35	38	45	48	54	29
2	0.3 mM SA	Initial	141	166	176	240		
	0.3 mM SA	Final	26	26	38	19	22	
3	0.3 mM SA + hematin, day 0	Initial	115	234	173	208	186	173
		Final	16	32	42	22	26	19
4	0.3 mM SA + hematin, day 4	Initial		186	144	96	147	352
		Final		38	10	26	13	22
1	None (control)	NADH	51	64	58	58	61	58
2	0.3 mM SA†	NADH	32	45	48	42	42	51
3	0.3 mM SA + hematin, day 0	NADH	25	61	54	35	48	48
4	0.3 mM SA + hematin, day 4	NADH		58	29	42	54	96

* In all experiments related to Table 1, hematin was added at a final concentration of 300 pmoles/ml to the medium in which the cells were grown. Oxygen uptake was determined polarographically, as described in Materials and Methods, and is expressed as nanoatoms of oxygen/min/10^7 viable cells. The final concentrations of menadione and NADH were 1 mM. These compounds were added after steady state conditions were obtained. Student's *t*-tests were performed using all values from each group in comparison with all values from the corresponding control group. The growth rates, heme contents and protein synthetic rates of cells in this table are presented in Fig. 2.

† The mean value of all the numbers in this group was significantly lower ($P < 0.001$) than the mean of controls. None of the other treatments produced significant differences from controls. Only values with $P < 0.01$ by Student's *t*-test are considered significant.

medium containing fetal bovine serum (Figs. 3 and 4), the values did not decrease to the extent seen in defined medium.

The effect of SA on cellular heme content of cells grown in defined medium, with and without heme supplementation, and of cells grown in medium containing fetal bovine serum is seen in Fig. 4. Addition of hematin, either directly or as the endogenous heme in serum, partially reversed the decrease of cellular heme produced by SA.

In addition to the measurements on growth and heme content shown in Fig. 2, the effect of SA treatment on the respiration of these MEL cells is shown in Table 1. Suspensions of SA-treated and control cells exhibited active endogenous respiration which was sensitive to cyanide and antimycin. Addition of menadione led to an initially rapid rate of oxygen consumption for a brief period (0.5–1.0 min), followed by a slower stable rate which did not differ significantly from the endogenous rate. When the slower, stable rate was re-established, NADH was added and was found to increase oxygen consumption. If NADH was added prior to menadione, a small increase in oxygen uptake was observed which was greatly augmented by the subsequent addition of menadione, indicative of the presence of an NADH: (quinone-acceptor) oxidoreductase (EC 1.6.99.5). NADPH was ineffective in these experiments. Addition of 0.1% Triton X-100 had little effect on the oxidation of NADH, showing that the MEL cells were permeable to the reduced nucleotide under these conditions. The initial rapid rate of oxygen uptake observed upon the addition of menadione to the respiring cells presumably is due to its rapid reduction by endogenous substrates followed by nonenzymatic oxidation. It is not known how much the endogenous substrates contribute to the observed oxygen uptake when menadione is added after NADH, although the kinetics of the oxygen consumption suggest that it is minimal.

Although SA produced no effects on the endogenous respiration of MEL cells, there was a significant decrease of NADH-stimulated O_2 consumption in MEL cells grown in 0.3 mM SA when compared with the NADH-stimulated O_2 consumption of control cells (Table 1). Furthermore, MEL cells grown in medium containing SA, but also supplemented with low concentrations of hematin (300 pmoles/ml), did not differ significantly from control cells in NADH-stimulated O_2 consumption (Table 1).

DISCUSSION

Kinetic and dialysis studies have shown SA to be an irreversible inhibitor of ALA dehydratase.* Profound inhibition of ALA dehydratase by SA at concentrations of 10^{-7} M is seen in both rat liver homogenates (1:4)* and MEL cells [5]. Since ALA dehydratase is normally present in most tissues in considerable excess above the rate-limiting enzyme of heme biosynthesis, it is necessary to inhibit profoundly ALA dehydratase before diminished heme synthesis occurs. When heme biosynthesis is inhibited, cellular heme concentrations can decline as a result of either cell division or intracellular heme turnover. As cellular heme concentrations decline, specific cell functions related to various heme-containing enzymes will be impaired sequentially. The order in which these functions are impaired will depend on the actual rate of decline of the individual heme-containing moieties, along with various other factors such as reserve capacity and variations in functional demand.

The present study has examined the effects of heme depletion on protein synthesis and respiration in MEL cells and has shown that the previously demonstrated growth inhibition produced by SA [5] can be reversed by hematin administration.

It has been shown previously that MEL cells, when grown in the presence of SA in defined medium, undergo about two cell divisions before growth ceases and cell death begins [5]. Although low concentrations of SA (0.1 mM) caused a significant decrease of cellular heme concentration (Fig. 1), these values did not decline sufficiently to produce growth inhibition. When higher concentrations of SA (0.3–1.0 mM) were added to the medium, heme concentrations declined to levels which inhibited growth (Figs. 1 and 2). Pronounced growth inhibition was not observed when cellular heme concentrations exceeded approximately 20 pmoles heme/mg protein (Figs. 1–3), except for cells grown in medium which was supplemented with hematin (300 pmoles/ml) on day 4. In this group, cellular heme concentrations ranged between 19 and 27 pmoles heme/mg protein on days 5–7 when growth inhibition equalled or exceeded that produced by SA alone (Fig. 2). It appears that growth inhibition always occurs when cellular heme is reduced to approximately 15 pmoles/mg protein or lower.

It is possible that SA might inhibit growth by multiple mechanisms, including some which do not involve cellular heme depletion. However, the fact that growth inhibition produced by SA could be partially or completely overcome by concentrations of hematin in the medium of 300 pmoles/ml to 1–2 nmoles/ml strongly suggests that the major mechanism of growth inhibition by SA is related to cellular heme depletion. Furthermore, SA did not inhibit growth of MEL cells in medium containing fetal bovine serum (Fig. 3), which was shown to contain significant concentrations of heme (269 pmoles/mg protein) [5]. As seen in Fig. 4, the order of increasing cellular heme concentrations of cells grown under various conditions was: (a) defined medium (gelatin in place of serum) containing 1.0 mM SA, (b) defined medium containing 0.5 mM SA, (c) medium containing fetal bovine serum and 1.0 mM SA, (d) defined medium containing 1.0 mM SA + hematin (300 pmoles/ml), (e) defined medium containing 0.5 mM SA + hematin (300 pmoles/ml), and (f) control cells grown in defined medium or medium containing fetal bovine serum. These findings indicate that, in the presence of varying concentrations of SA, intracellular heme concentrations are higher when hematin is present in the medium, either as hematin added to defined medium or as an endogenous component of fetal bovine serum. It appears that hematin can be taken up from the medium in the absence of albumin or specific carrier protein. The data on intracellular heme content in Figs. 1–4 suggest that exogenous heme uptake is most obvious in cells depleted of heme by the effect of SA. The mean value of heme in control cells grown in the presence of exogenous heme (contained in fetal bovine serum) was actually slightly lower than that of cells grown in the absence of exogenous heme. It is possible, therefore, that exogenous heme uptake is most obvious in heme-depleted cells. This uptake allowed cells to overcome much of the growth inhibitory effects of SA (Fig. 2). No attempt was made to differentiate between intracellular heme and any heme that might have remained adsorbed to the cell membrane after three washes in Tris–saline when heme measurements were performed in cells grown in medium supplemented with exogenous hematin. The fact that measured heme levels correlated well with the partial restoration of protein synthesis and cell growth in heme-depleted cells grown in medium supplemented with exogenous hematin strongly suggests that the cellular heme measurements reflect functional cell heme.

Declining intracellular heme concentrations affected protein synthesis rates. Protein synthesis was diminished after day 3 in cells grown in 0.3 mM SA (Fig. 2), as well as in cells grown in 0.5 and 1.0 mM SA (Fig. 2). Intracellular heme concentrations declined below 15 pmoles/mg protein on these days in the presence of the above three concentrations of SA (Figs. 1 and 2). These levels have been associated with growth inhibition.

The inhibition of protein synthesis and cell growth during cellular heme depletion is best explained by the recent studies which have shown heme to be involved in initiation of protein synthesis [2–4]. This may involve dissociated heme which may decrease during cellular heme depletion more rapidly than much of the tightly bound heme. Dabney and Beaudet [10, 11] have previously presented evidence that heme is capable of stimulating globin synthesis in MEL cells. In their first study [10], addition of 50 μM hemin to cells cultured in the presence of 0.5% dimethylsulfoxide increased globin synthesis. When ALA dehydratase was inhibited by 3-amino-1,2,4-triazole [12], an inhibitor less active than SA, globin chain synthesis was reduced in MEL cells in which hemoglobin synthesis had been fully induced by dimethylsulfoxide. The inhibition of globin synthesis by 3-amino-1,2,4-triazole was prevented by simul-

* D. P. Tschudy, R. A. Hess and B. C. Frykholm, unpublished observations.

taneous addition of 25 μM hemin to the cultures. In later studies [11], hemin was shown to stimulate the translation, in extracts of MEL cells, of both endogenous mRNA and exogenous globin mRNA in both dimethylsulfoxide-induced and uninduced cells. Ross and Sautner [13] had previously shown induction of globin mRNA synthesis by hemin in MEL cells. These studies indicate that heme plays a role in stimulating globin synthesis in MEL cells, perhaps by a mechanism similar to that which has been studied extensively in reticulocytes [2–4]. In addition, however, some of the data indicated an effect of heme on increasing the levels of globin mRNA in MEL cells [10]. More recent studies have shown hemin to increase hemoglobin synthesis in MEL cells [14, 15].

In the present studies, it has not been proved that the profound inhibition of growth which results from heme depletion in uninduced MEL cells is specifically caused by the observed decreases of protein synthesis. It is possible that the diminished protein synthesis is a result of, rather than the cause of, decreased growth. However, if heme depletion is inhibiting growth by diminishing protein synthesis, it would seem likely that the synthesis of proteins other than globin is inhibited.

Although endogenous respiration was unaffected by SA, there was a significant decrease of NADH-stimulated O_2 consumption in cells grown in medium containing SA, which was not seen when the SA-containing medium was supplemented with hematin (Table 1). As seen in Fig. 2, the heme content was lowest in those cells in which a significant decrease of NADH-stimulated O_2 consumption was detected. The data suggest that respiratory reserve capacity may be diminished in these cells during profound heme depletion. The present data indicate that heme depletion produced by SA in MEL cells decreases protein synthesis before endogenous respiration is affected. Therefore, it is most likely that impaired growth in these cells does not result from impaired respiration, but may be related to diminished protein synthesis, although the proof of this hypothesis remains to be determined. Details of the effects on protein synthesis and respiratory chain components of heme depletion and its relationship to growth inhibition require further study.

REFERENCES

1. B. Lindblad, S. Lindstedt and G. Steen, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4641 (1977).
2. S. Ochoa and C. de Haro, *A. Rev. Biochem.* **48**, 549 (1979).
3. V. Ernst, D. H. Levin and I. M. London, *Proc. natn. Acad. Sci. U.S.A.* **76**, 2118 (1979).
4. S. Saffer and W. F. Anderson, *CRC Crit. Rev. Biochem.* **5**, 261 (1978).
5. P. S. Ebert, R. A. Hess, B. C. Frykholm and D. P. Tschudy, *Biochem. biophys. Res. Commun.* **88**, 1382 (1979).
6. P. S. Ebert, I. M. Wars and D. N. Buell, *Cancer. Res.* **36**, 1809 (1976).
7. N. G. Ibrahim, P. J. Spieler and M. L. Freedman, *Br. J. Hemat.* **41**, 235 (1979).
8. S. Sassa, S. Granick, C. Chang and A. Kappas, in *Erythropoiesis, Proceedings of the Fourth International Conference on Erythropoiesis* (Eds. K. Nakao, J. W. Fischer and F. Takaku), p. 383, University of Tokyo Press, Tokyo, Japan (1975).
9. E. C. Weinbach and J. Garbus, *J. biol. Chem.* **241**, 3708 (1966).
10. B. J. Dabney and A. L. Beaudet, *Archs. Biochem. Biophys.* **179**, 106 (1977).
11. B. J. Dabney and A. L. Beaudet, *J. biol. Chem.* **253**, 7124 (1978).
12. D. P. Tschudy and A. Collins, *Science* **126**, 168 (1957).
13. J. Ross and D. Sautner, *Cell* **8**, 513 (1976).
14. G. Rovera, J. Vartikar, G. R. Connolly, C. Magarian and T. W. Dolby, *J. biol. Chem.* **253**, 7588 (1978).
15. J. L. Granick and S. Sassa, *J. biol. Chem.* **253**, 5402 (1978).