Determination of a Skin-Related Erythropoietic Substance (32833)

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(Introduced by J. A. Wells)
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The proposal of a feedback control of erythropoiesis by the products of destruction of the red blood cell was advanced by Kepinow (1) and independently by Isaac (2). Recently, it was shown that some compounds involved in the synthesis and breakdown of the red blood cell, especially the heme fraction, elicit an erythropoietic response when injected into a fasting "dehydrated" rat (3). The present report is a continuation of that work and describes the isolation of an erythropoietically active extract from skin tissues at the site of hemin injection. Several investigators, including Rambach *et al.* (4), Naets (5), Jacobson *et al.* (6) and Lucarelli *et al.* (7) have reported on the effect of various tissues on the erythropoietic response; and Ryan (8) has suggested that porphyrins play a part in the formation of an amorphous substance in the skin of patients with porphyria. However, this is the first evidence that the skin may be actively involved in the control of erythropoiesis.

Methods. Preparation of tissue extract. Generally, albino female rats (300–400 gm) of the Sprague-Dawley (Holtzman) strain, were used as tissue donors. These were maintained on either a complete nutritional diet or in a modified fasting "dehydrated" state (3). In a few cases, animals of other species were used as donors. Daily subcutaneous injections of a 10 mg/ml hemin (Mann, N.Y.) solution (adjusted to pH 7.2 with NH_4OH) were made into donor rats on the basis of 3 mg/100 gm of body weight. Three successive injections were given. Control animals were injected with an equal amount of similar dilute NH_4OH. Twenty-four hours after the third subcutaneous injection the animals were sacrificed. The discolored skin at the injection site was then removed, cut into pieces about 1 cm^2 in area and combined in a common donors'-skin pool. From this pool, 141-gm portions were treated as follows: 47 gm were mixed with 50 ml of 0.9% NaCl solution and homogenized for 1 min at 14,500 rpm in a Servall Omni-mixer.

The fluid material expressed from the homogenate was used for successive homogenizations of the second and third 47-gm portions of skin. An additional 10 ml of fresh saline was added each time. The pooled fluids were stored in the refrigerator. The mince was serially washed with three 20-ml portions of saline and each wash was added to the refrigerated pool. The combined supernatant pool was centrifuged at room temperature at 3500*g* for 15 min. The floating pellicle was discarded. The supernatant was collected and centrifuged a second time; both pellet and pellicle were discarded. The supernatant was then dialyzed against 15 liters of distilled water, with shaking, at 4°C for two 16-hour periods.

The dialysand was centrifuged at 3500*g* for 15 min at 4°C. The resultant supernatant was spun at 37,000*g* for 30 min at 0°C in a Servall RC2 refrigerated centrifuge, and the subsequent supernatant was centrifuged at 145,000*g* for 15 min. The final supernatant and pellet or precipitate (hereafter referred to as HSS and HSP respectively) were then lyophilized. The procedure used in preparing the extracts is outlined in Fig. 1.

Bioassay procedures. For assay, dried test materials were taken up in either water, 0.9% saline, 0.1 *M* phosphate buffer (pH 7.6), or dilute NH_4OH (pH 8.5) at concentrations of 0.07–4.0 mg/ml as needed. These were injected subcutaneously for 3 days into fasting "dehydrated" recipient rats (160–200 gm) as previously described (3). Two hours after the last subcutaneous injection, ferrous citrate-^{59}Fe was injected intravenously. Twenty-four hours later blood was taken from the tail for hematocrit and reticulocyte counts and blood was drawn from the abdominal aorta and counted (3). Total blood volume was computed on the basis of 5.18 ml/100 gm of the final body weight (9).

TABLE I. Effect of Hemin-Injected Skin Extract on Fasting "Dehydrated" Rats.[a]

Treatment[b]	No. of rats	HCT (%)[d]	^{59}Fe uptake (%)	Reticulocyte (%)
C[c] Supnt.	5	46.0 ± 2.0	8.6 ± 2.8	0.5 ± 0.2
H[e] Supnt.	6	46.0 ± 1.1	8.8 ± 2.3	0.6 ± 0.3
C[c] Ppt.	5	48.8 ± 6.3	7.2 ± 1.7	0.7 ± 0.2
H[e] Ppt. (HSP)	6	49.0 ± 5.1	24.9 ± 6.5	1.8 ± 0.4

[a] Rat wts. at sacrifice: 170–180 gm.
[b] Recipient rats received 3 daily 1.0-mg injections.
[c] Indicates extract from test solvent-injected donors.
[d] ± Standard deviation.
[e] Indicates extract from hemin-injected donors.

In one experiment, test material was assayed in polycythemic rats by a modification of the method of Jacobson *et al.* (10). Twelve rats weighing about 200 gm were twice injected intraperitoneally, 24 hours apart, with 3.5 ml of a normal saline solution containing 80% saline-washed packed homologous red blood cells. Thirty hours after the first intraperitoneal injection, the first of two subcutaneous injections of 4.0 mg of HSP was administered to 6 rats. Six rats received the control solvent. The second subcutaneous injection followed at 50 hours. Radioiron was injected intravenously at 72 hours and blood was taken for hematocrits, reticulocytes, and radioiron uptake (as above) at 144 hours. All polycythemic rats were maintained on a normal diet throughout the experiment.

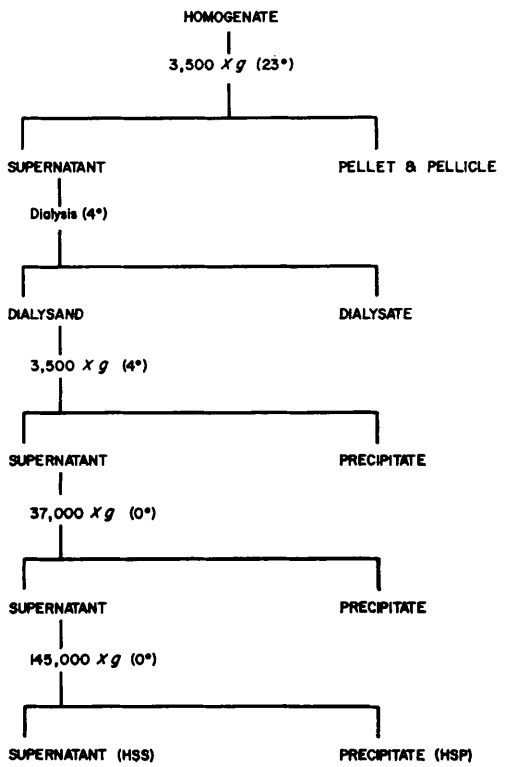

FIG. 1. Preparation of final extracts from hemin-injected skin tissues. Donor skins were homogenized in 0.9% NaCl.

Results. Table I shows the activities of the HSS and HSP obtained from the steps diagrammed in Fig. 1. Fasting "dehydrated" assay animals were injected with 1.0 mg of sample material (in 1.0 ml of saline) from donors that had been injected with hemin or the solvent in which hemin was dissolved. The data show increased ^{59}Fe uptake and reticulocytosis produced by the hemin-injected skin precipitate fraction as opposed to the supernatant fraction from the same source and both extracts from control rats.

To evaluate the response of test animals to various levels of HSP, a starting solution of 4.0 mg/ml of HSP in dilute NH_4OH was prepared such that the final pH was 8.5. Samples of this solution were diluted with NH_4OH (pH 8.5) to the required HSP concentration. One ml was then injected for 3 days into test rats as before and the protocol followed for ^{59}Fe uptake, hematocrits, and reticulocytes. The relationship between the log dose of HSP injected subcutaneously (on 3 successive days) into fasting "dehydrated" rats and the radioiron present in the peripheral red cells is shown in Fig. 2. The changes observed in the reticulocytes present in the peripheral blood are also given. The plots of log dose and linear response shown a sigmoid relationship for radioiron uptake and peripheral reticulocytes.

TABLE II. Comparison of Erythropoietin Standard B to HSP.

Treatment[b]	A. Response of fasting "dehydrated" rats[a]			
	No. of rats	HCT (%)[c]	^{59}Fe uptake (%)	Reticulocyte (%)
Standard B (0.65 EPF units)	4	53.2 ± 3.0	19.5 ± 1.9	1.0 ± 0.1
HSP (1.0 mg)	4	53.0 ± 1.8	21.0 ± 2.1	1.1 ± 0.1
Control	3	52.7 ± 2.5	5.9 ± 0.5	0.6 ± 0.2

Sample	B. Readings on spectrophotometer[d]		
	D_{260}	D_{280}	D_{260}/D_{280}
Standard B	0.595	0.700	0.85
HSP	1.350	1.400	0.96

[a] Rats wts. at sacrifice: 160–170 gm.
[b] Recipient rats received 3 daily subcutaneous injections.
[c] ± Standard deviation.
[d] Readings made using saline as a control. Concentrations were 1.0 mg/1.5 ml of saline.

A comparison (Table II) showed that 1.0 mg of HSP from the rat produced ^{59}Fe uptake, hematocrit, and reticulocyte responses that are comparable to 0.65 units of erythropoietin Standard B from human urine (Dept. of Biol. Standards, Nat'l. Inst. for Med. Research, London). Although the spectra were not completely identical, the D_{260}/D_{280} ratio for the HSP fraction and the human urine standard were similar.

To determine the effect of heating on the HSP active fraction, pyrex test tubes were used in the preparation of 15-mg samples of HSP in 3.0-ml aliquots of 0.1 *M* Sörensen buffer at pH 7.6 (11). As a standard, a sample was maintained at room temperature. Another aliquot and a tube containing 3.0 ml of buffer only were agitated in a boiling water bath for 10 min and then were allowed to cool to room temperature. The volume in each tube was next increased to 22.5 ml with additional buffer. On 3 successive days, 1.5 ml was injected subcutaneously into fasting "dehydrated" recipient test rats as before. Heating HSP showed no significant effect on the ^{59}Fe uptake or reticulocyte response.

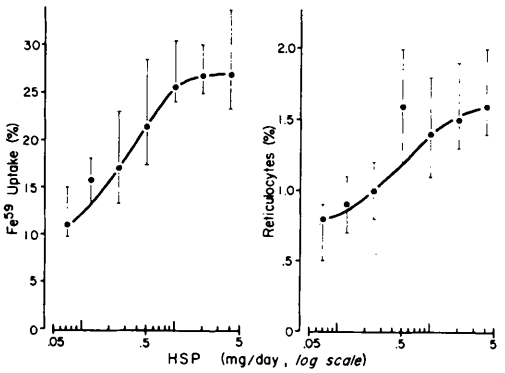

FIG. 2. Log dose response curves. Recipient rats were injected subcutaneously for 3 consecutive days with HSP. Each point represents the average response value for 6 rats. Wt. of rats at sacrifice was 150–160 gm.

Table III shows the response of recipient rats to HSP from the skins of hemin-injected (3 mg% of hemin/total body wt.) donors from several species. All donors were maintained on a normal diet. A dose of 0.7 mg HSP in 1.5 ml saline was injected, as before, into fasting "dehydrated" rats. In one experiment, 1.0 mg of mouse HSP was injected into somewhat larger recipient rats. A 0.9% saline solution was injected into control animals. The data confirm the activity of the HSP fraction from the hemin-injected skin of the cat, guinea pig, rabbit, rat, and mouse.

To investigate the effect of inflammation on the production of erythropoietic substances in the skin, donors were injected with substances known to produce inflammation (turpentine (12), talcum). Fasting "dehydrated" donor rats weighing 200 gm were subcutaneously injected on 3 consecutive days with 6.0

TABLE III. Response to HSP from Various Species.[a]

HSP donor	No. of rats	HCT (%)[c]	^{59}Fe uptake (%)	Reticulocyte (%)
A. Recipient rats received 0.7-mg sample daily for 3 days[b]				
Cat	6	55.0 ± 2.3	15.3 ± 0.7	0.9 ± 0.2
Guinea pig	6	52.0 ± 2.0	16.1 ± 3.3	1.1 ± 0.2
Rabbit	5	53.8 ± 0.6	10.9 ± 1.0	1.0 ± 0.1
Rat	6	54.1 ± 1.8	16.8 ± 1.2	1.1 ± 0.3
Control	6	49.2 ± 2.6	4.7 ± 0.8	0.4 ± 0.1
B. Recipient rats received 1.0-mg sample daily for 3 days[b]				
Mouse	4	50.7 ± 2.8	24.3 ± 4.7	1.5 ± 0.3
Control	4	50.3 ± 2.5	7.6 ± 1.6	0.6 ± 0.2

[a] Recipients were fasting "dehydrated" rats.
[b] Rat wts. at sacrifice: Expt. A. 150–160 gm; Expt. B. 180–190 gm.
[c] ± Standard deviation.

TABLE IV. Effect of Skin Extracts from Other Inflammation Producing Substances.

Donor treatment	Recipient[a] treatment (mg)	No. of rats	Mean HCT (%)	^{59}Fe uptake (%)[b]
Turpentine (0.5 ml/day for 3 days)	1.0	5	54.5	5.8 ± 0.6
	1.5	5	53.0	6.3 ± 1.5
Talcum (6 mg/day for 3 days)	1.0	5	55.0	6.9 ± 1.1
	Control (saline)	5	54.5	5.0 ± 0.3

[a] Fasting "dehydrated" rats received 3 daily subcutaneous injections of lyophilized skin ppt. Rat wts. at sacrifice: 160–170 gm.
[b] ± Standard deviation.

TABLE V. Effect of HSP on Polycythemic Rats.[a]

Treatment[b]	Polycythemic rats	HCT (%)[c]	Uptake (%)	Reticulocytes (%)
HSP	6	61.5 ± 2.3	24.0 ± 3.9	.42 ± .06
Control	6	61.7 ± 1.6	14.6 ± 0.4	.12 ± .05

[a] Rat wts. at sacrifice: 210–220 gm.
[b] Polycythemic rats received 2 daily 4.0-mg injections.
[c] ± Standard deviation.

mg of talcum or 0.5 ml of turpentine. The skins were extracted as before and samples (1.0 mg and 1.5 mg) of the precipitate fractions were tested in recipient rats. The results (Table IV) indicate that the subcutaneous injection of turpentine and talcum did not produce erythropoietically active material in the skin.

Table V shows the results of an experiment, using recipient polycythemic rats, to establish the activity of HSP in a system other than the fasting "dehydrated" rat. Significant increases ($p = < .002$) are produced in both radioiron uptake and reticulocytes.

Discussion. Extraction of the skin tissues from the site of hemin injection involves 5 general steps from the homogenate to the erythropoietically active HSP fraction. It should be noted that these steps do not effect complete separation since slight erythropoietic

effects were found in recipient rats upon the injection of an excessive (10 mg) amount of lyophilized HSS.

For dose response, all test animals were treated from a portion of a single HSP solution that was diluted to the required concentration for injection since specific activities were found to vary from extract to extract. The log dose response curves resemble the sigmoid curves of Schlueter *et al.* (13), rather than the straight line curves of Hodgson *et al.* (14).

Microscopic examinations on thin sections of rat skin, obtained from the site of hemin injection, showed a nonspecific inflammatory infiltration with many polymorphonuclear leukocytes, a scattering of plasma cells and an occasional lymphocyte. These facts presented the possibility that inflammation alone could produce an erythropoietically active substance. Since the examination of tissues at the site of injection of turpentine and talcum revealed a similar inflammation reaction, both of these substances were used for the preparation of extracts in the same way that HSP was prepared. Since the resultant extracts induced no erythropoietic activity when tested, it was concluded that the production of an erythropoietically active substance was not inflammation-dependent per se.

Other studies in this laboratory indicate that the production and effect of HSP may be highly specific for skin. Since this specificity is not due to the inflammatory cells, a rational explanation of the interaction of subcutaneous hemin and the skin, as well as the mode of action of HSP, must await the results of continuing studies on isolation and characterization.

Summary. A method is presented for the extraction of a hemin-stimulated, heat stable erythropoietically active fraction (HSP) from the skin of subcutaneously injected animals. The production of HSP is not species specific; similar substances were produced by the enderonic tissues of the cat, guinea pig, rabbit, rat, and mouse. While the production of HSP is coincident with inflammation produced by hemin injection, the production is not inflammation-dependent per se since other inflammatory substances tested do not stimulate production. The HSP compares favorably with erythropoietin Standard B (from human urine) for erythropoietic activity in the fasting "dehydrated" rat. Its activity has also been confirmed in the polycythemic rat. The dose–response curve for HSP administration shows a definite response to as little as 70 μg. The maximal response plateaus at a dose of about 1 mg.

1. Kepinow, L., Biochem. Z. **30**, 160 (1910).
2. Isaac, S., Therap. Halbmon **34**, 341 (1920).
3. Brown, J. R., Altschuler, N., and Cooper, J. A. D., Proc. Soc. Exptl. Biol. Med. **112**, 840 (1963).
4. Rambach, W. A., Alt, H. L. and Cooper, J A. D., Proc. Soc. Exptl. Biol. Med. **108**, 793 (1961)
5. Naets, J. P., Proc. Soc. Exptl. Biol. Med. **103**, 129 (1960).
6. Jacobson, L. O., Goldwasser, E., Fried, W., and Plzak, L. F., Nature **179**, 633 (1957).
7. Lucarelli, G., Howard, D., and Stohlman, F., Jr., J. Clin. Invest. **43**, 2195 (1964).
8. Ryan, E. A., Brit. J. Dermatol. **78**, 501 (1966).
9. Rambach, W. A., Alt, H. L., and Cooper, J. A. D., Blood **12**, 1101 (1957).
10. Jacobson, L. O., Goldwasser, E., Plzak, L. F., and Fried, W., Proc. Soc. Exptl. Biol. Med. **94**, 243 (1957).
11. Colowick, S. P. and Kaplan, N. O., eds., "Methods in Enzymology," Vol. 1, p. 143. Academic Press, New York, 1955.
12. Rigby, P. G., Strasser, H., Emerson, C. P., Betts, A., and Friedell, G. H., J. Lab. Clin. Med. **59**, 244 (1962).
13. Schlueter, R. J., Norgello, H., and White, W. F., Proc. Soc. Exptl. Biol. Med. **103**, 43 (1960)
14. Hodgson, G., Perreta, M., Yudilevich, D., and Eskuche, I., Proc. Soc. Exptl. Biol. Med. **99**, 137 (1958).

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