was most probably due to a combination of spontaneous breakdown and adsorption on the cells or cell products and that this constituted a non-specific phenomenon common to various cell types. Conjugation or metabolization could not be demonstrated. While macrophages, L-929 fibroblasts and Chang liver cells are not known to conjugate or metabolize bilirubin, one might have expected conjugation to occur with fetal and 3-week-old rat liver cells(2). The failure to do so *in vitro* may have been due to the scanty growth of the liver cells in tissue culture. On the other hand the drop of total bilirubin with these inadequate cultures of liver cells was equal to that with the heavy cultures of fibroblasts and Chang cells. It appeared, therefore, that the amount of bilirubin adsorbed on the cells was independent of the number of living cells in the culture. It might have actually been adsorbed onto dead cells and cell products as well.

The presence of 10 mg% of bilirubin in the medium did not produce any visible ill effect on the 3 cell types tested—higher concentrations were not tried in this experiment. In the previous study, concentrations of up to 30 mg% of bilirubin failed to affect the macrophages(1).

Summary. The effect of L-929 fibroblasts, Chang liver cells and rat liver cells on 10 mg% unconjugated bilirubin bound to albumin and introduced into tissue culture medium was studied. In the presence of these cells incubated at 4°C or 37°C there was a drop in concentration of bilirubin which exceeded by far that occurring spontaneously. This drop was observed as early as one minute after the introduction of bilirubin in the medium and did not vary appreciably during the next 48 hours. Conjugation or metabolization of bilirubin could not be demonstrated. These results are similar to those previously obtained with chick spleen macrophages and indicate that bilirubin in tissue culture is readily adsorbed on various cells or cell products. The bilirubin had no ill effects on the cells studied.

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Received March 29, 1965. P.S.E.B.M., 1965, v119.

Some Effects of Acute Manganese Excess in Rats.* (30351)

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The chronic effects of a high dietary manganese intake have been studied in a number of animals(1-14) and chronic manganese intoxication has been described in man(15-20). However, the effects of an acute manganese load on the organism have not been extensively investigated. To our knowledge only Paterni(21) has reported on this problem.

In the present study rats have been injected subcutaneously with various dose levels of manganese and observations of the effects on a number of physiological parameters made at various time periods.

Materials and methods. Albino Holtzman rats weighing 100 to 550 g were used throughout the study. Observations reported for any specific time period and/or dose levels are compared to controls of equal weight, age and sex. Manganous chloride was diluted in normal saline to give dose levels ranging from 5 to 150 mg of manganese. Controls received an equal volume of normal saline only. At various time periods following subcutaneous administration of manganese, animals were anesthetized with ether and 4 ml blood samples taken from the inferior vena cava. All

* This investigation was supported in part by USPHS Research Grant HE 07787 from Nat. Heart Inst., N.I.H.

TABLE I. Alterations in Blood Hemogram and Chemistry Values 20 Hours Following Acute Manganese Administration (15 mg/100 g Body Weight).

	Controls	Mn injected	
Hgb	13.7 ± .9 g %	16.3 ± 1.1	p <.001
Hct	42.7 ± 2.8 %	53.9 ± 2.9	p <.001
RBC	6.97 ± 1.90 ($\times 10^6$) cells/mm^3	6.66 ± 1.99 ($\times 10^6$)	
M.C.V.	65.0 ± 2.5 μ^3	72.8 ± 3.2	p <.001
M.C.H.	20.5 $\mu\mu$g	22.6	
M.C.H.C.	32.0 g %	31.0	
Serum protein	5.65 g %	5.77	
Blood volume	22.0 ml	23.0	
Plasma volume	11.8 ml	11.5	
Serum Mg	2.36 ± .39 mg %	3.29 ± .60	p <.01
" Cl	104 ± 4 mEq/l	123 ± 6	p <.001
" P	2.64 ± .16 mg %	3.26 ± .24	p <.01
" Ca	4.53 ± .60 mEq/l	3.14 ± .38	p <.001
" Fe	3.62 ± 1.02 μg/ml	.81 ± .20	p <.001

samples were heparinized with the exception of those samples used for serum electrophoresis. All measurements were made using standard techniques except where otherwise noted. Blood and plasma volumes were determined using iodine^{131} albumin dilution methods. Serum magnesium values were determined using the molybdivanadate method of Simonsen, Westover and Wertman(22). All animals were maintained on identical diets but were not pair fed.

Results. As shown in Table I, hemoglobin, hematocrit and mean corpuscular volume were significantly increased in animals receiving 15 mg/100 g body weight of manganese, as were serum chloride, phosphorus and magnesium. Serum calcium and iron were markedly depressed.

The peak increase in hematocrit and hemoglobin occurred between 12 and 18 hours (Fig. 1), and was maintained for several hours. Maximum response was induced by a dosage of 17 to 30 mg of manganese per 100 g of body weight and a measurable response was observed at levels of 5 mg per 100 g of body weight.

Examination of peripheral blood smears, following administration of 30 mg manganese per 100 g of body weight, revealed anisocytosis, basophilic stippling and hypochromia of red blood cells. Alterations in leucocytes were not observed. Necrotic changes were noted in hepatic tissue 18 hours following the injection of a single manganese dose of 17 mg per 100 g of body weight or more. As seen in Fig. 2, these changes are most pronounced in the periphery of the hepatic lobule. Microscopic examination revealed an apparent increase in liver and spleen iron content in several rats 48 hours following injection of a single dose of 30 mg manganese per 100 g of body weight. However, quantitative observations of iron content will be necessary to confirm this finding.

Rats weighing 140 ± 10 g maintained on an elevated dietary manganese intake (1% of manganese) failed to demonstrate an elevation of hematocrit or hemoglobin when compared to pair fed controls.

Discussion. The possibility of an hematinic action of manganese has been considered by several previous workers. Indeed, elevated hemoglobin values have been reported in

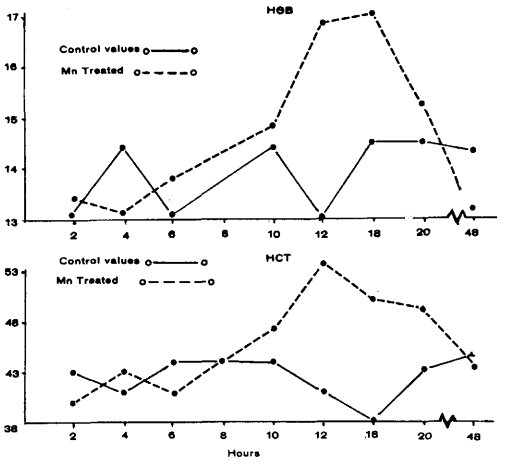

FIG. 1. Effect of acute administration of 50 mg of manganese on blood hemoglobin and hematocrit in 160 g rats. Each value represents the mean of at least 5 rats. These values are not included in Table I because of the difference in dosage of manganese.

workers exposed to fumes containing a high level of manganese(20). However, the elevated hemoglobin and hematocrit values found in the present study cannot be considered as evidence supporting the role of manganese in hematopoiesis. It is difficult, indeed, to consider that elevated manganese levels are capable of stimulating activity of bone mar-

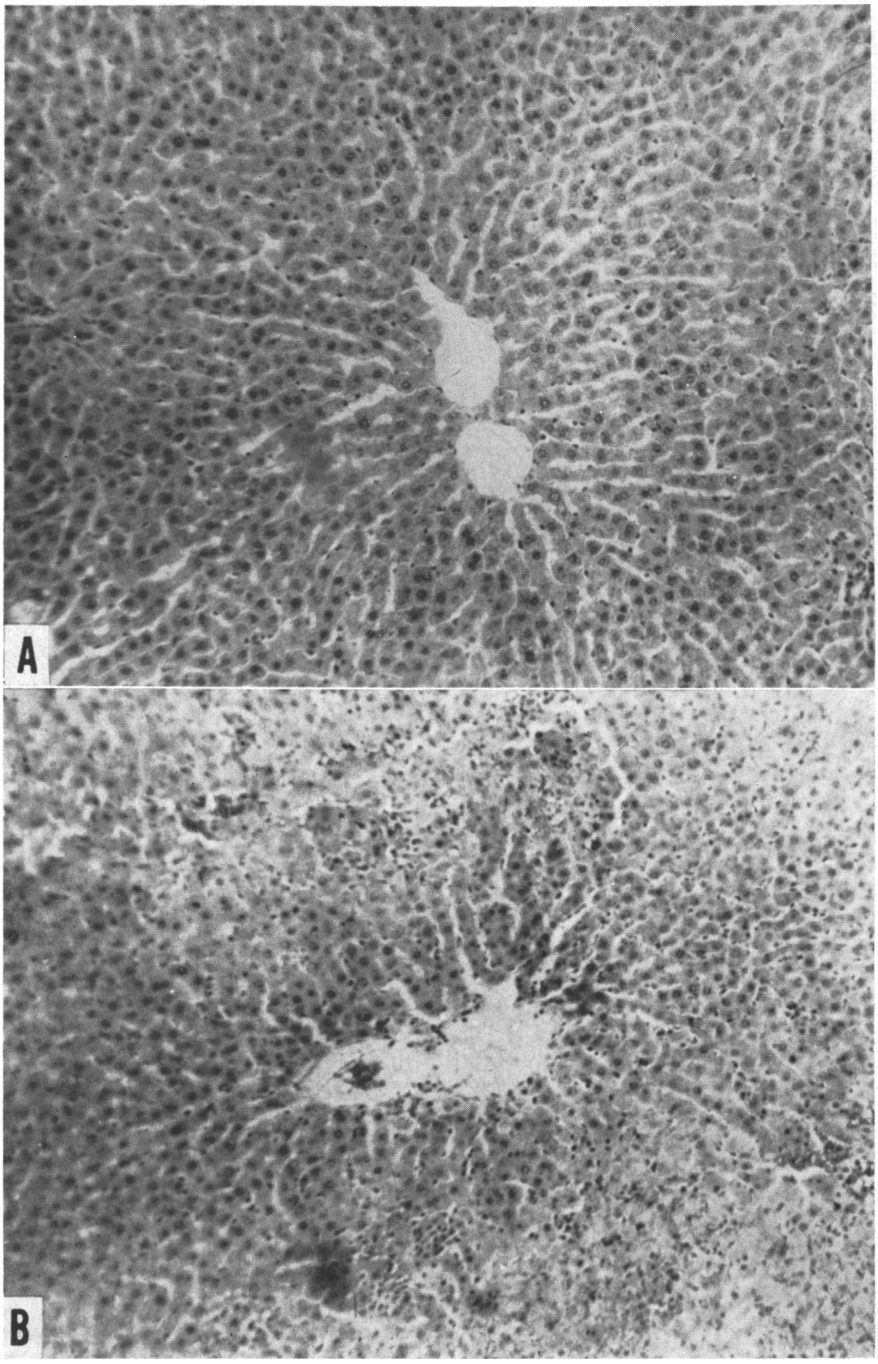

FIG. 2. Necrotic lesions in the liver of a manganese treated rat. A. Control. B. MN Treated.

row within the time period involved in our study. Lack of alteration in blood or plasma volume in manganese-treated animals, as well as the relatively unchanged serum protein values, virtually eliminates the possibility of fluid loss from the vascular space as being the primary cause of the elevation of hematocrit and hemoglobin. The possibility remains that a sudden release of erythrocytes from an extramedullary site, such as the spleen, is responsible for the erythrocytosis. In fact, preliminary studies have revealed an approximate 30% decrease in splenic weights in the manganese-treated group(23). Whether this finding is due to a primary effect of manganese or to a manganese-induced release of epinephrine is being studied.

In contrast to the elevated serum magnesium levels observed in our acute study, decreased serum magnesium values have been reported in sheep(7) and cows(4,8,9) chronically maintained on elevated dietary manganese intake. Both elevated and depressed magnesium levels are reported for rabbits maintained on high dietary manganese(6). It would then appear that the influence of manganese excess upon serum magnesium levels varies with the duration of effect, mode of manganese administration, or both. The elevated magnesium levels reported for the rabbit were observed at early stages in the course of intoxication. The relationship of manganese to magnesium in various *in vitro* and *in vivo* systems has been studied(24) but further studies will be necessary to determine the exact relationship of elevated manganese levels to magnesium metabolism.

The elevated serum phosphorus and depressed serum calcium observed may well be related to the same mechanism producing the hypermagnesemia. The interrelationship of these 3 elements and their role in animal metabolism has been well documented. Chornock (3) has shown that an excess oral intake of manganese resulted in increased fecal losses of calcium. However, it seems unlikely that subcutaneous manganese administration would have a similar effect. Conrad and Baxter have recently demonstrated a doubling of intracellular uptake of calcium^{45} in myocardium following subcutaneous administration of manganese(25). The increased calcium was found in the microsomal fraction of the myocardial cells. The rapid appearance of hypermagnesemia and hyperphosphatemia suggests that there is an outflux of these cations from soft tissue cells, bone or both. Whether this is a general catabolic effect of manganese excess cannot be determined from the present study.

Available knowledge of iron metabolism and its relationship to other metals of biological significance offers little basis for speculation concerning the mechanism responsible for the depressed values observed in this study. Somers and Shive(26) suggest that an excess of manganese may induce signs of iron deficiency in plants by oxidizing the available iron into the physiologically inactive ferric form.

Hartman and co-workers(7) found that high dietary intake of manganese in lambs interfered with iron absorption. Again, the rapidity of the effect in the present study using injected manganese makes an explanation based on a gastrointestinal absorption defect untenable. The demonstrable increase in hepatic and splenic iron content concurrent with the fall in serum values suggests the possibility of an intracellular shift of the metal. Further studies will be necessary to clarify the actual mechanisms involved.

Summary. Rats were injected subcutaneously with 5 to 120 mg of manganese as the chloride and various parameters were studied at periods from 1 to 72 hours. Blood hemoglobin, hematocrit and erythrocyte mean corpuscular volume increased within 10 hours after the injection, with a peak effect between 12 and 18 hours. Serum magnesium, chloride and phosphorus also increased but serum calcium and iron decreased. Blood volume was unchanged. Peripheral blood revealed anisocytosis, basophilic stippling and hypochromia of erythrocytes. Necrotic areas in the liver were observed within 18 hours and an apparent increase in iron content of liver and spleen was noted.

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Received March 31, 1965. P.S.E.B.M., 1965, v119.

Positive Direct Coombs' Test Induced by Various Azo Dyes.* (30352)

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In a previous study, subcutaneous injection of the diazo dye trypan blue, was found to produce an anemia with positive Coombs' tests in rats(1). Since a positive antiglobulin reaction was also produced *in vitro* when normal rat erythrocytes were exposed first to the dye and then to plasma, it was postulated that the protein binding property of trypan blue might be responsible for the *in vivo* coating of the erythrocytes. The capacity of other azo dyes to bind with protein has been related to their chemical structure(2). The present study was undertaken to correlate the molecular configuration of a selected group of these dyes with their ability to bind with serum proteins and to relate the serum concentration of both protein bound and free dye to the development of a Coombs' positive anemia.

Materials and methods. The compounds initially tested are listed in Table I with their commercial sources which are of importance since the actual dye content of different brands has been found to vary from 40 to 80% depending on the manufacturer. Aqueous solutions were injected subcutaneously into male and female Wistar strain rats (Simonsen Laboratories) weighing approximately 350 g.

The concentration of free and protein bound dye in the serum was measured by an adaptation of the method of Judah and Willoughby(3). The protein in 1 ml of serum was precipitated with an equal volume of 10% trichloracetic acid and the residue treated first with 50% ethanol and then with absolute ethanol to remove lipids. Any color in the supernatant was regarded as free dye. The dye bound to protein was extracted by suspending the precipitate in 3 ml of a 25% solution of aqueous pyridine at 70°C for 40 minutes or until extraction was complete. The color of the supernatant was measured in the Coleman Jr. Spectrophotometer at the wave length representing the peak of the absorption spectrum for each dye, and the dye concentration determined by reference to predetermined

* This investigation was supported in part by research grant CA-03309, from Nat. Cancer Inst., Nat. Inst. Health, U.S.P.H.S.