THE INCREASED SUSCEPTIBILITY TO HEMOLYSIS BY INDOL IN DOGS FED DEFICIENT DIETS

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Evidence has been presented in previous communications (1, 2) that indol, orally administered in suitable amounts, is hemolytic in dogs and that when the animals are fed deficient diets anemia results. The same amount of indol fed to dogs taking a normal diet has little or no effect in causing anemia. Since the hemolytic effect of indol has been proved, two possible ways exist in which anemia might result when the deficient diet is fed: (*a*) the hemolytic effect of the indol may be more marked in the presence of a deficiency or (*b*) the hemolytic effect may be constant and the bone marrow may be less capable of forming erythrocytes when the diet is unsuitable. To settle this question prolonged studies of the excretion of bile pigment and of the erythrocyte and hemoglobin levels in dogs receiving indol and fed normal diets, deficient diets, and deficient diets supplemented by the lacking factor have been made.

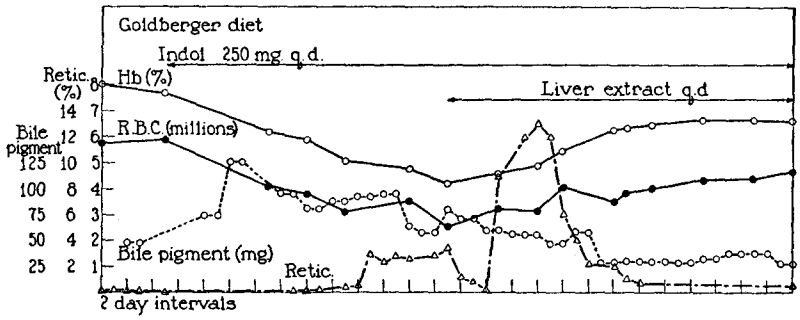
Methods

The technic employed for collecting the total bile in a sterile condition has been described (1, 2). It is a modification of that developed by Rous and McMaster (3). The measurements of the content of bilirubin in the bile have been made by the method of McMaster, Brown, and Rous (4). Although the method is open to certain theoretical objections the experimental results are valid since the interpretation depends upon the comparative values over different periods. The normal diet fed is a mixture of cooked beef, bread, and dog biscuit known empirically to be adequate in its content of vitamins. The Goldberger diet has been described repeatedly in previous publications (5), and is known to produce black tongue regularly in from 5 to 12 weeks. Since that effect can be prevented uniformly by feeding autoclaved yeast and other substances which are rich in their content of vitamin B_2 (G) and cannot be prevented by flavin (Koehn and Elvehjem, 6), its effect is supposed to be due to a lack of a part of the vitamin B_2 (G) complex other than the rat acrodynia factor or flavin.

Fresh dog bile was refed in 50 cc. amounts twice weekly to all the animals. Blood was taken at regular intervals from the jugular vein in standard amounts of potassium oxalate. Counts were made using standardized pipettes and counting chambers. The hemoglobin was estimated by the Sahli method, using calibrated tubes. The indol was a commercial crystalline product. It was fed by hand in ordinary absorbable capsules. All dogs were dewormed with hexylresorcinol and castor oil 1 month before they were put on experiment. The liver extract was Lilly (N.N.R.) powder, 4 gm. of which are derived from 100 gm. of liver. It was made up with water to a 25 per cent solution. In most instances the animals took it avidly.

EXPERIMENTAL

Experiment 1 (Text-Fig. 1).—This animal was fed the deficient diet for 4 weeks before the biliary fistula was formed. The output of bilirubin was allowed to



TEXT-FIG. 1. Dog 1

TEXT-FIGS. 1 to 7. Levels of reticulocytes, erythrocytes, hemoglobin, and daily excretion of bilirubin in dogs fed indol during periods of good and bad diets.

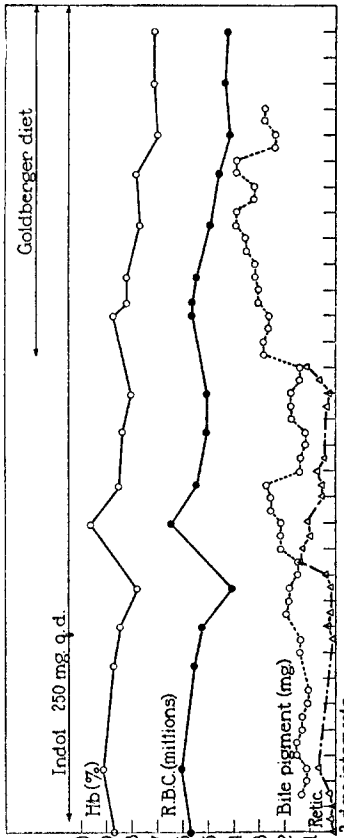
stabilize at about 50 mg. daily before indol was fed. Throughout the period depicted in the text-figure 250 mg. of indol were fed daily. 4 days after the first indol a daily output of bilirubin of 126 mg. was obtained which decreased slightly thereafter to stabilize at a figure between 80 and 90 mg. A progressive decrease in the blood levels from 5,780,000 erythrocytes and 80 per cent hemoglobin to 2,500,000 erythrocytes and 42 per cent hemoglobin took place without any significant elevation of reticulocytes. Over an 18 day period 1,568 mg. of bilirubin were excreted, an average of 87 mg. per day. The diet was then supplemented with 5 gm. of liver extract daily. On the 4th day the reticulocytes increased to 9 per cent and on the 7th day to 13 per cent followed by a progressively rising erythrocyte count to 4,360,000 and hemoglobin values to 63 per cent. Nothing was changed except for the addition of liver extract. The rate of excretion of bilirubin dropped sharply to 50 mg. daily, concurrently with the rise in numbers of reticulocytes, and then continued to decrease slowly. In the 18 days of treatment

859 mg. of bilirubin were excreted, an average of 47 mg. per day, a reduction of nearly 50 per cent of the untreated levels.

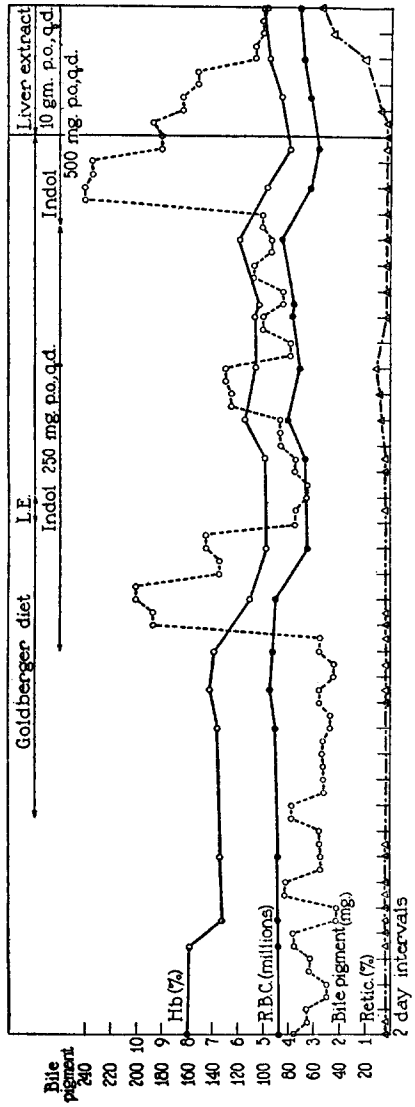
Experiment 2 (Text-Fig. 2).—In contrast to Experiment 1, indol was administered first to this animal while a normal diet was taken. As shown in Text-fig. 2 there was no increase in the excretion of bilirubin following this amount of indol, although the animal was observed for a control period of 33 days. No change in the levels of the blood or the reticulocytes took place. For the 15 days before the diet was changed 635 mg. of bilirubin were excreted, an average of 42 per day. The diet was then changed to that causing black tongue and a remarkably prompt and sustained increase in the excretion of bilirubin became evident. During the 15 days after the change 1,213 mg. of bilirubin were excreted, an average of 80 mg. daily or about double the levels of the control period. Concurrently there was a progressive drop in the blood values from 5,630,000 erythrocytes and 87 per cent hemoglobin to 4,100,000 erythrocytes and 70 per cent hemoglobin. At this point the bile became infected and the experiment was discontinued.

Experiment 3 (Text-Fig. 3).—The black tongue diet was fed for 14 days during which the excretion of bilirubin was extremely constant and the blood levels were stable. The total excretion was 753 mg. and the daily average 52 mg. Indol 250 mg. daily was then administered. The output of bilirubin rose promptly to a peak of 190 mg. daily and then stabilized at just over 100 mg., about double the output during the control period. The total output for the 14 days while indol was given was 1,701 mg. and the daily average 121 mg. The blood decreased from 4,370,000 erythrocytes and 67 per cent hemoglobin to 3,200,000 and 49 per cent hemoglobin. There was no significant change in the number of circulating reticulocytes. When the excretion of bilirubin and the blood levels had apparently stabilized, the amount of indol was increased to 500 mg. daily. This move resulted in an increase in the output of bilirubin to a total for 7 days of 1,406 mg., a daily average of 200 mg. At this point the blood had dropped to 2,700,000 erythrocytes and 39 per cent hemoglobin, again with no increase of reticulocytes. The diet was then supplemented with 10 gm. of liver extract daily, the administration of 500 mg. of indol daily being continued. Following the liver extract the reticulocytes rose on the 6th day to 9 per cent, and on the 8th day to 22 per cent followed by a progressive increase in blood levels. The output of bilirubin dropped progressively to an average of 100 mg. daily when infection intervened and the experiment was terminated. Here again the addition of liver extract reduced, by about 50 per cent, the hemolysis by indol.

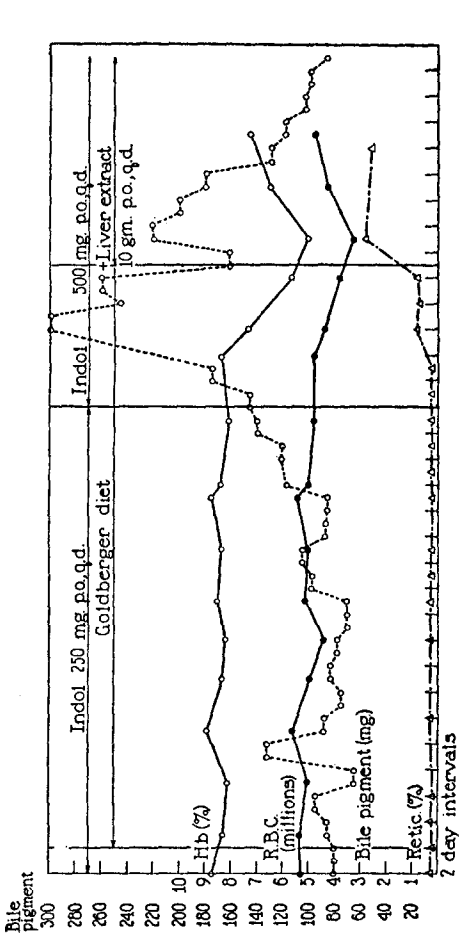
Experiment 4 (Text-Fig. 4).—In this experiment the administration of indol, 250 mg. daily, was begun while a normal diet was fed. During the control period without indol of 22 days, 1,643 mg. of bilirubin were excreted, a daily average of 74 mg. During a corresponding period of 22 days during which 250 mg. of indol were fed daily, the total output of bilirubin was 2,262 mg., a daily average of 101 mg. There was no apparent change in the blood. This animal showed a slight though distinct susceptibility to the hemolytic effect of indol even though on a normal diet. As the animal became adjusted to the presence of the toxin the



TEXT-FIG. 2. Dog 2



TEXT-FIG. 3. Dog 3



TEXT-FIG. 4. Dog 4

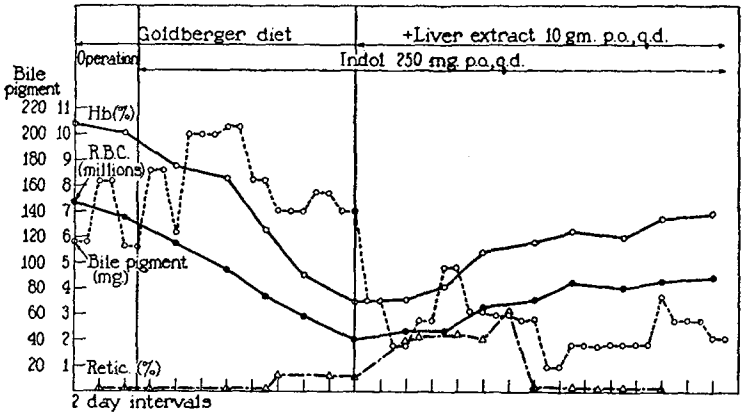
TEXT-FIG. 5. Dog 5

normal levels of the output of bilirubin were again restored, an average of 81 mg. a day for 20 days. At the beginning of Text-fig. 4, the diet was then changed to the deficient régime and the indol was continued. There ensued a slow but steady increase in the excretion of bilirubin over a period of 33 days. The total output for the first 10 days of this period was 868 mg. and for the last 10 days 1,160 mg. Coincidentally the blood dropped from 5,320,000 erythrocytes and 83 per cent hemoglobin to 4,800,000 and 81 per cent without any increase of reticulocytes. The daily dose of indol was then increased to 500 mg. The output of bilirubin for the next 10 days was 2,150 mg. and the blood dropped to 3,250,000 erythrocytes and 50 per cent hemoglobin. Without any other change 10 gm. of liver extract were administered daily. On the 2nd day the reticulocytes rose to 28 per cent followed by a rise in blood levels in 6 days to 4,200,000 erythrocytes and 65 per cent hemoglobin. The output of bilirubin dropped steadily to give a total of 1,657 mg. for the 10 days after liver extract, and to 608 mg. for the subsequent 6 days, an average of 101 mg. daily as compared to 215 daily before liver was given.

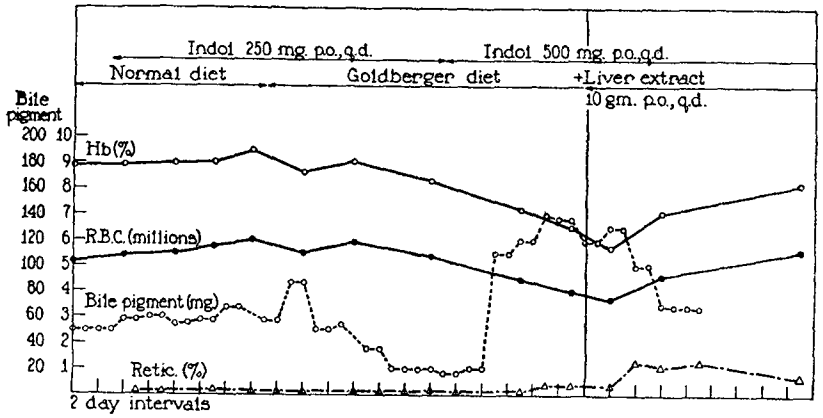
Experiment 5 (*Text-Fig. 5*).—This animal was fed the deficient diet for 1 month before indol was begun in a dosage of 250 mg. daily. During a control period of 12 days on this diet a total of 722 mg. of bilirubin were excreted, an average of 60 mg. daily. During the first 12 days of the indol feeding, 1,232 mg. of bilirubin were excreted, an average of 100 mg. daily. This increased hemolysis was compensated for by erythropoietic activity since no decrease of blood levels appeared, although the reticulocytes increased slightly in numbers. After 30 days the dose of indol was increased to 500 mg. daily. The output of bilirubin jumped to 1,706 mg. for the next 12 day period, an average of 140 mg. daily. The blood dropped from 4,390,000 erythrocytes and 67 per cent hemoglobin to 3,200,000 erythrocytes and 54 per cent hemoglobin without any increase in reticulocytes. Liver extract, 10 gm. daily, was then given. On the 2nd day following the reticulocytes were 9 per cent and on the 4th day 18 per cent. The output of bilirubin decreased somewhat more slowly than had been the case previously. For the first 10 days after the institution of liver therapy 1,208 mg. were excreted, a decrease of only 14 per cent, but in the next 7 days the output was 530 mg., an average of 75 mg. daily, a decrease of 50 per cent. Concurrently the blood rose in 6 days to 4,420,000 erythrocytes and 64 per cent hemoglobin and in the next 10 days to 5,400,000 erythrocytes and 75 per cent hemoglobin.

Experiment 6 (*Text-Fig. 6*).—This experiment is particularly instructive since the animal disliked the black tongue diet and took almost none of it after the biliary fistula was set up 1 month following the beginning of the diet feeding. Indol 250 mg. daily was started on the 6th postoperative day while the output of bilirubin was still in the 100 mg. range. The excretion of bile pigment rose sharply to a total for the next 17 days of 2,822 mg. or 166 mg. daily. The blood dropped from 6,700,000 erythrocytes and 101 per cent hemoglobin to 2,000,000 erythrocytes and 35 per cent hemoglobin. There was a slight rise of reticulocytes to 6 per cent. Liver extract, 10 gm. daily, was then administered, but the animal still refused to take the diet. The output of bilirubin dropped sharply neverthe-

less to a total of 646 mg. for the next 10 day period. On the 4th day the reticulocytes rose to 19 per cent, and on the 12th day to 30 per cent, then dropped quickly to normal levels. In the first 10 days the blood rose to 3,270,000 erythrocytes and 54 per cent hemoglobin, and in the next 10 days to 4,400,000 erythrocytes and 69 per cent hemoglobin.



TEXT-FIG. 6. Dog 6



TEXT-FIG. 7. Dog 7

Experiment 7 (Text-Fig. 7).—In this experiment after a primary control period of the normal diet 250 mg. indol daily were fed for 14 days. There was no increase in the excretion of bilirubin over the period without indol. The deficient diet was then fed and the small dose of indol continued. Deficiency developed so slowly however that no increase in the excretion of bilirubin was evident over a 15 day period. The indol was then increased to 500 mg. daily. During the previous

10 days the total output of bilirubin was 310 mg. During the next 10 day period the total output increased to 902 mg. or over 3 times, with a coincidental drop in blood levels from 5,490,000 erythrocytes and 82 per cent hemoglobin to 3,780,000 erythrocytes and 57 per cent hemoglobin, without any significant increase of reticulocytes. Liver extract was then given as a supplement to the diet in 10 gm. amounts daily without changing any other factor. The reticulocytes rose on the 5th day to 8 per cent and on the 7th day to 10 per cent. The output of bilirubin dropped steadily from the 4th day after liver was begun to an average of 78 mg. daily for the next 10 days. The blood rose to 4,640,000 erythrocytes and 70 per cent hemoglobin.

DISCUSSION

From the experiments the evidence is clear that an amount of indol which is well tolerated on a normal diet is causative of hemolysis and anemia when a deficient diet is fed. Furthermore the hemolytic as well as the anemia-producing effect of the combination of indol and a deficient diet may be prevented by supplementing the diet with liver extract. This is clear from the fact that following such supplement the output of bilirubin decreases to normal levels coincidently with the rise of reticulocytes and the improvement of the blood levels. It is to be inferred that the anemia results at least in part from hemolysis since that process is clearly conditioned by the dietary deficiency. The possibility exists, however, that a double process is operative, (*a*) the described increase of the hemolytic effect of indol on the deficient diet, and (*b*) a decrease in the erythropoietic power of the bone marrow. There is little evidence however that liver extract has the power to increase the rate of formation of erythrocytes since it is practically without effect in the standardized anemic dogs of Whipple (7). The anemia can be explained quite as well on the basis of simple destruction as by invoking two factors.

The mechanism of the increased hemolytic effect of indol in the presence of dietary deficiency is not clear. One possibility concerns the indol. (*a*) Under conditions of vitamin deficiency indol may be more freely absorbed than normally, (*b*) it may be metabolized to indoxyl more slowly than normally, or (*c*) an abnormal metabolism may be present by which some lytic intermediate product is formed. Experiments (8) have been made which show that no change in the rate of absorption or the conversion to indoxyl can be demonstrated even in a deficiency severe enough to cause black tongue. Moreover

it has not been possible to show any hemolytic effect of pure indol on washed erythrocytes *in vitro*. The possibility of the formation of pathological intermediate products is now under investigation.

A second possibility concerns a change in the erythrocyte itself as a result of the deficiency. It could become susceptible to lysis by indol although not normally so or it could be rendered by indol more susceptible to a normally existing hemolytic process. Both of these possibilities are now being studied.

An interesting phenomenon is the tendency of the organism to develop without treatment a slight but definite resistance to the hemolytic effect of indol. Reference to the figures shows that the increase in the rate of excretion of bile pigment is more marked immediately after the first administration of indol and then drops somewhat to a sustained effect. This factor does not affect the validity of the conclusions however since sufficiently long periods were observed to rule out any spontaneous changes.

The changes in the reticulocytes in these experiments are also of interest. It is known from the work of Steele (9) and previous workers that bleeding or the hemolysis resulting from phenylhydrazine is attended by persistently elevated levels of reticulocytes. The factor of hemolysis in the experiments with indol is indisputable but essentially no elevation of reticulocyte numbers is encountered. One possible explanation is that the hemolysis by indol involves all the hemoglobin-containing cells including the reticulocytes, whereas bleeding or phenylhydrazine removes only adult cells, leaving younger forms in the circulation. Dock (10) states that doses of saponin may be administered which cause severe anemia with low numbers of reticulocytes. Were a reticulocytolytic action of indol operative the increase in numbers of those cells with the cessation of hemolysis following liver extract would be easily explained. They are being formed actually in greater numbers than normally but are not visible because they are destroyed as fast as formed. When lysis ceases, however, they appear for a brief period until the erythropoietic activity of the bone marrow slows down following the cessation of the constant drain upon it.

The explanation is suggested then that the anemia resulting from a deficiency of the vitamin and the administration of indol is a hemo-

lytic anemia, due possibly to an increased susceptibility to lysis of all hemoglobin-containing cells, including reticulocytes. The rise in blood levels following the administration of liver extract as well as the temporary increase of reticulocytes seems to reflect decreased hemolysis and a decreased, rather than an increased production of cells. No experiment made so far, however, has ruled out conclusively the possibility that a double factor is operative, (*a*) an increased rate of cell destruction and (*b*) a lessened rate of cell production, referable either to the toxic effect of indol or to the deficient diet. Further experiments bearing on this point are in progress.

CONCLUSIONS

1. Indol is more hemolytic in the presence of a deficiency complex than when a normal diet is fed.
2. The hemolytic effect can be abolished by supplementing the deficient diet with liver extract curative of pernicious anemia in man.
3. The hemolysis affects all hemoglobin-containing cells, including reticulocytes.
4. The repair of the anemia resulting from the administration of indol in the presence of a deficiency represents the cessation of a hemolytic process.
5. An abnormally low rate of production of erythrocytes may well be a factor in the production of the anemia.

BIBLIOGRAPHY

1. Rhoads, C. P., and Barker, W. H., *J. Exp. Med.*, 1938, **67**, 267.
2. Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1938, **67**, 273.
3. Rous, P., and McMaster, P. D., *J. Exp. Med.*, 1923, **37**, 11.
4. McMaster, P. D., Brown, G. O., and Rous, P., *J. Exp. Med.*, 1923, **37**, 395.
5. Rhoads, C. P., and Miller, D. K., *J. Exp. Med.*, 1933, **58**, 585.
6. Koehn, C. H., and Elvehjem, C. A., *J. Nutrition*, 1936, **11**, 67.
7. Robscheit-Robbins, F. S., and Whipple, G. H., *J. Exp. Med.*, 1929, **49**, 215.
8. Miller, D. K., and Rhoads, C. P., *J. Exp. Med.*, 1937, **66**, 367.
9. Steele, B. F., *J. Exp. Med.*, 1933, **57**, 881.
10. Dock, W., The importance of hemolysis in the pathogenesis of macrocytic anemia, in Medical papers dedicated to Dr. Henry A. Christian, Baltimore, Waverly Press, 1936, 552.