2) Although fetal bovine serum does not elicit under these conditions the rapid increase in GS activity, it does not inhibit the process when added to the medium with horse serum. Therefore, the precocious elicitation of the rise in GS activity may be due to a factor present in horse serum but, apparently, low or lacking in fetal serum (*8*). Since other *adult* sera can also elicit the precocious increase in GS activity, the postulated factor appears not to be restricted to adult horse serum (see below).

3) While fetal serum appears to lack an effective concentration of this essential factor, it has a promoting effect on the GS-forming system: when added to the medium with horse serum, fetal serum stimulates increase in GS activity to values significantly higher than additive, and greater than can be obtained by doubling the concentration of horse serum. Furthermore, when 10-day retina is transferred to horse-serum medium following a 24-hour exposure to fetal-serum medium, GS activity increases more rapidly than in retinas maintained continuously in horse-serum medium, as if the fetal serum had "primed" the retina for a response to horse serum. Fetal serum may thus contain a "priming" factor demonstrable by the simultaneous or subsequent exposure of the retina to horse serum.

4) When 10-day retina is cultured for only 24 hours in horse-serum medium and is then transferred to fetal-serum medium or to a serum-free medium, there is only a slight additional increase in GS activity; however, after 48 hours cultivation in horse serum, transfer to fetal bovine serum permits a substantial further increase (Fig. 2) suggesting that, during 48 hours of culture in the horse-serum medium, the GS-forming system in the 10-day retina becomes independent of continuous elicitation by this adult serum.

Data obtained in 86 similar cultures of 16-day retina are summarized in Figs. 2 and 3. Generally, the results are similar to those obtained with 10-day retina, with this exception: after only 24 hours of culture the GS-forming system of the 16-day retina becomes independent of the requirement for horse serum. It is of considerable interest that in both the 10-day and the 16-day retina the development of independence of horse serum parallels the development of insensitivity to actinomycin (Fig. 2). This correspondence may be coincidental or may indicate stabilization of the GS-forming system caused by an effect of horse serum.

Precocious enhancement of the GS-forming system in the 10-day retina in vitro can also be caused by other *adult* serums: chicken, bovine, and human. In this effect, the adult serums may be considered to simulate conditions affecting the retina in the late chick embryo at the time of the normal increase in retinal GS activity. On the other hand, serum and extracts from early chick embryos resemble fetal serum in failing to elicit in vitro the precocious effect, and simulate in this respect the conditions affecting the retina in the early chick embryo prior to the normal rise in GS activity.

As a step toward testing the above assumptions we have isolated an active fraction from horse serum which causes a precocious increase in retinal GS activity in vitro. Its characterization should facilitate further studies on its site and mode of action in "turning on" or stabilizing the GS-forming system in retinal cells, with reference to retinal differentiation.

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8. This activation is similarly observed with dialyzed horse serum; it is not readily explained on the basis of variations in glutamate or glutamine concentrations, although these amino acids have some modifying effects on the amount of enzyme activity in culture (*2*).
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Granulocytosis-Promoting Extract of Mouse Tumor Tissue: Partial Purification

Abstract. *A granulocytosis-promoting extract of mouse tumor tissue was partially purified by differential precipitation with cold 20- to 30-percent ethanol at pH 6.5, "salting out" with 50- to 75-percent saturated ammonium sulfate, and filtration through Sephadex G-75 dextran gel. In intact mice, single doses of the extract (at least 12 micrograms per gram of body weight) induced up to a 30-fold increase in circulating granulocytes and a two- to threefold decrease in circulating mononuclear cells within 3 to 7 hours of intravenous injection.*

A variety of human and animal tumors have been reported to elaborate humoral substances unrelated to the type of tumor tissue. Adrenocorticotropic hormone (*1*), antidiuretic hormone (*2*), thyroid-stimulating hormone (*3*), a hypoglycemic factor (*4*), a lipid-mobilizing factor (*5*), a parathyroid-stimulating factor (*6*), erythropoietin (*7*), and leukopoietin (*8*) figure among these substances.

Previously, we reported (*9*) that massive granulocytosis associated with extramedullary granulopoiesis and a trend toward reduction of platelet numbers was evoked in CE and (BALB/C × CE)F_1 hybrid mice by a transplantable mouse tumor, CE 1460 mammary cancer, but that no such changes could be induced in BALB/C or (BALB/C × CE)F_1 hybrid mice by another transplantable mouse tumor, BALB/C 2301 mammary cancer. Instead, the latter tumor was associated with normal numbers of leukocytes and with fluctuations of platelet numbers between "baseline" levels (comparable to those found in normal mice) and elevated levels (up to two and one half times above the "baseline"). Our present investigation was based on the hypothesis that the CE 1460 tumor tissue is the source of a granulocytosis-promoting factor (GPF) which induced the granulocytosis and perhaps also the extramedullary granulopoiesis observed in mice bearing CE 1460 tumors.

Fresh-frozen CE 1460 tumor tissue was homogenized with cold 0.4*N* perchloroacetic acid in a Vir-Tis homogenizer. The acid-insoluble residue was washed successively with 75-percent, 95-percent, and 100-percent ethanol. The residue was then boiled for 60 minutes in 15 volumes of 10-percent

Table 1. The increase in the potency of GPF with progressive purification of the extract from CE 1460 tumor tissue. Bioassays were conducted in intact (BALB/C × CE)F_1 hybrid mice. The extract obtained from 500 mg of tumor tissue (wet weight) was injected intravenously as a single dose into each 20-g mouse; ppt., precipitate.

Stage of GPF purification	Yield of GPF (dry wt) per gram of tumor (wet wt) (mg)	Solubility	GPF bioassay response				
			Hours			Max. No. peripheral granulocytes/mm³	
			Onset	Peak*	Duration	Peak	24 hours
0- to 70-percent ethanol at *p*H 6.5 ppt.	630.6	Insol.	2–4	3–5	9–12	68,600	6,000
20- to 30-percent ethanol at *p*H 6.5 ppt.	17.0	H_2O, 60°C	2–5	3–6	9–12	123,000	12,000
Sephadex G-75 dextran gel eluate	16.0	H_2O, 23°C	2–5	3–7	9–>24	172,000	37,000
50- to 75-percent saturated $(NH_4)_2SO_4$ ppt.	2.1–5.6	H_2O, 23°C	2–5	3–7	9–>24	172,000	37,000

* Peak: time of greatest granulocyte response.

NaCl buffered at seven different *p*H values with either 0.3*M* sodium phosphate or 0.3*M* sodium acetate plus acetic acid. The *p*H values used ranged in 0.5-unit intervals from 4.0 to 7.0. The *p*H values of the individual supernatants decreased by 0.3 units during the boiling process, and were therefore readjusted to the original values before proceeding to the next step. The extracted material was precipitated from each of the *p*H-adjusted supernatants, first with 40-percent and then with 70-percent ethanol at −20°C. In another procedure, CE 1460 tumor tissue was extracted as above, but the boiling 10-percent saline was buffered at only four different *p*H values (*p*H 4.5, 5.5, 6.5, and 7.0) and differential precipitation of GPF from each of the *p*H-adjusted supernatants was attempted at −20°C with ethanol concentrations increasing progressively, at 10-percent intervals, from 10 to 70 percent. The precipitates resulting from these procedures were dehydrated with cold 100-percent ethanol, redissolved in small amounts of distilled water at 60°C, and bioassayed.

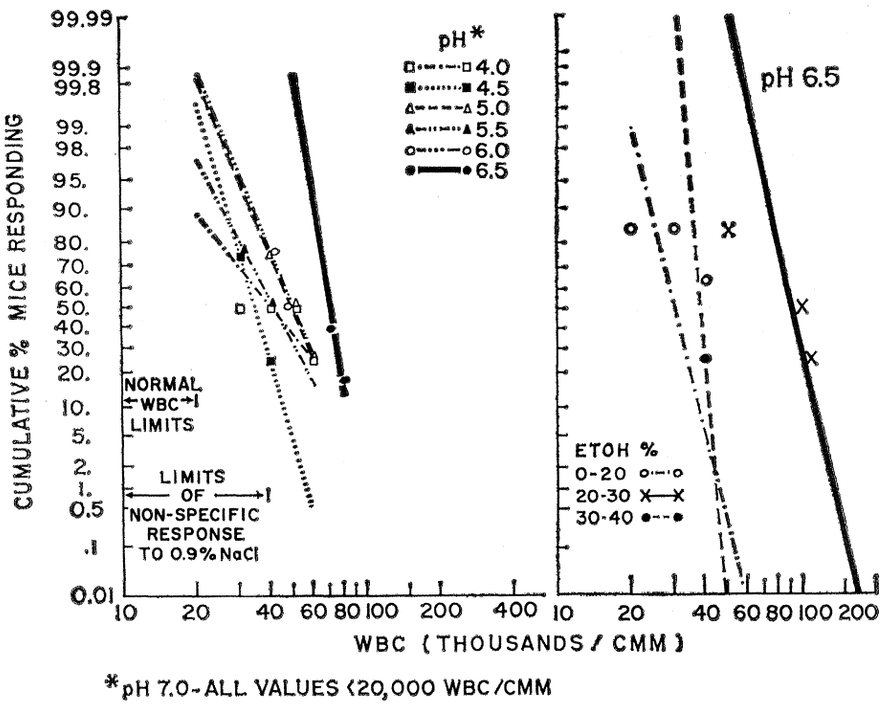

Fig. 1. Determination of the optimum *p*H and optimum concentration bracket of ethanol for the purification of GPF extracted from CE 1460 tumors of mice. (Left) Sensitivity of GPF to *p*H, as measured by the response of mice to intravenous injections of single doses of GPF-rich extracts equivalent to 500 mg tumor tissue (wet weight). The extracts were boiled in buffered 10-percent NaCl and precipitated with cold 0- to 40-percent ethanol at seven different *p*H values. (Right) Successful differential cold-ethanol precipitation of GPF at *p*H 6.5, as measured by the response of mice to intravenous injections of single doses of GPF-rich extract equivalent to 500 mg of tumor tissue (wet weight).

Minimum significant GPF activity was defined in a bioassay as induction of a transitory peripheral granulocytosis; the total numbers of circulating granulocytes gradually rise from the normal 1200 to 6000 per cubic millimeter (6000 to 20,000 leukocytes per cubic millimeter, with 15 to 30 percent granulocytes) before treatment to "peak" numbers of at least 36,000 granulocytes per cubic millimeter (at least 40,000 leukocytes per cubic millimeter, with 90 to 98 percent granulocytes) within 3 to 7 hours after intravenous or intraperitoneal injection of a single dose of the extract, and gradually return to the values before treatment within nine or more hours. The greatest degree of granulocytosis observed in a given animal after a single dose of bioassay material was termed the "peak" response. For all the dose-response studies, we used 500-mg "tumor wet-weight-equivalent" doses—that is, the final amount of extract obtained from 500 mg of wet tumor tissue at any stage of the purification procedure. Marked increases in solubility, "peak" granulocyte values, and durations of significant response were obtained at progressive stages of GPF purification (Table 1).

The GPF-rich material failed to pass through dialysis casing (Visking), suggesting that the active principle was either a large molecule or bound to a large molecule.

After we had established that a *p*H of 6.5 and a 20- to 30-percent concentration of ethanol provided the critical as well as optimal conditions for extraction of GPF in boiling saline and for differential precipitation of GPF with cold ethanol (Fig. 1), further purification was achieved by "salting out." The solution was brought successively to 50-percent, 55-percent . . . 90-percent, and 95-percent saturation of $(NH_4)_2SO_4$ by addition of the appropriate volumes of a saturated solution of $(NH_4)_2SO_4$. The precipitate from each step was dissolved in a small amount of distilled water at 23°C. The $(NH_4)_2SO_4$ was removed by absorption on dry Sephadex G-25 (coarse), and the eluate was bioassayed.

Equal portions of the GPF-rich fraction, found in the precipitate obtained

from "salting out" with the 50- to 75-percent saturated solutions of $(NH_4)_2SO_4$, were added to columns of Sephadex G-25, G-50, and G-75 dextran gel equilibrated with 0.05*M* NaCl plus sodium phosphate buffer (*p*H 6.5), and were eluted with the same buffer at 4°C. The eluate peaks, determined at 260 mμ in a Beckman spectrophotometer, were rapidly dehydrated at 60°C in a closed rotary "flash-evaporator" (partial vacuum) and stored at −40°C until bioassayed. The GPF was not absorbed by the Sephadex G-75 column, a finding which suggests a molecular weight of over 50,000. Since GPF activity was enhanced by passing the GPF-rich solution through Sephadex G-75 (Table 1), it is suggested that this technique removed small contaminating molecules whose presence partly inhibits or masks GPF activity. At this stage of purification, GPF retained full potency for over 6 weeks at −40°C.

For determination of dry-weight yield and for amino acid analysis, the GPF-rich eluate obtained from the Sephadex G-75 gel column was freed of NaCl and sodium phosphate by absorbing these salts with dry Sephadex G-25 (coarse). The final yield from the last step of purification varied between 2.1 and 5.6 mg of GPF (dry weight) per gram of tumor (wet weight) (Table 1). Analysis of a hydrolysate of this material on an automatic amino acid analyser (*10*) showed that GPF contains no amino acids.

The minimal effective dose of the most purified fraction of GPF was 0.8 μg per gram of body weight. Doses of 12 μg, or more, per gram of body weight evoked "peak" responses of 80,000 to 172,000 granulocytes per cubic millimeter (82,000 to 174,000 leukocytes per cubic millimeter, with 96 to 98 percent granulocytes), representing up to a 30-fold increase over the baseline granulocyte levels. By 7 hours after administration, 7 to 12 percent of the granulocytes consisted of immature forms including stab cells and metamyelocytes A to C. The granulocytes were predominantly neutrophilic. Eosinophils totaled 1 to 3 percent, representing an absolute but no relative numerical increase. Maximal residual response as high as 37,000 granulocytes per cubic millimeter (up to 59,000 leukocytes per cubic millimeter and 64 percent granulocytes) lasted up to 24 hours and longer. At this stage of the response, immature forms including metamyelocytes totaled 0 to 5 percent of the circulating granulocytes. Throughout the period of granulocyte response, circulating mature erythrocyte numbers remained unchanged and normoblasts were absent from the circulating blood. Platelet levels failed to manifest any consistent change. Circulating mononuclear cell numbers manifested a transitory two- to threefold decrease. The evidence points to a specific mobilization by GPF of both marginated and extravascular granulocytes from the intra- and extramedullary reserve compartments. Until completion of bone marrow studies, nothing can be concluded about the possible granulopoietic effect of GPF.

As preliminary controls for the specificity of CE 1460 tumor tissue as a source of GPF, we tested the effects of physiologic saline as well as of an extract of BALB/C 2301 mammary tumor tissue processed by the GPF-purification method. No significant granulocytosis-promoting effect was observed.

Preliminary data indicated that multiple intraperitoneal doses of GPF extracted from CE 1460 tumor, injected into hypertransfused mice at the peak of inhibition of hemopoietic activity, failed to elicit even a transitory granulocytosis but were capable of inducing permeation of the spleen with large undifferentiated myeloid cells and the formation of multiple small foci of both immature and mature granulocytic forms in the liver. Possibly, GFP evoked these effects in the tissues either by favoring extramedullary colonization by the transfused leukocytes or by stimulating endogenous extramedullary hemopoietic precursor cells. The lack of peripheral response to GPF in these animals may be explained on the basis of the hypothesis that there are high concentrations of a theoretic "granulocyte release-inhibitor" substance in hypertransfused animals, which counteract the granulocytosis-promoting effect of GPF. The absence of similar tissue transformation in hypertransfused mice following multiple injections of control material (physiologic saline and BALB/C 2301 tumor extract) militates for the specificity of GPF in inducing the tissue response.

The GPF extracted from CE 1460 tumor tissue has several physicochemical characteristics in common with those reported by Lowy and Borsook (*11*) and Winkert *et al.* (*12*) for erythropoietin. These include thermostability, *p*H stability, the $(NH_4)_2SO_4$ concentration for "salting out," failure to dialyze, and nonabsorption by Sephadex G-75 dextran gel. Like Bierman's (*13*) leukopoietin-G, GPF is thermostable and *p*H stable, and has a very low protein content; unlike the former, GPF cannot be dialyzed. This could indicate either that GPF is not identical with leukopoietin-G but is merely a molecule evoking similar effects, or that GPF is a leukopoietin-G-like molecule bound to a larger molecule from which it may eventually be separated. If, as one might postulate from the available data, leukopoietin and GPF form a family of molecules together with erythropoietin and thrombopoietin, differential ethanol precipitation—achieved for erythropoietin (*11*) and for the GPF extracted from CE 1460 tumor tissue (Fig. 1)—may eventually become one of the tools for separating these molecules from each other.

The evidence presented here thus suggests that CE 1460 tumor is a highly concentrated source of a granulocytosis-promoting factor, possibly related to leukopoietin, and that the method reported here is a promising intermediate step in the eventual complete purification and isolation of this molecule.

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