ported which inhibit hepatic synthesis of cholesterol and lower blood cholesterol; α-phenylbutyric acid(7,8), α-p-bi-phenylbutyric acid (9,10) and vanadium salts(11,12).

It is not known if an increased hepatic synthesis of cholesterol is a contributing factor in the etiology of the various abnormal metabolic states in which cholesterol has been implicated. The clinical study of benzmalecene in these conditions should be useful in obtaining information on their etiology and may offer a practical method of combating hypercholesteremic states.

Summary. Benzmalecene inhibited *in vitro* incorporation of 2-C^{14}-mevalonic acid into cholesterol by rat liver homogenates. The inhibition proved to be non-competitive. Oral administration of this compound to normal rats resulted in a significant reduction in plasma cholesterol.

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Erythropoietin. I. Analysis of Radioiron Assay in Fasted Rats, Using Cobalt as a Reference Standard.* (25406)

R. J. SCHLUETER, H. NORGELLO AND W. F. WHITE
(Introduced by R. E. Thompson)
Central Research Labs., Armour and Co., Chicago, Ill.

In the past few years research on erythropoietin has expanded rapidly and the number of papers in this field has greatly increased. Unfortunately, however, it has been difficult to compare assay results between laboratories because there has been no reference standard. Gordon(1) documented the need for such a material. We attempted to remedy the situation by choosing cobalt† as a readily available primary reference standard in the radioiron assay developed by Fried *et al.*(2). Using the starved rat as subject, 5 μmoles of cobalt gives a substantial response above the saline blank and therefore has been designated as the one unit response. Further studies have been made in the 1 to 2 unit range, comparing cobalt to various erythropoietin fractions. Finally, a partially purified fraction from anemic sheep plasma has been carefully standardized against cobalt and has been adopted by our laboratory as a day-to-day working standard.

Methods. Normal, 2 month old, male Sprague-Dawley rats, weighing 175 to 200 g were used. The rats were acclimated to the animal room for 4 days, and maintained on Rockland R-4 rat diet. At start of assay all food was withdrawn, but water was supplied *ad lib.* Samples were prepared in 0.05 *M* $NaPO_4$, 0.15 *M* NaCl, pH 6.8 (saline). After 30 hours fasting, the first 2 ml sample was injected intravenously (tail vein) under light

* This paper is based on work done under Contract with Atomic Energy Comm.

† Here the simple term "cobalt" is understood to mean "cobaltous ion". Reagent grade cobalt chloride ($CoCl_2 \cdot 6H_2O$) has been used as source of cobaltous ion.

TABLE I. Analysis of Effect of Cobalt on Fe^{59} Uptake in Fasted Rats in 7 Assays. Consecutive experiments in which both levels of cobalt were assayed.

Saline		5 μM Co^{++} dose		10 μM Co^{++} dose		Slope	
No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	b*	Lambda
6	2.86 ± .45	6	8.71 ± 1.70	6	12.93 ± 5.02	14.02	.268
6	4.41 ± .91	6	6.31 ± 1.07	6	9.46 ± 1.64	10.45	.132
6	3.01 ± 1.12	6	6.96 ± 1.23	6	8.04 ± 1.93	3.60	.450
6	3.11 ± .89	6	8.97 ± 2.49	6	13.57 ± 5.95	15.26	.299
6	3.94 ± 1.20	6	10.19 ± 3.39	5†	13.76 ± 1.55	11.87	.230
6	3.08 ± .63	7	6.44 ± 1.80	7	10.77 ± 2.28	14.39	.143
6	3.76 ± .45	7	6.44 ± 2.48	7	10.86 ± 3.33	14.67	.200
‡ 42	3.45 ± .96	44	7.66 ± 2.47	43	11.26 ± 3.81	11.22	.291

* Based on log dose-response coordinates assuming 5 μM Co^{++} equal to 1 unit response.
† One response was less than the blank, hence its value was discarded.
‡ Total.
± = stand. dev.

ether anesthesia. Twenty-four hours later, a duplicate injection was made. When cobalt was tested, it was given in 2 subcutaneous injections in 1 ml doses, in neutralized 0.15 *M* NaCl.‡ Twenty-four hours after final sample injection, 1 ml of Fe(ic)^{59} chloride (1 to 2 μc) was introduced into the tail vein. Sixteen hours after radioiron administration, a 1 ml sample of blood was taken by cardiac puncture. Activity of Fe(ic)^{59} in the blood sample was determined by counting in a well-type scintillation counter. Calculations were based on the assumption of blood volume being 5% of final body weight(3). Potencies were calculated as described by Bliss(4). The saline response did not enter into the calculations; however, only responses significantly greater than those of saline were considered valid. *Materials.* In addition to cobalt we tested plasma, phenylhydrazine-anemic plasma (5) and fractions therefrom. Most fractions were made by procedure outlined in Fig. 1(6), Two fractions in particular will be discussed: Step 1 material, which is the fraction eluted from the first DEAE-cellulose column; and Step 3 material, which is the fraction eluted from second DEAE-cellulose column. In dialyzed lyophilized form, these fractions represent yields of approximately 0.35 g and 0.06 g l of plasma, respectively.

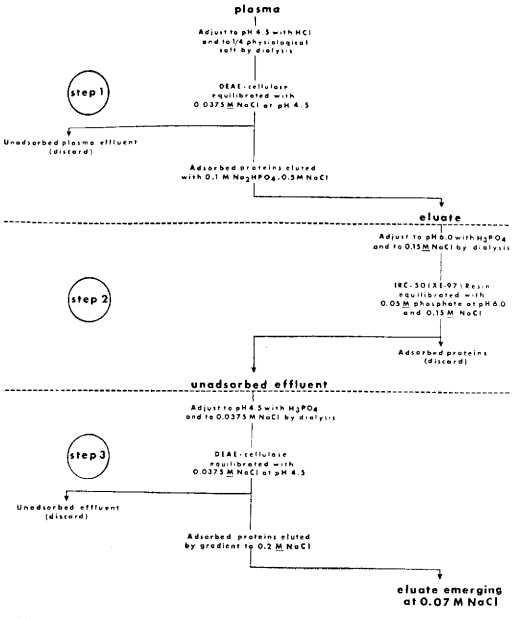

FIG. 1. Three step process for production of erythropoietin.

‡ Phosphate is omitted from NaCl used for preparation of $CoCl_2$ solution.

Results. (A) *Cobalt*: Earlier work by Goldwasser *et al.*(7,8) had indicated that cobalt stimulated incorporation of iron at dose levels of a few μmoles/rat. Preliminary experiments indicated that 5 to 10 μmole doses gave responses substantially greater than the saline blank. Table I shows typical responses to these dose levels on consecutive assays. The pooled λ§ value for assays in Table I is 0.291. Later work with our assay indicated that purified fractions from anemic sheep plasma gave slopes comparable to that of cobalt in the same response range (Table II) with λ values averaging 0.229. Cobalt therefore was accepted as the primary reference

§ λ is defined as standard deviation of a single response in terms of log-dose. It is calculated by dividing the standard deviation by the slope of the dose-response curve.

TABLE II. Analysis of Effect of Standard Sheep Plasma Erythropoietin on Fe^{59} Uptake in Fasted Rats in 7 Assays. Consecutive experiments in which both levels of sheep plasma erythropoietin standard were assayed.

Saline		1 mg standard		2 mg standard		Slope	
No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	No. rats	% uptake Fe^{59}	b	Lambda
6	2.61 ± 1.67	5	6.18 ± 2.43	5	10.78 ± .73	15.24	.081
6	3.10 ± .47	6	8.52 ± .68	6	9.85 ± .24	4.41	.272
6	3.57 ± 1.42	12	9.18 ± 1.61	6	12.90 ± 4.15	12.40	.230
	*	5	8.50 ± .64	4	12.49 ± 2.85	13.28	.136
6	3.56 ± .59	5	8.32 ± 2.84	5	11.12 ± 2.69	10.62	.248
6	3.78 ± 1.00	6	7.47 ± 1.62	5	11.03 ± 2.71	11.98	.179
6	3.77 ± 1.07	6	9.82 ± 2.57	6	12.08 ± 2.48	7.53	.336
† 36	3.40 ± .86	45	8.47 ± 1.80	37	11.45 ± 2.60	9.99	.229

* One rat was eaten by the others in the group thus the responses were all aberrant.
† Total. ± = stand. dev.

standard for low level responses with the 5 μmole response designated as one unit(6). It was acknowledged that the relationship between responses of cobalt and erythropoietin fractions might not hold true at higher levels or with different injection schedules. However, it was believed that these complications could be avoided by selecting a reasonably pure erythropoietin fraction for use as a day-to-day working standard. Such a fraction would have to be carefully standardized against cobalt and then checked occasionally for maintenance of potency.

(B) *Plasma*: On the basis of the cobalt standard, plasmas from individual sheep made anemic by phenylhydrazine(5) gave potencies in the range of 0.06 to 7.4 units/ml. However, pools of plasma from 25 to 50 sheep varied only from 0.2 to 1.6 units/ml. Plasmas from normal sheep, even at maximum dose of 4 ml/rat, have not given significant responses. However, by assaying Step 1 fraction from normal plasma at high concentrations, it has been calculated that normal blood contains about 0.01 unit/ml.

(C) *Purified fractions*: Step 1 fractions have shown potencies from 0.3 to 0.9 unit/mg, and Step 3 fractions have been in the range of 1 to 4 units/mg. Both types of fraction have given dose-response slopes similar to those for cobalt in the 1 to 2 unit range. Further fractionation has given materials with indicated potencies to 50 units/mg. However, since Step 3 material was already reasonably homogeneous by ultracentrifugal and electrophoretic criteria, and since it showed no toxic effects even at very high doses, it appeared to be a suitable material for use as a working standard. Table II shows results of a series of assays on a Step 3 fraction pool. By comparing results with those of cobalt in Table I, this fraction was very nearly 1 unit/mg. It has been used as the working standard for our assays and for development of dose-response curve, Fig. 2. Stored in lyophilized form in the refrigerator, it has not shown any appreciable loss of activity over several months.

Discussion. The advantage of increased

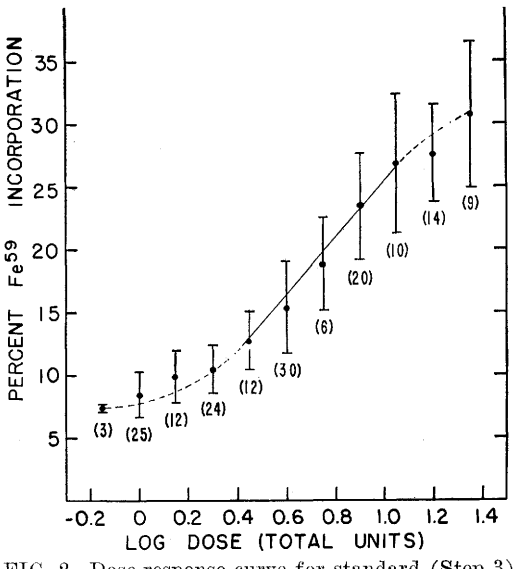

FIG. 2. Dose-response curve for standard (Step 3) erythropoietin. Data was accumulated from 7 consecutive assays, exclusive of assays used for data in Tables I and II. Figures in parentheses indicate total No. of animals assayed at a given dose level and vertical lines show stand. error of the mean. The line drawn is by inspection. One response on the 0.5 unit dose was omitted because of apparent aberrance.

TABLE III. Analysis of Dose-Response Curve.

Unit dose interval	Slope	Lambda	~ % stand. error*
1-2	6.8	.278	77.1†
2-4	16.6	.182	45.5
4-8	27.5	.139	33.1
11.2-22.4	13.3	.431	142.6

* Using 5 rats/level. Greater accuracy is naturally gained by use of more animals, thus, if 30 rats were used in the 1 to 2 unit range, the approximate stand. error would be lowered to 25%.

† Individual assays in the 1 to 2 unit range have varied in stand. error from 18 to 96%.

sensitivity of the radioiron assay in the fasted rat over that of the normal(2) or even hypophysectomized(9) rat has been demonstrated. As expected, however, degree of sensitivity in the fasted rat is a function of dose level. It is evident from Fig. 2 that the log dose-response curve shows an increasing slope, at least as high as the 11.3 unit dose (26.8% radioiron incorporation). Two other groups(9,11) working with purified urinary erythropoietin have studied the dose-response relationship in fasted rats but have not noted the sigmoid nature of the curve. An analysis of our curve between increasing dose levels (Table III) shows that the standard error of the assay can be decreased considerably at dose levels higher than the 1 to 2 unit interval. It would be more desirable to assay at these higher levels although there are several disadvantages, the most obvious being the limited amounts of erythropoietin available. Likewise, low potency materials would have to be injected in enormous volumes and/or concentrations. Under the procedure described, 4 ml of whole plasma is the practicable injection limit. Even at this concentration some mortality is incurred with resultant cannibalism, hence responses of remaining rats in the group are invalidated. The possibility of using subcutaneous dosage for eliminating some of these problems is now being considered. Korst and Bethell(10) indicate that route of administration is not critical. For purposes of following fractionation procedures, the accuracy in the 1 to 2 unit response range with 5 animals/level has proven adequate. Routinely standards are obtained at both these levels on each assay, but the samples are performed at only a single intermediate level. Critical samples, however, are generally performed at 2 levels, and reassayed if response values fall outside of limits established by the standard. It should be pointed out that curve in Fig. 2 cannot be applied directly as a standard curve for evaluation of potencies. Variations within each assay must be compensated for by performing at least 2 levels of standards in the desired response range.

Summary. Radioiron assay of erythropoietin has been analyzed and for purposes of inter-lab comparison of erythropoietic activity, the response to 5 μmoles of cobalt, given by subcutaneous injections, is suggested to represent 1 unit of activity. A house standard of partially purified sheep erythropoietin, having a potency of 1 unit/mg, has been used to establish a dose-response curve up to 22.4 units/rat. In this range the curve shows an increasing slope to 11.3 units/rat. On the basis of this assay, the potency of several fractions of sheep erythropoietin has been given.

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