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Presence of An Active Erythropoietic Factor (Erythropoietin) in Plasma of Rats after Prolonged Cobalt Therapy.* (24374)

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Ability of cobalt to stimulate erythropoiesis has been demonstrated repeatedly(1). The mechanism of its action on red cell formation, however, is not clearly understood. Warren *et al.*(2) have presented evidence that cobalt does not stimulate bone marrow by local anoxia as had been previously believed. Recently Goldwasser *et al.*(3) demonstrated that cobalt would stimulate production of erythropoietin, or similar erythropoietic plasma factor, and suggested that in this manner cobalt exerts its effect on erythropoiesis. These workers demonstrated recently(4) that erythropoietin could be found in plasma in greatest amounts 12 hours after single injection of cobalt and returned to normal 72 hours later. This material had gross properties the same as those of active substances from phenylhydrazine treated rats. Our experiment was conducted to determine whether or not erythropoietin production was still being stimulated after prolonged cobalt therapy, and to compare any effect obtained to the activity of plasma of rats in which erythropoietin production had been stimulated by bleeding.

Methods and materials. Rats used were females of Sprague-Dawley strain. Hematocrit determinations were made using Van Allen tubes (no diluent) and spun 1 hour at 2,000 rpm. Plasma from cobalt treated rats was dialysed to remove excess cobalt as follows. Ten parts plasma plus 1 part buffered NaCN solution (50 ml 0.2M K_2HPO_4 + 260 mg NaCN diluted to 100 ml) was dialysed against a dilute NaCN solution of pH 7.5 (same as above but diluted to 1 l with 0.9% NaCl). Dialysis was carried out in mechanical agitator 4 days at 2°-4°C; the dialysate was changed daily and saved. The plasma mixture was then dialysed against 0.9% NaCl 7 more days to remove cyanide. A portion of normal plasma was similarly treated. To determine effectiveness of dialysis on cobalt removal, chemical analyses‡ were made on samples of dialysed plasma, and dialysates from treated rats, and plasma from normal rats as follows. A 20 ml sample was placed in platinum dish, covered and heated in muffle oven for 16 hours at 500°C. After cooling to room temperature, 3-5 ml of 70% perchloric acid were added dropwise to the charred residue and mixed. This was dried slowly to a grey-white residue which was returned to the partially cooled oven, heated 1 hour at 600°C, then cooled to room temperature. 5 ml of 50% HCl and 2 ml of distilled water were

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‡ The method described is a modification, devised by D. M. Hubbard of Kettering Laboratories, Cincinnati, O., of nitroso-R-salt technic. The authors wish to thank Mr. Hubbard for use of his facilities and his supervision of analyses.

added; this was neutralized, using phenolphthalein indicator, with 20% NaOH. 2 ml Spekker acid, 10 ml 0.1% aqueous nitroso salt solution and 10 ml of 50% sodium acetate trihydrate were added, the solution brought to boil, then 5 ml of concentrated HNO_3 were added and boiling continued 2 minutes. This mixture was diluted to 50 ml with distilled water, placed in Beckmann spectrophotometer set at 510 mμ, and optical density differences between sample and reagent blank were obtained. A series of standard solutions of 0 to 100 μg, in 10 μg intervals, were used to establish a standard curve from which the amount of cobalt in the sample was estimated. All chemicals were reagent grade Mallinckrodt compounds. Cobalt concentration for sample of normal plasma was 0.18 μg/ml as compared to 0.20 μg/ml for the dialysed plasma from cobalt treated rats indicating essentially normal levels had been obtained by dialysis. Since plasma was limited, only one determination was made in each case. Analyses of dialysates showed more cobalt/unit volume of plasma from cobalt treated rats than from normal, showing excess quantities of cobalt were contained in treated rats' plasma and had been removed by dialysis. Erythropoietic activity was determined in hypophysectomized female rats. Plasma was concentrated in a Todd freeze dry apparatus to a small volume and diluted with distilled water until 1 ml of final concentrate equalled 6.6 ml original plasma. Twenty-four hours prior to testing, 0.1 ml cardiac blood was taken to establish control levels. 1 ml of concentrated plasma was injected subcutaneously for 3 days; 24 and 48 hours later 0.1 ml cardiac blood was taken for study. Reticulocyte counts of smears stained by the New Methylene Blue method of Brecher(5) were used as indices of erythropoietic activity.

Procedure and results. Plasma was obtained *via* heart puncture from 4 groups of donor rats. (A) Normal control rats—average hematocrit 43% (range 39-46%); (B) hemorrhagic anemic animals—plasma obtained one day after 3 daily bleedings from the tail of approximately 3 ml—average hematocrit 18.5% (range 13-25%); (C) cobalt treated rats—injected subcutaneously daily

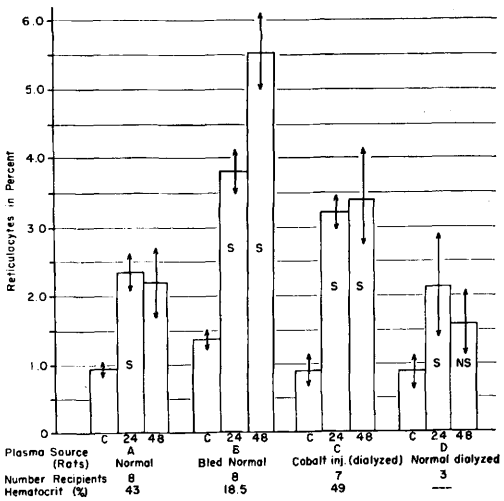

FIG. 1. Reticulocyte response of hypophysectomized rats to injections of 3 cc of non-dialysed plasma concentrated 6.6 times obtained from normal rats (A), and bled normal rats (B), and concentrated dialysed plasma from cobalt injected rats (C), and normal rats (D). C = control values (before inj.); 24 = 24 hr after last inj.; 48 = 48 hr after last inj. Vertical arrows = standard error; S = significantly (Fisher's "t") different than controls; NS = not significantly different than controls. Note: one rat in group C showed markedly elevated reticulocyte levels of 10.5 and 6.3% and was not included in the averages.

with 0.2 mg elemental cobalt (as cobaltous chloride) for 10 days and increased to 0.3 mg for 16 more days—plasma taken within 8 hours of last injection—average hematocrit 49% (range 45-57%); (D) control rats for group C—plasma treated identically.

Fig. 1 shows results of injections of concentrations of above plasmas into hypophysectomized recipients. The results show that concentrates of normal plasma (group A) caused a reticulocyte increase of 148 and 132% above pre-injection levels 24 and 48 hours respectively following the end of injections. Dialysed plasma from normal non-treated animals (group D) showed increases of 137% above normal 24 hours after final injection and 78% after 48 hours. The latter figure is not statistically significant. These figures are similar to those for normal non-dialysed plasma and indicate that cyanide dialysis in itself did not have a significant effect on erythropoietic potential of plasma. Concentrated plasma from hemorrhagic anemic animals (group B) increased reticulocyte levels 61.5 and 151% above the response to normal plasma (group

A) after 24 and 48 hours respectively. These differences were statistically significant. Plasma from cobalt treated rats (group C) caused elevation in reticulocyte level 36.4 and 54.5% above the response to normal plasma (group A) for the 2 time intervals studied. The difference between the 2 groups at 24 hours was statistically significant; the difference at 48 hours, although similar in magnitude, was not statistically significant.

Discussion. Our data substantiate the finding by Goldwasser *et al.*(3) that cobalt stimulates formation of a circulating erythropoietic active substance, and furthermore demonstrate that erythropoietin is still being formed after prolonged treatment. That the response is due to increased levels of cobalt in plasma of treated rats has been eliminated. Goldwasser *et al.* added cobalt to normal plasma to establish control values. Utilizing the fact that erythropoietin is non-dialysable (6) we reduced cobalt levels to normal by cyanide dialysis.

Reticulocytosis induced by plasma from cobalt treated rats was smaller than that by plasma from bled rats. This probably indicates a circulating level of erythropoietin less than in acutely anemic rats but greater than in normal. This observation correlates well with the fact that weeks are required to induce significant elevations of hematocrit by cobalt therapy. Evidence that erythropoietin is being formed after prolonged cobalt treatment suggests this is the mechanism whereby cobalt induces a polycythemia. Anemia of bleeding will repair in time, presumably due to action of increased levels of erythropoietin. It seems possible that a similar mechanism is working in the case of cobalt polycythemia. Here cobalt given to an animal already having a normal blood picture, or even an anemia(7), may stimulate erythropoietin production and if continued long enough increases the erythrocyte picture above normal. Whether the 2 erythropoietic substances in the 2 instances are identical is still problematical.

Injection of both dialysed and non-dialysed samples of concentrated normal plasma induced significant increase in reticulocyte levels. Perhaps this response is due to the presence of active erythropoietic factor normally found in the plasma, the presence of which has been revealed by the near 7-fold concentration of the plasma.

Summary. 1) Plasma was obtained from hemorrhagic anemic rats and from rats injected with cobalt for 26 days for determination of erythropoietic activity. Cobalt concentration of plasma from cobalt treated rats was lowered to normal levels by dialysis against isotonic phosphate-cyanide solution of pH 7.5. The level of cobalt in plasma was determined by modification of the nitroso-R-salt method. 2) Plasma to be injected was concentrated 6.6 times *in vacuo*. The erythropoietic activity was tested, using reticulocyte changes as index of activity, in hypophysectomized rats injected subcutaneously with 1 cc of concentrated plasma for 3 consecutive days. Reticulocyte counts were made 24 and 48 hours after end of injections. Plasma from anemic animals caused increase of reticulocytes to 61.5 and 151% (24 and 48 hours respectively) above values obtained from injections of plasma from normal rats. Injections of "cobalt treated" plasma increased the level of circulating reticulocytes to 36.4 and 54.5% above values obtained from injecting plasma from normal rats for similar time intervals, and demonstrates that erythropoietic activity of plasma is increased in rats receiving prolonged cobalt therapy, and that the activity is not due to increased levels of cobalt.

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