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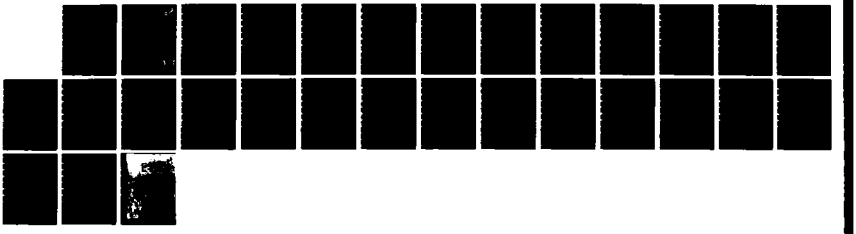

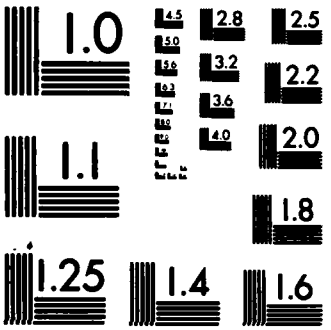

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TECHNICAL REPORT 83-15

INHIBITION OF BABOON MARROW CFU-GEMM, CFU-GM, BFU-E and CFU-E BY ADRENOCHROME, AN EPINEPHRINE METABOLITE

by

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CFU-GM	Red cell production	Blood

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(CFU-E > BFU-E > CFU-GEMM).

The addition of 0.2 mM hemin alone produced a slight but not statistically significant depression of baboon marrow BFU-E growth. The combination of hemin and 0.5 mM adrenochrome produced no growth of BFU-E, but did produce a significant increase in the CFU-GEMM as compared to 0.5 mM adrenochrome alone.

These data suggest that substances such as adrenochrome may be involved in the suppression of red cell production observed in patients subjected to traumatic injuries.

ABSTRACT

Using tissue culture systems designed for the growth of human bone marrow cells, studies were done to assess the effects of adrenochrome, a metabolite of epinephrine, and of hemin on baboon bone marrow CFU-GEMM, CFU-GM, CFU-E and BFU-E. In all the assay systems studied, growth of CFU-GEMM, CFU-GM, CFU-E and BFU-E was suppressed by the addition of 0.5 mM adrenochrome. For those clonal cell assays expressing erythroid growth in culture, the degree of suppression by 0.5 mM adrenochrome increased with increasing cellular maturity (CFU-E > BFU-E > CFU-GEMM).

The addition of 0.2 mM hemin alone produced a slight but not statistically significant depression of baboon marrow BFU-E growth. The combination of hemin and 0.5 mM adrenochrome produced no growth of BFU-E, but did produce a significant increase in the CFU-GEMM as compared to 0.5 mM adrenochrome alone.

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INTRODUCTION

Clinicians have long observed that seriously wounded military and civilian persons require larger volumes of blood to restore their blood volumes to normal than can be explained merely by their blood loss.[1-5] The cause of the unexpectedly low blood volumes, of the so-called "missing blood syndrome", has intrigued many workers who have sought to discover the mechanism for this unexplained hypovolemic anemia. One of the many speculations suggests that circulating toxic substances are involved which inhibit red blood cell production.[1-3]

In studies by Valeri and Altschule from 1968 to 1972 of more than 300 patients who had sustained war-related injuries in South Vietnam, a clinical pattern of hypovolemic anemia was observed that was characterized by reduced red cell and plasma volumes and normal or slightly reduced peripheral blood hematocrit values and hemoglobin concentration levels.[6] The reduction in red cell volume was due to blood loss, to a reduction in red cell survival, and to suppression of red cell production. This syndrome was identified as "the hypovolemic anemia of trauma". Because plasma epinephrine concentrations were known to rise above the normal range following traumatic shock,[7] these authors investigated the possible suppressive effects of epinephrine and its metabolites. Adrenochrome (2,3-dihydro-3-hydroxy-N-methylindole-5,6-quinone), a metabolite formed in the oxidation of epinephrine, was observed in the blood from 25% of the patients with the hypovolemic anemia of trauma and was found to produce hemolysis in vitro.[8] Toxic substances produced from plasma catecholamines

liberated during traumatic injury were also thought to inhibit erythropoiesis.

We now have available clonal cell assays for pluripotential hematopoietic stem cells producing colonies containing granulocytic, erythroid, monocyte-macrophage, and megakaryocytic elements in methylcellulose (CFU-GEMM),[9-11] for granulocyte-macrophage progenitors in agar (CFU-GM),[12,13] and for erythroid progenitors in plasma clot (BFU-E, CFU-E),[14,15] making it possible to study the alterations in blood cell production caused by substances liberated during traumatic injuries.

The purpose of this study was to assess the effect of adrenochrome on baboon marrow CFU-GEMM, CFU-GM, BFU-E and CFU-E. Hemin, the chloride form of hematin (ferric chloride protoporphyrin IX), has been shown to regulate the synthesis of hemoglobin.[16] It has also been shown to enhance the in vitro growth of primitive murine erythroid progenitors.[17] Since red blood cell destruction in the presence of catecholamine metabolites may produce porphyrins and iron,[6,18] the effects of hemin on the hematopoietic progenitor cells, both alone and in conjunction with adrenochrome, were studied.

MATERIALS AND METHODS

Marrow Samples

Each normal baboon was anesthetized with ketamine hydrochloride, and a 3-5 ml volume of bone marrow aspirated from posterior iliac crests was drawn into heparinized 20 ml syringes which were then stored on ice. Each sample was diluted with an equal amount of Iscove's modified Dulbecco's medium (ImDm) (GIBCO), layered over a ficoll-sodium metrizoate gradient (Lymphoprep, Nyegaard), and centrifuged at 800 X g for 30 minutes at 0-4 C. The mononuclear cells were collected, washed by dilution with ImDm, and then centrifuged at 500 X g for 10 minutes at 4 C. The supernatant solution was discarded and the cells were washed a second time. The cells were counted using a Coulter counter (Coulter Electronics).

Preparation of Feeder Layers for CFU-GEMM and CFU-GM

Human venous peripheral blood was used as the source of colony-stimulating activity (CSA) and burst-promoting activity (BPA), according to the procedure of Pike and Robinson.[13] Briefly, blood was collected from normal volunteers into heparinized 50 ml syringes and allowed to stand for 1-2 hours at room temperature. The buffy coat was isolated, and the nucleated cells were counted. Feeder underlays were prepared by adding 1 X 10^6 cells/ml of McCoy's 5A medium (GIBCO) with 20% fetal bovine serum (Sterile Systems) and 0.5% boiled agar (Difco). Each 1 ml aliquot was pipetted into a 35 mm tissue culture plate (Lux) and allowed to gel at room temperature. The plate then received 1200 rads of gamma irradiation (^{137}Cs, Gammacell).

Preparation of PHA-LCM

2 X 10^6 human peripheral blood leukocytes/ml were stored in Dulbecco's minimum essential medium (MEM) for 7 days in the presence of 1% (v/v) phytohemagglutinin (GIBCO) and 15% fetal bovine serum at 37 C, 5% CO_2 in a humidified atmosphere, as described by Aye et al.[19]

Preparation of Hemin and Adrenochrome Solutions

A 2 mM solution of bovine hemin (Type I, Sigma) was prepared according to the method of Stenzel et al.[20] 10.4 mg hemin, 0.4 ml 1N KOH (Fisher), and 2.0 ml of filtered 0.2 M Tris-HCl (pH 7.8) were added to distilled water to a volume of 8 ml. The pH was adjusted to 7.5 and the solution was heated in a boiling water bath for 5 minutes. The 2 ml aliquots of this preparation were frozen at -80 C until the time of experiment. A 5 mM solution of adrenochrome (ICN Pharmaceuticals) was prepared on the day of experiment by adding 8.95 mg of adrenochrome to ImDm to a total volume of 10 ml. The unfiltered solution was agitated for 3 minutes prior to use. Hemin was added to yield a final concentration of 0.2 mM. Adrenochrome was added in the final concentrations of 0.005, 0.05, and 0.5 mM.

CFU-GEMM Culture Conditions

CFU-mixed and CFU-GM colonies were grown in methylcellulose, according to the procedure of Ash et al.[11] Bone marrow cells in a concentration of 1 X 10^5 were suspended in 1% methylcellulose in ImDm supplemented with 30% fetal bovine serum, 5% PHA-LCM, 1 U/ml human urinary erythropoietin (EPO, Toyobo), and 5 X 10^{-5} M 2-mercaptoethanol (Sigma). Four 1 ml aliquots were

layered over feeder underlays and incubated at 37 C in a 5% CO_2 humidified atmosphere for 14 days.

CFU-GM Culture Conditions

CFU-GM colonies were grown in agar-gel, following the procedure of Pike and Robinson.[13] Bone marrow cells in a concentration of 2 X 10^5/ml were suspended in 0.3% agar in McCoy's 5A medium supplemented with 20% fetal bovine serum. Four 1 ml aliquots were layered over feeder underlays and incubated for 14 days.

Examination and Scoring of CFU-GEMM and CFU-GM Cultures

Clusters of 50 or more cells, observed unfixed and unstained under a dissecting microscope, were scored as colonies. CFU-GM colonies contained no erythroid cells, whereas mixed colonies contained both non-erythroid and erythroid elements as indicated by their reddish color. Selected mixed colonies which were scored on this basis were examined and found to contain mature granulocytes, erythrocytes, macrophages, and frequently megakaryocytes. The number of CFU-mixed and CFU-GM colonies were reported in the CFU-GEMM assay.

BFU-E Culture Conditions

BFU-E bursts were grown in clotted bovine plasma according to the method of Axelrad et al.[14] One hour before cell plating, an aliquot of bovine plasma (Flow) was placed in a 37 C incubator to allow cold insoluble fibrinogen to dissolve. Bone marrow cells in a concentration of 0.75 to 1.0 X 10^6/ml were suspended in ImDm supplemented with 30% fetal bovine

serum, 1.5% beef embryo extract (GIBCO), 1% bovine serum albumin (Sigma), 0.2 mg/ml L-asparagine (GIBCO), and 10% warmed plasma. Eight 0.1 ml aliquots of the final mixture were cultured in microtiter wells (Dynatech) placed in 35 mm tissue culture plates and incubated for 12 days.

CFU-E Culture Conditions

CFU-E colonies were grown in clotted bovine plasma according to the method of Stephenson et al.[15] Bone marrow cells in a concentration of 2-2.5 X 10^5/ml were plated as above for BFU-E, except that a 0.25 U/ml final concentration of human urinary erythropoietin was used and the cells were cultured for 4 days.

Harvesting, Staining, and Scoring of BFU-E and CFU-E Cultures

Each clot was removed from its microtiter well using a metal microspatula and placed on a glass microscope slide. A strip of filter paper (Fisher) was placed over the slide, and clots were fixed with 5% glutaraldehyde (Ladd) and stained with hematoxylin (Fisher) and benzidine (Kodak). Aggregates of 8-64 benzidine-positive cells with characteristic erythroid morphology observed at a magnification of 250X under oil were scored as CFU-E. Isolated groups of 50 or more nucleated cells, at least one-half of which were benzidine-positive observed at a magnification of 100X under oil, were scored as BFU-E.

Statistics

The paired t-test was used to compare control groups to experimental groups

RESULTS

In our laboratory, colonies were grown using the BFU-E and CFU-E assays from baboon bone marrow mononuclear cells under experimental conditions similar to those used in human and murine systems using 2 U/ml EPO for the BFU-E assay and 0.25 U/ml EPO for the CFU-E assay.

The effect of three concentrations of adrenochrome on the growth of marrow CFU-GEMM, CFU-GM, BFU-E and CFU-E was studied. Adrenochrome in a concentration of 0.5 mM inhibited the growth of all hematopoietic progenitor cells studied (Table 1). The degree of suppression varied among the four assays, with the greatest suppression noted in the most mature erythroid progenitor cell (CFU-E: 2.5% control, $p < 0.005$), and the least in the early mixed hematopoietic cell line (CFU-GEMM: 50.6% control, $p < 0.01$). Adrenochrome in lower concentrations did not significantly inhibit growth of CFU-GEMM, CFU-GM, BFU-E or CFU-E ($p > 0.05$).

The effect of 0.2 mM hemin on the growth of marrow CFU-GM, BFU-E, CFU-E and CFU-GEMM and CFU-mixed using the CFU-GEMM assay was also studied. Hemin had no effect on the growth of CFU-GM or CFU-E when compared to untreated controls. In addition, hemin had no effect on these assays in combination with adrenochrome when compared to samples treated with adrenochrome alone (Figures 1 and 2). In contrast, CFU-mixed colonies in the CFU-GEMM assay showed a slight but not statistically significant ($p > 0.05$) increase in number in the presence of hemin alone (Figure 3). In the presence of hemin and 0.5 mM adrenochrome, significant enhancement of mixed colony growth was observed ($p < 0.05$) compared to adrenochrome

FIG.
FIG.
FIG.

alone. At lower adrenochrome concentrations, this effect was not statistically significant ($p>0.05$).

Growth of BFU-E colonies on day 12 appeared to be suppressed by hemin (Figure 4), but this effect was not statistically significant ($p>0.2$). However, in the presence of hemin and 0.5 mM adrenochrome, no BFU-E growth was observed in any of the experiments.

FIG. 4

DISCUSSION

Maintenance of mammalian homeostasis depends on the differentiation of several classes of hematopoietic progenitors which appear to arise from the uncommitted stem cell. Among these classes is the pluripotent stem cell which is capable of producing mature granulocyte, erythrocyte, monocyte-macrophage, and megakaryocyte elements in culture (CFU-GEMM), and the CFU-GM, BFU-E, and CFU-E which are derived from it. Our data demonstrate that colonies derived from these four classes of hematopoietic progenitors were grown in vitro from baboon bone marrow mononuclear cells in tissue culture systems designed for the growth of human cells. The number of baboon bone marrow BFU-E colonies on day 12, CFU-E colonies on day 4, CFU-GM colonies on day 14, and CFU-GEMM colonies on day 14, observed in our laboratory were within the range of values reported from other laboratories using human and murine bone marrow.[17,21-23] This study assessed the effects of adrenochrome, a catecholamine metabolite, and hemin, both alone and in combination, on baboon marrow hematopoietic progenitor cells.

The suppression of colony growth from CFU-GM, CFU-GEMM, BFU-E and CFU-E by the addition of 0.5 mM adrenochrome was observed in all four assay systems studied, although the degree of suppression varied among the assays. In the clonal cell assays capable of expressing erythroid growth in culture, the degree of suppression by 0.5 mM adrenochrome increased with increasing cellular maturity (CFU-E > BFU-E > CFU-GEMM).

Earlier studies on the incubation of adrenochrome and other catecholamine

metabolites in human blood plasma have shown that plasma soluble rheomelanins form after 24 hours at 37 C.[24] These rheomelanins have strong free radical activity,[25,26] which appears to be implicated in red blood cell destruction[8] and which may be damaging to red blood cell production as well. Free radical reactions are known to disrupt cellular metabolism by inactivating enzymes, cross-linking DNA, and altering membrane functions.[27] More recent work has indicated that rheomelanins exhibit several characteristics similar to lipofuscins.[28] Therefore, it seems likely that in our study several chemical changes involving adrenochrome occurred in the course of the 4- to 14-day incubation periods.

Although the concentrations of adrenochrome employed were certainly neither pharmacologic nor pathophysiologic, the inhibition of colony growth in vitro suggests a possible role of adrenochrome or one of its metabolites in the anemia of trauma.

Previous observations on the effect of hemin on in vitro growth of murine marrow erythroid progenitors have indicated that hemin in a concentration of 0.2 mM augments the growth of murine marrow BFU-E,[17] but our findings with baboon marrow are in contrast to those observations. Growth of baboon bone marrow BFU-E showed a slight, but not statistically significant, suppression by the addition of hemin. This did not appear to be a non-specific cytotoxic effect on baboon hematopoietic progenitors, since growth of CFU-GEMM was slightly although not significantly enhanced, and growth of CFU-GM and CFU-E was unaffected by the presence of hemin. The reasons for the discrepancies in these results are not clear. They may be due simply to some inherent difference between baboon and murine

marrow hematopoietic cells and their responses to bovine hemin.

Adding hemin to cultures of CFU-GEMM incubated with 0.5 mM adrenochrome increased the colony numbers, an interesting observation insofar as no BFU-E growth was observed in the presence of this same combination. One difference between the two systems is the presence of BPA. Colony growth in the CFU-GEMM assay using methylcellulose contained sources of BPA in the human feeder underlays and in the PHA-LCM, whereas colony growth in the BFU-E assay using plasma clot did not. Since enhanced growth of CFU-GEMM but not BFU-E colony growth occurred in the presence of hemin, it is possible that hemin has no stimulatory effect on baboon marrow erythropoietic progenitors per se, but only brings about an effect through stimulation of human peripheral blood cells to produce increased amounts of BPA. The role of hemin, both alone and in combination with adrenochrome, warrants further study.

FIGURE 1

The effect of adrenochrome on CFU-GM colony formation in agar gel in the presence or absence of 0.2 mM hemin. Values are expressed as the number of CFU-GM colonies per 10^5 marrow cells plated and represent the mean of 5 experiments $\pm$ SEM. No significant difference between cultures with and without hemin ($p > 0.05$).

FIGURE 1

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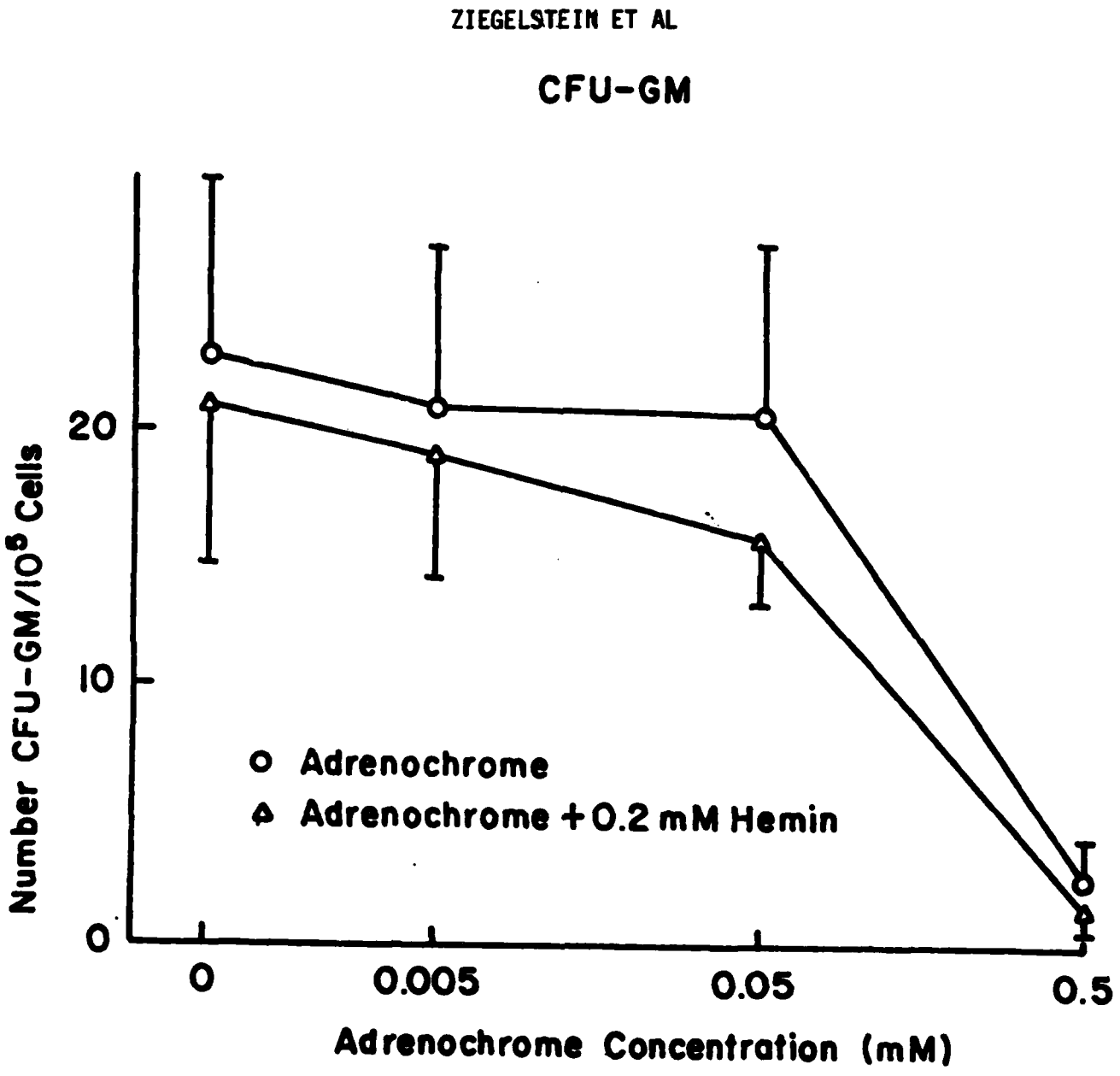

FIGURE 2

The effect of adrenochrome on CFU-E colony formation in plasma clot in the presence or absence of 0.2 mM hemin. Values are expressed as the number of CFU-E per 10^5 marrow cells and represent the mean of 5 experiments ± SEM.

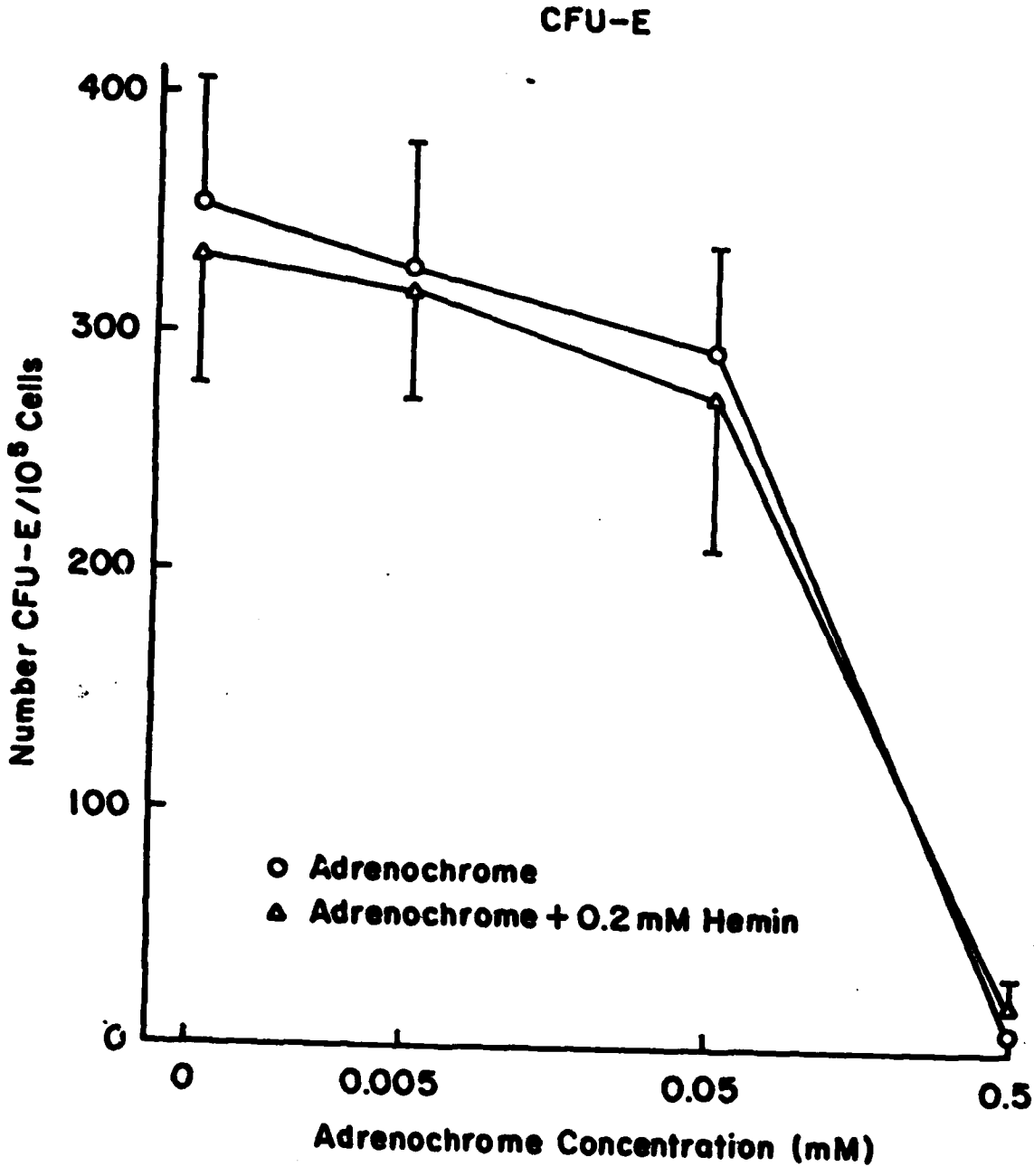

FIGURE 2

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FIGURE 3

The effect of adrenochrome on CFU-GEMM-CFU-MIXED colony formation in methylcellulose in the presence or absence of 0.2 mM hemin. Values are expressed as the number of CFU-GEMM per 10^5 marrow cells and represent the mean of 5 experiments ± SEM, except where otherwise noted. No significant difference between cultures with and without hemin except in the presence of 0.5 mM adrenochrome ($p < 0.05$). † = mean of 4 experiments.

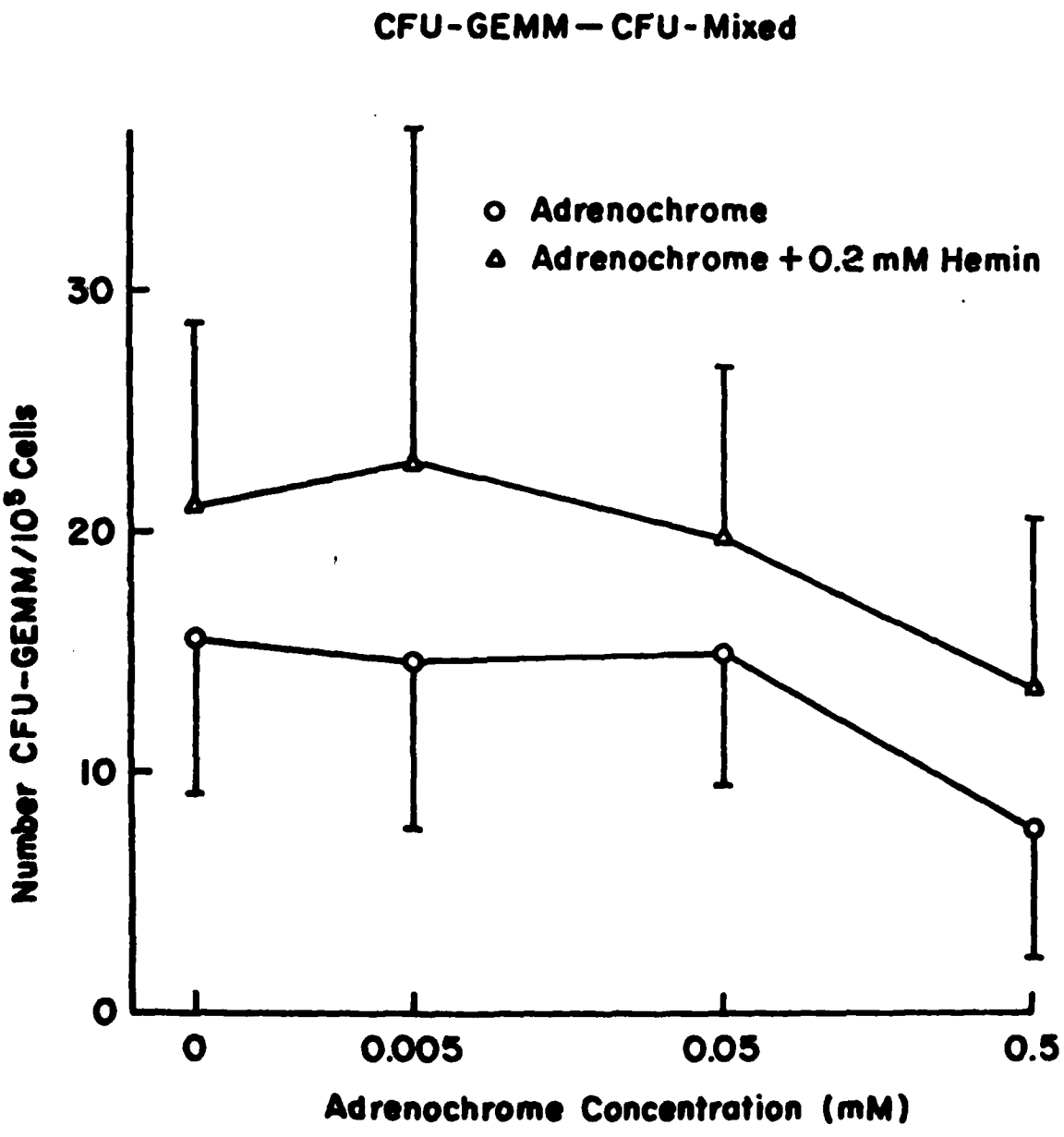

FIGURE 3

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FIGURE 4

The effect of adrenochrome on BFU-E colony formation in plasma clot in the presence or absence of 0.2 mM hemin. Values are expressed as the number of BFU-E per 10^5 marrow cells and represent the mean of 5 experiments ± SEM. No significant difference between cultures with and without hemin.

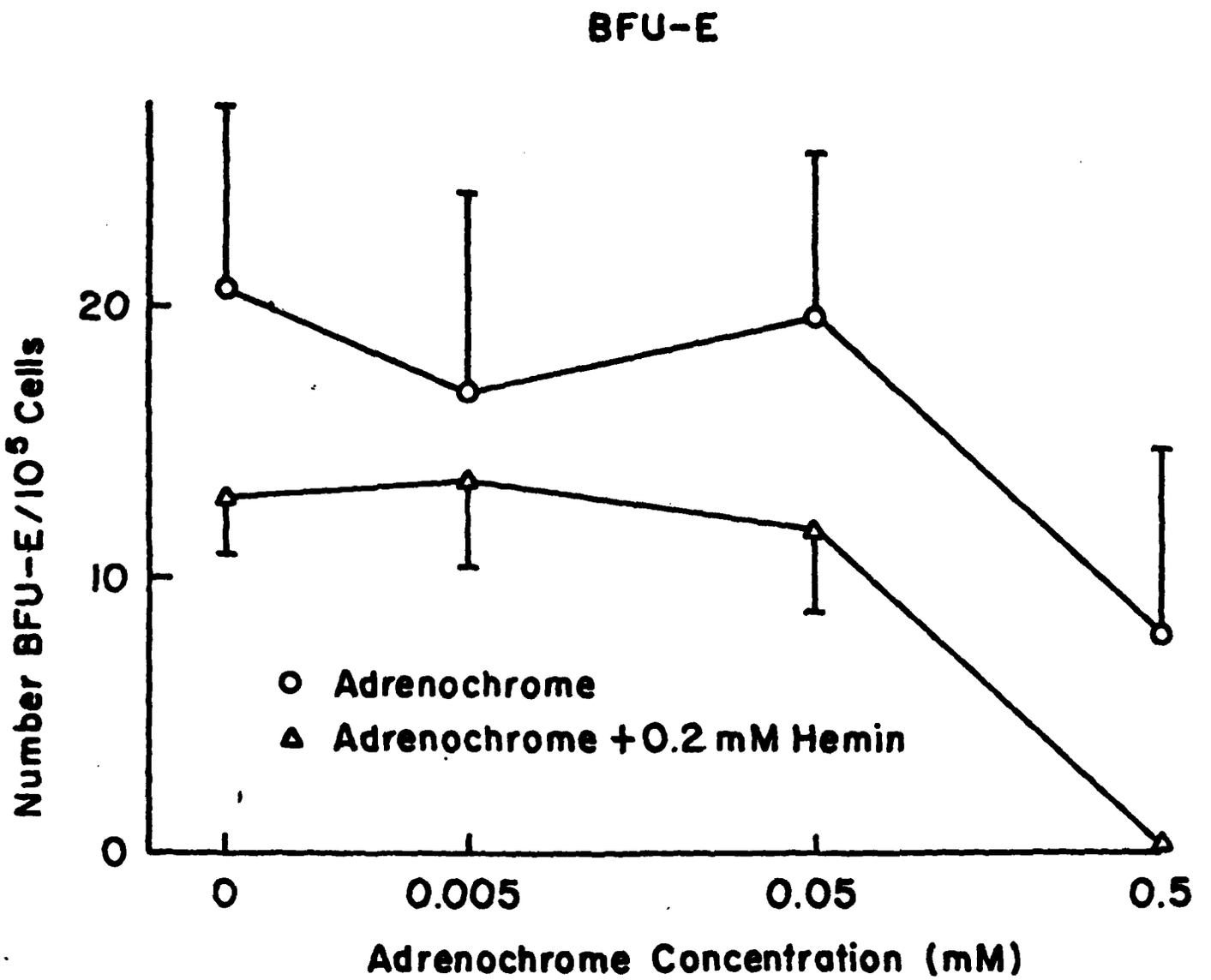

FIGURE 4

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TABLE 1

EFFECT OF ADRENOCHROME ON CFU-GEMM, CFU-GM, BFU-E and CFU-E*

COLONIES/10^5 MARROW CELLS

Concentration of Adrenochrome (mM)	CFU-GM	CFU-GEMM		BFU-E	CFU-E
		MIXED	GM		
0	22.9 ± 6.8	15.6 ± 6.4	31.4 ± 8.4	20.6 ± 6.9	353.2 ± 52.4
.005	20.8 ± 6.6**	14.8 ± 7.0**	36.2 ± 12.0**	16.6 ± 7.7**	327.7 ± 52.6**
.05	20.7 ± 6.5**	15.0 ± 5.5**	28.5 ± 6.0**	19.6 ± 6.2**	294.9 ± 45.3**
.5	2.7 ± 1.6† (11.8%)	7.9 ± 5.5‡ (50.6%)	5.4 ± 2.9‡† (17.2%)	8.0 ± 6.6‡† (38.8%)	8.8 ± 7.8‡‡ (2.5%)

*Data presented as the mean of 5 experiments ± SEM; paired t-tests were done between samples with and without adrenochrome. Percent control values for the 0.5 mM adrenochrome groups appear in parentheses.

**No significant difference ($p > 0.05$)

†Significant difference ($p < 0.05$)

‡Significant difference ($p < 0.01$)

‡†Significant difference ($p < 0.025$)

‡‡Significant difference ($p < 0.005$)

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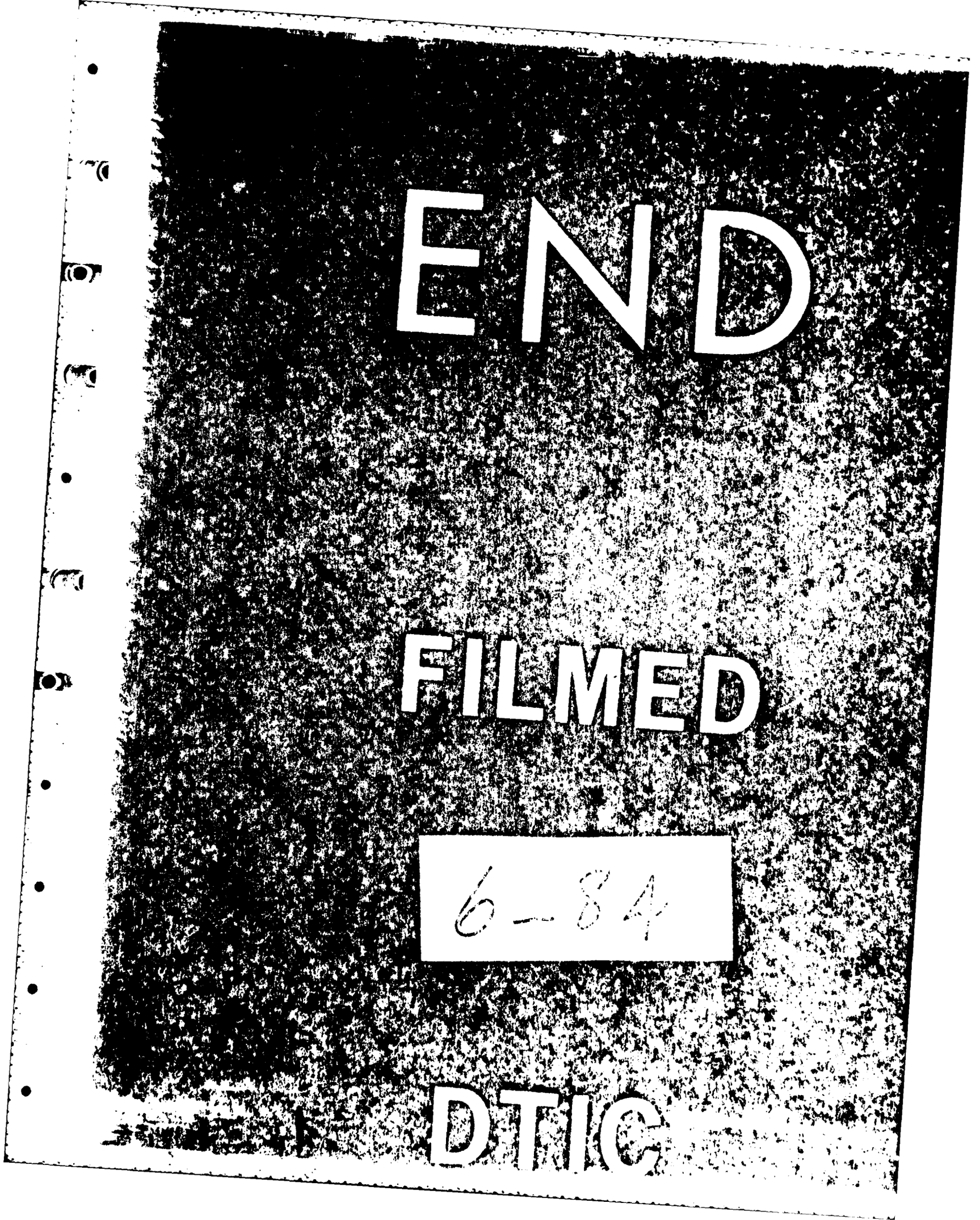
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